Cytogenetic and molecular characterization of lacertid lizard species from the Iberian Peninsula

Verónica Rojo Oróns

Doctoral Thesis

2015

Supervisors: Dr Horacio Naveira Fachal, Dr Andrés Martínez Lage

Department of Cell and Molecular Biology

UNIVERSIDADE DA CORUÑA

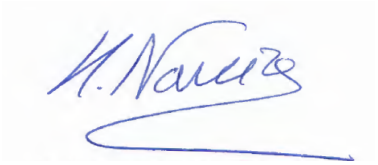
UNIVERSIDADE DA CORUÑA

D. HORACIO NAVEIRA FACHAL, DOCTOR EN BIOLOGÍA Y CATEDRÁTICO DE UNIVERSIDAD DEL ÁREA DE GENÉTICA DEL DEPARTAMENTO DE BIOLOGÍA CELULAR Y MOLECULAR DE LA UNIVERSIDADE DA CORUÑA, Y D. ANDRÉS MARTÍNEZ LAGE, DOCTOR EN BIOLOGÍA Y PROFESOR TITULAR DE UNIVERSIDAD EN EL ÁREA DE GENÉTICA DEL DEPARTAMENTO DE BIOLOGÍA CELULAR Y MOLECULAR DE LA UNIVERSIDADE DA CORUÑA,

INFORMAN

QUE el trabajo titulado "Cytogenetic and molecular characterization of lacertid lizard species from the Iberian Peninsula", presentado por Dña. **VERÓNICA ROJO ORÓNS** para optar al título de Doctora en Biología con Mención Internacional por la Universidade da Coruña, ha sido realizado bajo nuestra dirección. Considerándolo finalizado, autorizamos su presentación y defensa.

A CORUÑA, A 22 DE SEPTIEMBRE DE 2015



Fdo. Dr. Horacio Naveira Fachal

Fdo. Dr. Andrés Martínez Lage

Fdo. Verónica Rojo Oróns

To my dear Rodri

To my granny

Acknowledgements

Firstly, I would like to thank my supervisors, Horacio Naveira and Andrés Martínez, for trusting me and for the opportunity to work on such an interesting project. Thank you for your patient, guidance, and for sharing your knowledge and experience with me!

Many thanks to Ana González and Pedro Galán for their significant contributions to this research. Ana, thanks for your friendship and all your support in scientific and non-scientific questions. Pedro, thank you for collecting all the specimens for this thesis, and for your invaluable wealth of knowledge about lacertids.

I am very grateful to all the people who generously hosted me during my fruitful internships abroad: Ettore Olmo, Vincenzo Caputo Barucchi, Massimo Giovannotti, Paola Nisi Cerioni, Andrea Splendiani and Paolo Ruggeri, at the Universitá Politecnica delle Marche (Ancona); Malcolm Ferguson-Smith, Jorge Pereira, Fumio Kasai and Willem Rens, at the University of Cambridge. These research internships have been an exciting learning experience, and essential for the completion of this thesis. My special thanks to Massimo, for his infinite patient to show me the tips and tricks of cell cultures and FISH. Thank you for your support and valuable advice over all these years. Thanks also to Adriana Canapa and Marco Barucca for their warm welcome, and to Çinel, Bruna, Sara and Sabrina, for our good moments in Ancona.

Back to Coruña, thanks to my colleagues at the Evolutionary Biology Group and, especially, to my wonderful lab partners (past, present, and newcomers): Álex, David, Joaquín, Rosa, my Panoquita, Elba, Neus, Nuria, Luis, Iván, Inés, Eva...I've been really lucky to work with you! Thank you very much for the many good times we enjoyed (and I'm sure we will still enjoy) in and outside the lab. Many thanks to Rosa García Díaz and her magic hands, for their brilliant technical skills, and to Nani, Raquel, and Miriam, from the University Research Support Services, for their willing assistance on DNA sequencing. I would also like to thank the great AllGenetics team and, especially, Joaquín, Álex and Antón, for giving me the opportunity to work and learn from them.

To my friends (you know who you are), for being always there and for helping me forget about everything when I needed it. And, of course, for your interest in this lizards' "thing"!

I am forever grateful to my amazing family. To Rosa, Pedro, and Lucas, for their energizing support throughout this odyssey, and for bringing back happiness into our lives. To Rubén; thank you for your incredible patient and understanding, and for always making me end the day with a smile. To Cris, for sharing with me a bit of her talent and making this beautiful cover design. And, especially, to my parents, for their unconditional love, encouragement and support. This thesis, and everything I achieved, is thanks to you.

Finally, I wish to thank the thesis committee and the external reviewers who read and commented the draft of this thesis report.

I was supported by a FPU (Formación de Profesorado Universitario) grant, from the Spanish Ministry of Education, Culture and Sport, and by a Research Internship Grant, from the Universidade da Coruña.

Contents

Abbreviations

2n	Diploid chromosome number
4xT	4x SSC, 0.05% Tween-20
µL	Microlitre
µm	Micromolar
ºC	Degree Celsius
π	Nucleotide diversity
ACSL1	Acyl-CoA synthetase long-chain family member 1
ADAM12	ADAM Metallopeptidase Domain 12
Ag-staining	Silver-staining
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
BLAST	Basic Local Alignment Search Tool
C19orf47	Chromosome 19 open reading frame 47
CCD	Charge coupled device
CDH8	Cadherin 8, type 2
CGH	Comparative genomic hybridization
CMA_3	Chromomycin A3
COL5A1	Collagen, type V, alpha 1
Cy3	Cyanine 3
DAPI	4′,6-Diamidino-2-phenylindole
DDBJ	DNA DataBank of Japan
DMRT1	Doublesex and mab-3 related transcription factor 1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate

DOP-PCR	Degenerate oligonucleotide primer–polymerase chain reaction
dUTP	2'-Deoxyuridine 5'-triphosphate
EEF2	Eukaryotic elongation factor 2
EMBL	European Molecular Biology Laboratory
ESD	Environmental sex determination
FBXW11	F-Box And WD Repeat Domain Containing 11
FCA	Factorial correspondence analysis
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein Iso-Thyocianate
g	Relative centrifugal force (RCF)
GMPPA	GDP-mannose pyrophosphorylase A
GSD	Genotypic sex determination
h	Hour
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
iCGH	Interspecies comparative genomic hybridization
IgG	Immunoglobulin G
IPO5	Importin 5
ITS	Interstitial telomeric site
IUPAC	International Union of Pure and Applied Chemistry
KCl	Potassium chloride
L	Litre
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minute
mL	Millilitre
mM	Millimolar

mmol	Millimol
mya	Million years ago
MYST2	MYST histone acetyltransferase 2
myr	Million years
NaCl	Sodium Chloride
ng	Nanogram
NCBI	National Center for Biotechnology Information
NOR	Nucleolar organizing region
OAF	Out at first homolog
OCA2	Oculocutaneous Albinism II
OSGIN1	Oxidative stress induced growth inhibitor 1
PCR	Polymerase chain reaction
PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta
RAD-seq	Restriction size-associated DNA sequencing
RNase	Ribonuclease
RNF19B	Ring finger protein 19B
rRNA	Ribosomal ribonucleic acid
s	Second
satDNA	Satellite DNA
SBNO1	Strawberry notch homolog 1 (Drosophila)
SH3PXD2A	SH3 And PX Domains 2A
SKIL	SKI-Like Proto-Oncogene
SNP	Single nucleotide polymorphism
SOX5	(Sex determining region Y)-box 5
SS18	Synovial sarcoma translocation, chromosome 18
SSC	Saline sodium citrate
TKT	Transketolase

TOP2A	Topoisomerase (DNA) II alpha
TRIM37	Tripartite motif containing 37
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine
TSD	Temperature-dependent sex determination
UV	Ultraviolet
WAC	WW domain containing adaptor with coiled-coil
WDR43	WD Repeat Domain 43
WT1	Wilms Tumor 1
ZNF326	Zinc Finger Protein 326

SHORT ABSTRACTS

Abstract

Reptiles, with their great diversity of sex-determining systems, have long been regarded as a model group for studying the evolution of sex determination and sex chromosomes. They also hold a key phylogenetic position to elucidate the organization and evolution of amniote genomes. This PhD thesis aims to contribute to this understanding by investigating sex chromosomes and karyotype evolution in lacertid lizards, with a focus on rock lizard species (genus *Iberolacerta*) endemic of the Iberian Peninsula. Firstly, we applied classical and molecular cytogenetic methods to identify and characterize previously unknown ZW sex chromosomes in the species *I. monticola*. Secondly, we developed whole-chromosome paints from *I. monticola* to detect chromosomal rearragements and test the homology of sex chromosomes among closely related lacertid species. These results revealed a high degree of karyotype conservation, but a rapid and independent differentiation of sex chromosomes, and even a putative cryptic event of sex chromosome turnover. Finally, we explored the mode of evolution of two satellite DNA families shared by all eight *Iberolacerta* species. Both satellite DNAs showed complex and disparate evolutionary patterns, and a highly dynamic behaviour which may be correlated with chromosomal rearragements and karyotype diversification in this genus.

Resumen

Los reptiles, con su extraordinaria diversidad de sistemas de determinación del sexo, constituyen uno de los grupos más atractivos para el estudio de la evolución del genoma y los cromosomas sexuales en amniotas. Esta tesis pretende ampliar el conocimiento en esta área investigando la evolución del cariotipo y de los cromosomas sexuales en lacértidos, y principalmente en lagartijas del género *Iberolacerta* endémicas de la Península Ibérica. En primer lugar, se emplearon técnicas de citogenética clásica y molecular para caracterizar el par sexual ZW, previamente no identificado, en la especie *I. monticola*. En segundo lugar, se desarrollaron sondas cromosómicas en *I. monticola* para detectar reordenaciones cromosómicas y evaluar la homología de los cromosomas sexuales en otras especies de lacértidos. Estos resultados revelaron un alto grado de conservación de los cariotipos, pero también procesos independientes de diferenciación de los cromosomas sexuales, e incluso un posible evento de sustitución del par sexual. Por último, se analizó el modo de evolución de

dos familias de ADN satélite presentes en las ocho especies del género *Iberolacerta*. Ambas familias mostraron patrones evolutivos distintos y complejos, y un elevado dinamismo que podría estar asociado a la diversificación del cariotipo en este género.

Resumo

Os réptiles, coa súa extraordinaria diversidade de sistemas de determinación do sexo, constitúen un dos grupos máis atractivos para o estudo da evolución do xenoma e os cromosomas sexuais en amniotas. Esta tese pretende ampliar o coñecemento nesta área investigando a evolución do cariotipo e dos cromosomas sexuais en lacértidos, e principalmente en especies de lagartas do xénero *Iberolacerta* endémicas da Península Ibérica. En primeiro lugar, empregáronse técnicas de citoxenética clásica e molecular para caracterizar o par sexual ZW, previamente non identificado, na especie *I. monticola*. En segundo lugar, elaboráronse sondas cromosómicas en *I. monticola* para detectar reordenacións cromosómicas e avaliar a homoloxía dos cromosomas sexuais noutras especies de lacértidos. Estes resultados revelaron un alto grao de conservación dos cariotipos, pero tamén procesos independentes de diferenciación dos cromosomas sexuais, e mesmo un posible evento de substitución do par sexual. Finalmente, analizouse o modo de evolución de dúas familias de ADN satélite presentes nas oito especies do xénero *Iberolacerta*. Ámbalas dúas familias amosaron patróns evolutivos distintos e complexos, e un elevado dinamismo que podería estar asociado á diversificación do cariotipo neste xénero.

EXTENDED ABSTRACT

Extended abstract

Los reptiles (Sauropsida) son un clado de vertebrados amniotas que incluye tres grandes linajes: Lepidosauria, que comprende los órdenes Squamata (lagartos, serpientes y anfisbénidos) y Sphenodontia (tuátara); Archosauria, que engloba a cocodrilos y aves; y Testudines (tortugas), el grupo hermano de los arcosaurios. Debido a la posición clave que ocupan en la filogenia amniota, la caracterización de los genomas de reptiles es fundamental para comprender la organización y los patrones de evolución del genoma en vertebrados.

Desde el punto de vista cariológico, los reptiles son un grupo muy heterogéneo, con una gran diversidad en el número cromosómico, estructura del cariotipo y tasa de reordenaciones cromosómicas. El cariotipo típico de reptiles consta de hasta 10 pares de macrocromosomas y un número variable de microcromosomas, si bien algunos clados (crocodrilos, geckos y lacértidos) poseen un número reducido o incluso carecen de microcromosomas. En constraste con esta diversidad cariológica, trabajos recientes de genómica comparada, cartografiado genético y pintado cromosómico (*chromosome painting*) han revelado una extraordinaria conservación de la sintenía cromosómica en todos los linajes de saurópsidos, a pesar de que su divergencia se inició hace aproximadamente 275 millones de años.

Los cromosomas sexuales, en cambio, representan una conspicua excepción a este patrón. En general, los reptiles se caracterizan por poseer una variabilidad excepcional de mecanismos de determinación sexual, que incluye tanto sistemas de determinación dependientes de la temperatura como sistemas de determinación genética con cromosomas sexuales XY (machos heterogaméticos XY, hembras XX) y ZW (hembras heterogaméticas ZW, machos ZZ). Esta variabilidad no muestra una clara segregración filogenética, lo que sugiere que a lo largo de la historia evolutiva de este grupo ha habido múltiples transiciones entre distintos sistemas de determinación sexual. No obstante, los niveles de plasticidad en la determinación de sexo difieren considerablemente entre distintos grupos taxonómicos. Así, el dinamismo observado en los geckos o los lagartos agámidos contrasta notablemente con la elevada estabilidad de los cromosomas sexuales en aves o serpientes. La distribución filogenética de los mecanismos de determinación del sexo, con los datos actualmente disponibles, sugiere que algunos otros linajes podrían tener también sistemas de cromosomas sexuales conservados. Uno de estos linajes serían los lacértidos (familia Lacertidae), que aparentemente comparten un sistema ZW único. Sin embargo, la dificultad para identificar los cromosomas sexuales en algunas especies mediante técnicas citogenéticas convencionales, y la falta de estudios que evalúen la homología a nivel

molecular entre distintos sistemas de cromosomas sexuales, impiden reconstruir de forma inequívoca la historia evolutiva de la determinación del sexo tanto dentro como entre distintos grupos del reptiles.

Esta tesis pretende contribuir a esta reconstrucción realizando un análisis comparativo de los cromosomas sexuales en lacértidos y, principalmente, en el género *Iberolacerta*. Se ha examinado también homología cromosómica entre la especie *I. monticola* y otros grupos de reptiles, con el fin de trazar el proceso de reestructuración del cariotipo que condujo a la reducción en el número de microcromosomas en la familia Lacertidae. Por último, se investigó la plasticidad del genoma en las especies de *Iberolacerta* analizando la evolución y organización cromosómica de dos familias de ADN satélite, HindIII y TaqI. Por su particular modo de evolución, el ADN satélite representa uno de los componentes más dinámicos de los genomas eucariotas, pudiendo variar tanto en secuencia como en número de copias entre especies estrechamente relacionadas. Este elevado dinamismo puede estar asociado en ocasiones a reordenaciones cromosómicas, e incluso al aislamiento reproductivo y la especiación. Por ello, el estudio del ADN satélite puede ayudar a comprender no solo los factores que influyen en la evolución de este elemento repetitivo, sino también su posible papel en la diversificación del cariotipo dentro del género *Iberolacerta*.

El género *Iberolacerta* (Arribas, 1997) agrupa a todas las lagartijas serranas de la Península Ibérica y a una especie balcánica (*I. horvathi*). Atendiendo a las últimas revisiones taxonómicas, *Iberolacerta* consta de ocho especies, que pueden ser clasificadas en tres grupos geográficamente diferenciados: 1) el grupo ibérico, que engloba a todas las poblaciones de la Península Ibérica, excepto las de los Pirineos, e incluye las especies *I. cyreni*, *I. martinezricai*, *I. galani* e *I. monticola*; 2) el grupo pirenaico, considerado como subgénero *Pyrenesaura* (Arribas, 1998), en el que se incluyen *I. aranica*, *I. aurelioi* e *I. bonnali*; y 3) el grupo de Horvath, que agrupa las poblaciones de los Alpes y los Balcanes, situadas a más de 1000 kilómetros de la Península Ibérica, todas ellas pertenecientes a una misma especies, *I. horvathi*.

Los análisis citogenéticos publicados hasta la fecha describen como característica del género *Iberolacerta* la falta del típico par de microcromosomas, presente en todos los demás linajes de lacértidos. Así, el cariotipo de las especies de *Iberolacerta* cuenta con 2n=36 macrocromosomas acrocéntricos, a excepción de las del grupo pirenaico, que muestran frecuentes fusiones Robertsonianas que reducen su número de cromosomas hasta 23 o 24 (en hembras y machos, respectivamente, de *I. bonnali*). Con respeto al par sexual, la mayoría de las especies posee un sistema ZW, típico de la familia Lacertidae. Sin embargo, el grado de degeneración de cromosoma W parece variar considerablemente entre especies. *I. horvathi*, *I. cyreni* e *I. galani*

poseen un par ZW altamente heteromórfico, con un cromosoma W de menor tamaño que el Z y casi completamente heterocromático. Por el contrario, los cromosomas sexuales de *I. aranica*, *I. martinezricai* e *I. monticola* son aparentemente homomórficos e indistinguibles en base a diferencias en tamaño, morfología o grado de heterocromatinización. De nuevo, dos de las especies pirenaicas (*I. aurelioi* e *I. bonnali*) muestran una excepción a este patrón general, al compartir un sistema múltiple $Z_1Z_2W/Z_1Z_2Z_1Z_2$, donde el W es submetacéntrico y los homólogos Z_1 y Z_2 son acrocéntricos. Las diferencias en el grado de degeneración del cromosoma W se observan entre especies tan estrechamente relacionadas como *I.* galani, *I. monticola* e *I. martinezricai*, lo que sugiere que la diferenciación de cromosomas sexuales se ha producido de forma rápida e independiente en los diferentes *taxa*. Por ello, el género *Iberolacerta* constituye un excelente sistema para examinar en profundidad los procesos involucrados en el origen y diferenciación de los cromosomas sexuales.

- Con este propósito, en el Capítulo I se realizó un análisis citogenético,—aplicando técnicas de tinción convencionales y diferenciales, hibridación *in situ* fluorescente (FISH) e hibridación genómica comparada (CGH),— para caracterizar de forma detallada el cariotipo, y potencialmente detectar los cromosomas sexuales, en la especie *I. monticola*. Los resultados confirmaron el cariotipo previamente descrito para esta especie, compuesto por 2n=36 macrocromosomas acrocéntricos de tamaños gradualemte decrecientes. La tinción con plata (Ag-NOR) y la hibridación con una sonda de los genes ribosomales mayores (18S-5.8S-28S) localizaron las regiones organizadoras nucleolares (NORs) en la región subtelomérica del par cromosómico 6. La hibridación con la sonda telomérica de vertebrados $(TTAGGG)_n$ produjo señales claras en ambos telómeros de cada cromosoma, y también señales intersticiales en cinco pares cromosómicos. Estas señales intersticiales podrían ser "cicatrices" evolutivas; es decir, vestigios de reordenaciones cromosómicas (inversiones y fusiones) que se han producido durante la evolución del cariotipo. El bandeo C, seguido de la tinción diferencial con DAPI y CMA_3, mostró la presencia de bloques de heterocromatina constitutiva uniformemente teñidos por ambos fluorocromos en los centrómeros de todos los cromosomas, así como en regiones intersticiales de 10 pares cromosómicos largos. Se detectaron también bandas C tenues, CMA_3-positivas (y, por tanto, ricas en GC), en posición telomérica, en los 12 pares cromosómicos de mayor tamaño. Este último es un carácter compartido con *I. galani*, pero no con *I. martinezricai*. De hecho, en general, los patrones de bandas C descritos hasta la fecha en *Iberolacerta* muestran una gran heterogeneidad en la cantidad, distribución y composición de la fracción heterocromática en los distintos *taxa*. Si bien estos patrones pueden ser útiles para identificar

caracteres diagnóstico de especie, no reflejan necesariamente las afinidades filogenéticas entre especies.

Finalmente, a diferencia de trabajos anteriores, los resultados del bandeo C y de la CGH revelaron la existencia de un par sexual ZW, que fue detectado en todas las hembras de las cuatro poblaciones de *I. monticola* analizadas. Este par ZW presenta características citológicas similares a las de los cromosomas sexuales ya descritos en *I. horvathi*, *I. cyreni* e *I. galani*. El cromosoma W es uno de los más pequeños del cariotipo y completamente heterocromático, con excepción de una pequeña región de eucromatina intersticial. El cromosoma Z tiene un tamaño similar al de los pares 9 o 10, y puede ser diferenciado de los autosomas por poseer una banda C telomérica más aparente tras la tinción con CMA_3. Los resultados de la CGH confirmaron que los cromosomas Z y W están altamente diferenciados a nivel molecular, como resultado de la acumulación diferencial de secuencias repetitivas en la región distal del cromosoma W.

- En el Capítulo II se desarrollaron sondas cromosómicas de *I. monticola*, obtenidas mediante separación de los cromosomas por citometría de flujo. Una vez determinada la correspondencia de cada sonda con los cromosomas de *I. monticola*, aquella que contenía el cromosoma W fue utilizada en experimentos de *chromosome painting* sobre preparaciones metafásicas de otras especies de lacértidos, para evaluar la homología entre sus cromosomas sexuales. En particular, se seleccionaron cuatro especies con diferentes sistemas ZW: *I. galani e I. bonnali*, ya descritas anteriormente; *Timon lepidus,* con un micro-cromosoma W; y *Lacerta schreiberi*, con un par sexual aparentemente homomórfico e indistinguible citológicamente. La sonda W de *I. monticola* produjo una clara señal de hibridación en la región eucromática del cromosoma W de *I. galani*, y en el brazo largo del cromosoma W submetacéntrico de *I. bonnali*, demostrando que los cromosomas sexuales de las tres especies derivan de un mismo par ancestral. Además, fue posible determinar que el brazo corto del W de *I. bonnali* es homólogo al cromosoma 15 o 16 de *I. monticola*. Este resultado indica que la formación del neo-W de *I. bonnali* es el resultado de una fusión céntrica del cromosoma W original con uno de los autosomas de menor tamaño del cariotipo ancestral. Al contrario, no se detectó ninguna señal de hibridación de la sonda W sobre los cromosomas sexuales de *T. lepidus* y *L. schreiberi* (cuyo par ZW fue descubierto también en este trabajo). Experimentos recíprocos de CGH entre *I. monticola*, *T. lepidus* y *L. schreiberi* confirmaron que los cromosomas W de las tres especies están sumamente diferenciados entre sí, y probablemente evolucionaron de forma independiente mediante la rápida acumulación de secuencias repetitivas características de cada linaje. Para comprobar la homología del par ZW entre estos tres *taxa*, se realizaron pruebas adicionales de *chromosome painting* con la sonda que contiene el cromosoma Z de *I. monticola*. Esta sonda identificó el cromosoma Z de *T. lepidus*,

pero no el de *L. schreiberi* que, en cambio, fue detectado con la sonda que incluye los pares 15 y 16 de *I. monticola*. Si bien estos datos son preliminares, — puesto que en este estudio se analizaron únicamente hembras de cada especie, — la existencia de un par ZW en *L. schreiberi* distinto al de *I. monticola* y *T. lepidus* sugiere que los cromosomas sexuales en la familia Lacertidae han evolucionado de forma independiente, a partir de distintos autosomas, al menos en dos ocasiones. La sustitución de los cromosomas ZW originales parece ser un carácter derivado en *L. schreiberi*, y podría haber ocurrido, por ejemplo, tras la transposición o translocación del gen determinante del sexo a un autosoma, que ahora daría lugar al nuevo par ZW.

La hibridación de todas las demás sondas cromosómicas de *I. monticola* sobre metafases de *T. lepidus* y *L. schreiberi* mostró un alto grado de conservación evolutiva y pocas variaciones estructurales entre los cariotipos de las tres especies. Las principales reordenaciones cromosómicas observadas incluyen: 1) la formación de un par metacéntrico en *T. lepidus*, tras una fusión Robertsoniana de dos elementos acrocéntricos homólogos a los cromosomas 2 y 4 de *I. monticola*, y 2) la ausencia del par de microcromosomas en *I. monticola* (y en todas las demás *Iberolacerta*), resultado de su translocación al par cromosómico 11 o 12.

Finalmente, las fracciones cromosómicas de *I. monticola* fueron utilizadas también en un experimento de cartografiado genético mediante PCR, para deducir la homología de estos cromosomas con los de otras especies de reptiles filogenéticamente más distantes. En concreto, se hizo uso de la información disponible del genoma de *Anolis carolinensis* (Iguania, Squamata) para seleccionar al menos un marcador localizado en cada uno de los cromosomas de esta especie. El número cromosómico de *A. carolinensis* es idéntico al de *I. monticola* (2n=36) pero su cariotipo consta de 6 pares de macrocromosomas y 12 pares de microcromosomas y, por tanto, difiere sustancialmente del cariotipo de los lacértidos. Para el cartografiado de los marcadores seleccionados, se diseñaron cebadores degenerados a partir de las secuencias de *A. carolinensis* y de otros reptiles existentes en las bases de datos. Las parejas de cebadores se utilizaron en reacciones de PCR sobre cada una de las fracciones cromosómicas de *I. monticola*. La localización de un marcador en una fracción determinada se confirmó secuenciando el producto amplificado en la PCR. Pese a las limitaciones experimentales que presentó esta estrategia, la comparación de los resultados obtenidos con los mapas citogenéticos publicados para otras especies de reptiles arrojó algunas observaciones interesantes. Por ejemplo, el cromosoma 1 de *I. monticola* es al menos parcialmente sinténico con los cromosomas 3, 5 y 7 del gallo (*Gallus gallus*), un rasgo que parece estar conservado en la mayoría de los linajes de Squamata. Los resultados de la cartografía cromosómica también apoyan la hipótesis de que los

cromosomas sexuales de lacértidos y *A. carilinensis* no son homólogos, y sugieren que la reducción en el número de microcromosomas en Lacertidae es el resultado de múltiples fusiones entre los microcromosomas presentes en el cariotipo ancestral de los saurópsidos.

- En el Capítulo III, se estudió la dinámica evolutiva de dos familias de ADN satélite previamente identificadas en *Iberolacerta*. La primera de ellas, HindIII, forma parte de la heterocromatina constitutiva centromérica, y parece ser exclusiva de este género. La segunda, TaqI, se localiza en las bloques de heterocromatina intersticiales y muestra una distribución taxonómica más amplia, habiéndose aislado también en otras especies de lacértidos (géneros *Lacerta*, *Podarcis* y *Timon*). En este Capítulo, se realizó un análisis detallado de la variabilidad intraespecífica, la organización genómica y la localización cromosómica de ambas familias en todas las especies de *Iberolacerta*, con el objetivo último de dilucidar los patrones de variación y los factores que determinan su modo de evolución, así como la posible implicación de estos elementos repetitivos en la evolución del cariotipo.

El análisis de las secuencias aisladas para cada familia permitió identificar una serie de posiciones nucleotídicas diagnósticas, que definieron tres grandes grupos de monómeros o subfamilias, en el caso del satélite HindIII, y dos subfamilias, en el satélite TaqI. Estas subfamilias constituyen unidades evolutivas independientes y se distribuyen de forma diferencial en los distintos *taxa*, dando lugar a perfiles característicos en cada especie, que no muestran una clara correspondencia con la filogenia del género. En general, aquellas especies con un perfil de ADN satélite más diverso (es decir, con un mayor número de variantes HindIII o TaqI), mostraron bajas tasas de homogeneización y de evolución concertada de las secuencias, conservando en gran medida la variabilidad nucleotídica ancestral. Al contrario, aquellas especies con una subfamilia HindIII o TaqI predominante mostraron mayores tasas de homogeneización, y un patrón general de evolución concertada, que se traduce en menores niveles de variabilidad intraespecífica que de divergencia interespecífica. Las diferencias en las tasas de homogeneización entre especies pueden estar relacionadas con diversos factores que influyen sobre la actividad de los mecanismos moleculares de intercambio no recíproco, como la arquitectura del cariotipo (cromosómicas acrocéntricos *vs*. meta o submetacéntricos) o la organización intercalada de distintas subfamilias en un mismo tándem de ADN satélite. En conclusión, el patrón evolutivo de las familias HindIII y TaqI se ajusta al modelo de la biblioteca (*library hypothesis*), según el cual especies relacionadas comparten una colección de variantes monoméricas o subfamilias de ADN satélite, presentes ya en el genoma de la especie ancestral. Los perfiles de ADN satélite específicos de especie están definidos en este caso por variaciones en el número de copias de las distintas subfamilias, debido a su amplificación diferencial a partir

de esa biblioteca común. Además, como muestran los resultados de nuestro análisis, la amplificación preferencial de una determinada variante en una especie puede incrementar la eficacia de los mecanismos moleculares de homogeneización, acelerando la tasa de evolución de las secuencias de ADN satélite en esa especie en concreto. Por otra parte, las fluctuaciones en el número de copias pueden alterar rápidamente la abundancia de un ADN satélite en el genoma. Por ejemplo, los resultados de las hibridaciones *in situ* con la sonda HindIII muestran que, si bien este ADN satélite es el componente mayoritario de la heterocromatina centrómerica en *I. monticola* e *I. galani*, su abundancia se ha reducido de forma drástica en *I. horvathi* e *I. bonnali*. En esta última, y quizás también en las otras especies pirenaicas, la rápida dinámica del ADN satélite centromérico puede estar correlacionada con la alta tasa de reordenaciones cromosómicas características de este linaje.

Las principales conclusiones de esta tesis pueden resumirse en los siguientes puntos:

- El análisis mediante bandeo C e hibridación genómica comparada del cariotipo de *I. monticola* demostró la existencia de un par sexual ZW heteromórfico, previamente no identificado. La heterocromatinización del cromosoma W es en gran medida el resultado de la acumulación masiva de secuencias repetitivas específicas de este cromosoma. Este resultado sugiere que la presencia de un par ZW es la condición ancestral para el género *Iberolacerta*. Cabe esperar, por tanto, que las especies *I. martinezricai* e *I. aranica* posean también cromosomas sexuales diferenciados que, como en el caso de *I. monticola,* podrían ser detectados tras un análisis citogenético detallado y la aplicación de técnicas citogenéticas de alta resolución.

- Los experimentos de *chromosome painting* con sondas de *I. monticola* demostraron la homología de los cromosomas sexuales en el género *Iberolacerta* y en la especie *T. lepidus*, pero no en *L. schreiberi*. Es posible que, pese al alto grado de conservación de sus cariotipos, los cromosomas sexuales hayan evolucionado de forma independiente en distintos linajes de lacértidos. Asimismo, los cromosomas sexuales de *I. monticola* parecen no ser homólogos con los cromosomas sexuales de *A. carolinensis*. Los resultados del cartografiado cromosómico sugieren también que la ausencia de microcromosomas en lacértidos es el resultado de múltiples fusiones entre microcromosomas existentes en el cariotipo ancestral de los reptiles.

- El análisis de dos familias de ADN satélite en *Iberolacerta* reveló la compleja dinámica evolutiva de estos elementos repetitivos, que en general difiere del patrón esperado de evolución concertada. Pese a tener historias evolutivas dispares, los ADNs satélite HindIII y TaqI muestran ciertos rasgos comunes: (i) cada familia está constituida por una "biblioteca" de variantes monoméricas o subfamilias, presentes ya en el ancestro común de *Iberolacerta*; (ii) los perfiles de ADN satélite específicos de especie están prinicpalemente definidos por la amplificación diferencial de determinadas variantes a partir de la biblioteca común; (iii) la tasa de evolución de las secuencias de ADN satélite difiere incluso estre especies estrechamente relacionadas, resultando en niveles variables de homogeneidad intraespecífica y divergencia interespecífica. Las fluctuaciones en el número de copias de las distintas variantes monoméricas pueden provocar la amplificación preferencial de una determinada variante en una especie, o una reducción drástica en la abundancia global del ADN satélite en el genoma de otras especies. Como resultado de este complejo modo de evolución, los satélites HindIII y TaqI no son marcadores filogenéticos informativos para el género *Iberolacerta*.

INTRODUCTION

Introduction

1. Reptilian karyotype evolution

1.1 *Chromosome numbers and karyotypic structure*

Extant amniotes are represented by two major lineages: Synapsida (mammals) and Sauropsida (reptiles) which diverged from a common ancestor around 320 million years ago (mya) (Shedlock and Edwarsd 2009). Sauropsida, with approximately 18,000 extant species, comprises three main clades which separated ~275 mya (Shedlock and Edwards 2009): Lepidosauria, containing squamates (amphisbaenians, lizards and snakes) and the tuatara; Archosauria, including crocodiles and birds; and turtles, the sister group of archosaurs (Chiari et al. 2012; Crawford et al. 2012) (Fig. 1). Because of their pivotal phylogenetic position, characterization of reptilian genomes is fundamental for understanding the structural changes and patterns of genome evolution in amniotes.

Reptiles are a karyologically heterogeneous group exhibiting high diversity in chromosome numbers, morphologies and rates of chromosome changes (Olmo 2008). Karyotypes of birds and most non-avian reptiles (referred to as reptiles hereafter) consist of up to ten pairs of macrochromosomes and a varying number of microchromosomes (reviewed in Deakin and Ezaz 2014). Birds have a particularly large number of microchromosomes, which are relatively gene-rich compared to macrochromosomes (Burt 2002). In contrast to mammals, birds exhibit a slow rate of change in chromosome number and interchromosomal rearragements (Griffin et al. 2007; Ellegren 2010), most species having diploid chromosome numbers of 2n=76-84 (Rodionov 1997) consisting of 14-16 macrochromosomes and 60-64 microchromosomes (Fig. 1).

Among reptiles, crocodilians and turtles have the most conserved karyotypes (Cohen and Gans 1970; Olmo and Signorino 2005; Olmo 2008). Most turtles have bimodal complements, i.e., constituted of macrochomosomes and microchromosomes, and show low variability in chromosome morphology and G-banding patterns (Olmo 2008 and references therein). Chromosome numbers range from 2n=22 to 2n=66, but the most common diploid number is 2n=52, comprising 28 macrochromosomes and 24 microchromosomes (Graves and Shetty 2000) (Fig. 1). Variations in the diploid number often involve changes in the number of microchromosomes (Valenzuela and Adams 2011). The karyotypes of crocodiles are characterized by the lack of microchromosomes and reduced diploid numbers (2n=30-42)

derived from a single basic model with 2n=32 (Graves and Shetty 2000). As in turtles, G-banding patterns of macrochomosomes are highly conservative in this order (Olmo 2008).

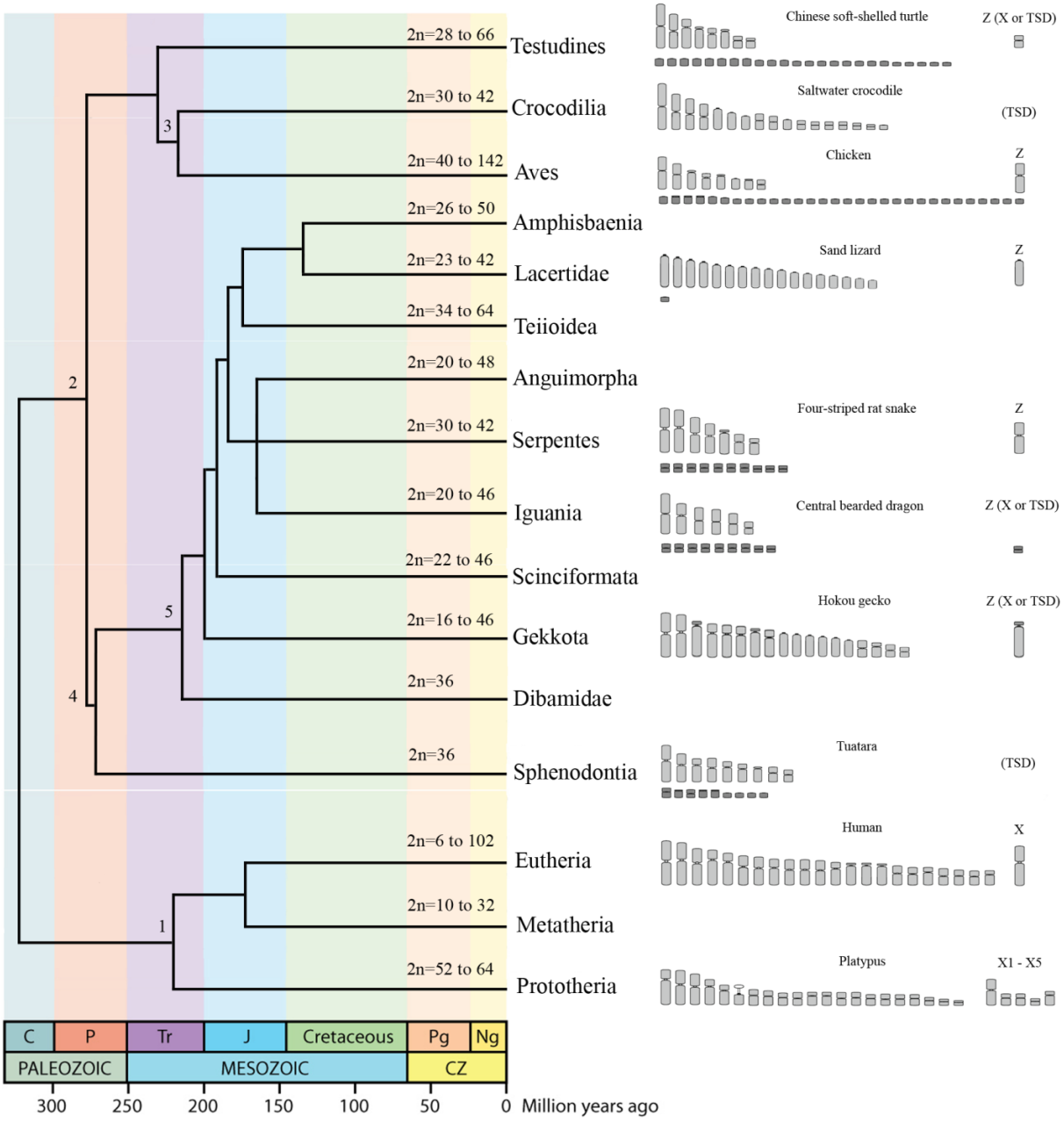


Figure 1. Amniote phylogeny showing haploid karyotypes for representative species (figure modified from Deakin and Ezaz 2014). The range of diplod chromosomes numbers for each lineage are indicated on the branches Microchromosomes are indicated in dark grey. The sex chromosomes present in the homogametic sex are shown for representative species and alternatives present in each lineage are indicated. TSD: temperature sex determination. 1: Mammalia; 2: Sauropsida; 3: Archosauria; 4: Lepidosauria; 5: Squamta. Data are from: Shetty and Graves (2000); Olmo and Signorino (2005); Valenzuela and Adams (2011); Deakin and Ezaz (2014). Amniote phylogeny and divergence time estimates follow Shedlock and Edwards (2009).

In contrast, squamate reptiles have a high level of karyotypic variability, both in chromosome numbers (2n=24-50) and morphologies, and a higher rate of change in chromosomes clearly related to the number of living species. Karyotypes with few or no microchromosomes are found in the lacertid lizards and in geckos, whereas karyotypes containing many microchromosomes are found in the remaining group of squamate reptiles (Deakin and Ezaz 2014) (Fig. 1). Snakes have relatively conserved chromosome numbers, which typically include eight pairs of macrochromosomes and ten pairs of microchromosomes (2n=36). However, karyotypes have undergone frequent rearrangements including fission, fusion and repeat accumulation (Mengden and Stock 1980; O'Meally et al. 2010). Larger variation in chromosome number is found in amphisbaenians (2n= 26-50) and lizards (2n=16-64) (Olmo and Signorino 2005), although a common karyotype consists of 2n=36, including 12 macrochromosomes and 24 microchromosomes. Especially in lizards—the group with the highest karyotypic variability among reptiles—chromosomal variation seems to have played an important role in the evolution of several taxa (Olmo 2008).

1.2 *Conservation of chromosomal synteny*

The availability of amniote genome assemblies (http://www.ncbi.nih.gov/genome/browse/), allows comparisons of the organization and function of amniote genomes across vast evolutionary distances. These comparisons have provided significant insight into the mechanisms of early genome evolution and subsequent lineage-specific evolution in all amniotes. For instance, a comparative analysis between the chicken (*Gallus gallus*) and the green anole lizard (*Anolis carolinensis*, Iguania)—the first reptilian genome sequenced (Alföldi et al. 2011)—revealed relatively high conservation of chromosomal synteny and few chromosomal rearrangements in the 280 million years since anole and chicken diverged. Indeed, 19 out of 22 anchored chicken chromosomes are each syntenic to a single *A. carolinensis* chromosome over their entire length, and all sequence anchored to microchromomes in *A. carolinensis* also aligns to microchromosomes in the chicken (Alföldi et al. 2011).

Althoug whole genomes are available or in progress for an increasing list of reptilian species [(the green anole lizards, *Anolis carolinensis*; the Chinese softshell turtle *Pelodiscus sinensis;* the green sea turtle *Chelonia mydas;* the western painted turtle *Chrysemys picta;* the Burmese python *Python molurus;* the king cobra *Ophiophagus hannah*; the Chinese alligator

Alligator sinensis; the American alligator *Alligator mississippiensis,* the gharial *Gavialis gangeticus;* the saltwater crocodile *Crocodylus porosus*, the Burmese python *Python molurus*, the King cobra *Ophiophagus hannah,* the speckled rattlesnake *Crotalus mitchellii*, and the corn snake *Pantherophis guttatus* (Tzika et al. 2015 and references therein)], none of these genomes, except that of the green anole, have been anchored to chromosomes.

So, to date, most information about genome evolution and chromosomal reorganization in reptiles comes from molecular cytogenetic analyses. Even if limited, these data are disclosing an unprecedented level of conservation of sauropsid genomes, which is in sharp contrast with the extraordinary high rates of interchromosomal rearrangements in mammals (Ferguson-Smith and Trifonov 2007). Recent comparative gene mapping of several reptile species (*Pelodiscus sinensis*, Testudines; *Crocodylus siamensis*, Crocodilia; *Lacerta agilis*, *Elaphe quadrivirgata*, *Varanus salvator macromaculatus*, *Leiolepis reevesii rubritaeniata*, *Pogona vitticeps*, and *Anolis carolinensis*, Squamata) with the chicken revealed extensive linkage homology between avian and reptilian chromosomes, despite the substantial diversification of reptilian karyotypes (Matsuda et al. 2005; Matsubara et al. 2006, 2012; Srikulnath et al. 2009, 2013, 2014; Alföldi et al. 2011; Uno et al. 2012; Young et al. 2013). From these results, it has been hypothesized that the ancestral amniote karyotype had at least 10 large linkage groups and many microchromosomes, which correspond to the chicken macro- and microchromosomes, respectively (Uno et al. 2012). Therefore, the karyotypes of lacertid lizards and geckos, with few or no microchromosomes, probably have resulted from repeated fusions of microchromosomes, which may have occurred independently in each lineage (Srikulnath et al. 2014).

Cross-species chromosome painting is also a powerful tool for genome-wide comparison of the chromosome constitution of different species. Yet, only a limited number of studies based on chromosome painting have been performed on reptiles. Among them, painting with chicken probes revealed a strong conservation of the chromosomes syntenic with the avian Z sex chromosome, as well as a conserved association of the avian ancestral chromosomes 3, 5 and 7, across most major squamate lineages (Pokorná et al. 2011, 2012). At a finer scale, chromosome painting demonstrated highly conserved karyotypes, but also species-specific rearragements, in skinks (Scincidae) (Giovannotti et al. 2009), and in Gekkotan lizards (Trifonov et al. 2011; Johnson Pokorná et al. 2015). Comparative analyses from additional reptile groups would be necessary to produce a robust reconstruction of ancestral karyotypes and to detect cytogenetic synapomorphies of particular lineages.

2. The evolution of sex-determining mechanisms in reptiles

Sex determination is the regulatory process that directs differentiation of the gonads in the early embryo to form either testes or ovaries. A variety of mechanisms of sex determination exist in vertebrates, and these can be divided in two basic systems: genotypic sex determination (GSD), in which the sex of an individual is determined by sex chromosomes, i.e., by sex-specific differences in genotype; and environmental sex determination (ESD), where sex chromosomes are absent and sex is determined by nongenic factors experienced within a discrete period after conception (Bull 1983; Valenzuela et al. 2003). In vertebrates, the most common form of ESD is temperature-dependent sex determination (TSD), in which the incubation temperature experienced during embryonic development is the critical environmental factor that determinines sex (Bull 1983; Janzen and Paukstis 1991).

Within amniotes, GSD is universal in mammals and birds (Bull 1983). Most therian mammals have male heterogamety, i.e., sex is determined by an XX female:XY male sex chromosome system in which the Y chromosome harbors the male-dominant testis-determining gene *SRY* (Sinclair et al. 1990; Koopman et al. 1991). Birds also have a stable chromosome system, but in this case female is the heterogametic sex (ZZ male:ZW female), and male development is determined by the double dosage of a Z-borne gene, *DMRT1* (Smith et al. 2009). In contrast with this stability, reptiles exhibit an extraordinary array of sex-determining modes, comparable to the variety observed in fish and frogs (for a review see Graves 2008). All crocodilians, the tuatara, most turtles and some lizards have TSD systems (e.g., Janzen and Krenz 2004; Valenzuela and Lance 2004). Among those groups with GSD, all snakes have female heterogamety (ZW, ZZW or ZWW) (Becak and Beçak 1969; Solari 1993), whereas both XY- and ZW-type sex chromosomes have been reported in lizards and turtles (King 1977; Solari 1993; Olmo and Signorino 2005). Although reptilian orthologs of mammalian sex-determining genes have been identified (reviewed in Rhen and Schroeder 2010), the master sex-determining gene and the specific molecular mechanism (dominance or dosage) of sex determination in reptiles remain unknown.

GSD and TSD have been traditionally considered as two alternative states, two dichotomous sex-determining systems that differ basicaly in the presence or absence of sex-specific genotypes (e.g., Bull 1983; Pokorná and Kratochvíl 2009). However, the finding that certain reptiles with GSD also respond to temperature (Shine et al. 2002; Valenzuela and Lance 2004; Quinn et al. 2007; Radder et al. 2008) have led to propose that there is no sharp

boundary between the two main modes of sex determination, and they may be rather viewed as two ends of a continuum of sex-determining mechanisms (Shine et al. 2002; Sarre et al. 2004). This continuum may be explained by the existence of a genotypic system sensitive to temperature, where sex is determined by gene-environmental interactions (Valenzuela et al. 2003). For instance, the dragon lizard, *Pogona vitticeps*, has a cryptic ZW genetic mode of sex determination (Ezaz et al. 2005) that is overridden by temperature at higher extremes (Quinn et al. 2007). Since there have been few attempts to examine the occurrence of gene-environment interactions in sex determination of reptiles, it is possible that cases such as that of *P. vitticeps* represent a much wider phenomenon, which might be connected with the evolutionary lability of sex-determining systems in this group (Sarre et al. 2011).

2.1 *The evolution of sex chromosomes*

Sex chromosomes and their evolution have attracted researchers' attention since their discovery in the late 1800s (Henking 1981; Stevens 1905; Wilson 1905). Sex chromosomes have evolved independently many times in animals and plants (Ohno 1967; Bull 1983; Graves and Peichel 2010); nevertheless, they are shaped by similar selective forces and share many common features. In mammals and most birds, the the two types of sex chromosome homologs (X and Y, or Z and W) are often morphologically distinguishable (heteromorphic), with the Y or W being largely heterochromatic, filled with repetitive DNA, and containing only a limited number of genes (Bull 1983). Existing theories of the early stages of sex chromosome evolution show how sex chromosomes first become non-recombining, and how this can lead to genetic degeneration of the heterogametic sex chromosome (Y or W) (reviewed in Charlesworth et al. 2005).

It is widely accepted that sex chromosomes evolved from an autosomal pair when one of the homologs acquired a sex-determining gene (Muller 1914; Ohno 1967; Charlesworth 1991). This can occur, for example, by mutation, duplication or translocation of a gene—either involved in the gonad differentiating pathway or not—which takes over the primary sex-determining function, converting the autosome into a nascent sex chromosome. For instance, the Y-linked *DMY* (*dmrt1bY)* in the medaka fish *Oryzias latipes* (Matsuda et al. 2002; Nanda et al. 2002), and the W-linked *DM-W* in the African clawed frog (*Xenopus laevis*) (Yoshimoto et al. 2008) evolved through duplication and neofunctionalization of *DMRT1*, a conserved regulator of gonadogenesis in all vertebrates studied and necessary for male sex determination

in the chicken (Smith et al. 2009). After acquisition of a sex-determining gene, sexually antagonistic selection (i.e., opposing selection pressures on the two sexes) leads to the accumulation of alleles beneficial for one sex but detrimental to the other in the vicinity of the sex-determining locus (Rice 1987). Once such genes have accumulated, there is a selective advantage to suppressing recombination between them and the sex-determining regions of the proto-sex chromosome. A gradual reduction of crossover frequencies, due to the spread of genetic modifiers of recombination rates, or chromosome rearrangements such as inversions (which can also cause heteromorphism of the sex chromosome pair), may favor cessation of recombination between the evolving sex chromosomes (Charlesworth et al. 2005; Bergero and Charlesworth 2009). Further accumulation of sexually-antagonistic genes, or genes simply adavantageous to the heterogametic sex, followed by further suppression of recombination, progressively extends the non-recombining region of the Y or W chromosome, and the adaptation of these chromosome to a sex-specific function. The fraction of the Y or W chromosome outside this region that continue to recombine with the X or Z homolog, is a pseudoautosomal region. Suppression of recombination can evolve in multiple steps along the proto-sex chromosomes, leaving evolutionary strata, i.e., regions along the sex chromosomes that lost recombination at distinct time points, as has happened in mammals (Lahn and Page 1999), birds (Handley et al. 2004), snakes (Vicoso et al. 2013a) or plants (Nicolas et al. 2004; Wang et al. 2012).

Cessation of recombination between large parts or all of the sex chromosomes is the ultimate cause of the degeneration of Y or W chromosomes. Lack of recombination among genes carried on Y or W chromosomes reduces the ability of selection to fix favorable mutations and to prevent the fixation of deleterious ones—due to Hill-Robertson interference processes such as Muller's ratchet, background selection and the hitch-hiking of deleterious alleles by favorable mutations—which eventually leads to pseudogenization and gene loss (reviewed in Charlesworth and Charlesworth 2000). Moreover, repetitive sequences (e.g. microsatellites, satellite DNA, rDNA sequences) and transposable elements are predicted to accumulate rapidly—even before genes start to degenerate— in the Y or W chromosomes after recombination stops (Charlesworth et al. 1994; Steinemann and Steinemann 2005), enabling the formation of heterochromatin (Zhou et al. 2013). This may be not only a result of suppression of recombination, but also its cause, further promoting the degeneration of the heterogametic sex chromosome. The loss of genetic material and the accumulation of repetitve sequences and heterochromatin on the Y or W chromosomes often leads to dramatic

differences in size and appearance between the two homologs, which can be obvious as cytological heteromorphism. This process of repeat accumulation is rapid and stochastic, and may generate varying degrees of sex chromosome differentiation (from homomorphy to extreme differentiation), as often observed between the sex chromosomes of closely related species (White 1973).

The genetic erosion of the heterogametic sex chromosomes results in an unequal number of functional copies of many genes between the sexes. The need to equalize the dosage of the expression of genes between the sexes and between the sex chromosomes and the autosomes can drive selection for long-term retention of homologous X-Y gene pairs in non-recombining regions of the sex chromosomes (e.g., Bellott et al. 2014), or to the evolution of dosage compensation, a regulatory mechanism that balances gene expression of sex-linked and autosomal genes in the heterogametic sex (Ohno 1967). Several, but not all, species with differentiated XY systems have evolved different strategies for global or complete sex chromosome compensation (see Mank 2013). On the other hand, all species investigated to date with ZW systems [including birds (Ellegren et al. 2007; Itoh et al. 2007), lepidopterans (Harrison et al. 2012) and snakes (Vicoso et al. 2013a)] lack a chromosome-wide dosage compensation mechanim and, instead, only some dosage-sensitive genes in the Z chromosome appear to be upreguated in females in a gene-specific manner (Mank et al. 2011).

2.1.1 *Long-term preservation of homomorphic sex chromosomes*

Most of our knowledge on the evolution of sex chromosomes comes from a few well-studied model organisms, especially mammals and *Drosophila melanogaster*, with evolutionary old and highly differentiated XY chromosomes (Bachtrog et al. 2014). However, some other groups, such as certain lineages of birds or snakes, possess homomorphic sex chromosomes (i.e., undifferentiated at the cytological level) that appear to have been maintained over relatively long periods of evolutionary time (e.g., Matsubara et al. 2006; Mank and Ellegren 2007; Vicoso et al. 2013a, b). For example, avian ZW sex chromosomes—which formed about 120 mya, and are thus similar in age to the mammalian sex chromosomes (about 165 mya)—are homomorphic in ratites (ostriches and their kin), but highly differentiated in neognathous birds (including the chicken) (see Vicoso et al. 2013b and references therein). Similarly, the old ZW sex chromosome pair of snakes is homomorphic in boids and phytons, moderately differentiated in colubrids and completely heteromorphic in elapids and vipers (see Vicoso et

al. 2013a). Homomorphic sex chromosomes in these clades resemble the ancestral autosomes and contain fewer repetitive sequences and more functional genes than their heteromorphic orthologs in related species (Matsubara et al. 2006; O'Meally et al. 2010).

These observations suggest that suppressed recombination can remain limited over long evolutionary times, and that degeneration is not necessarily the ultimate fate of sex chromosomes. Several hypothesis have been put forward to explain the variable rates of degeneration of the heterogametic sex chromosome (for review, see Bachtrog et al. 2014). For instance, the differentiation of sex chromosomes might be constrained by the absence of a dosage compensation mechanism, as recently proposed for ostriches (Adolfsson and Ellegren 2013). It is also possible that the rate of sex chromosome degeneration is be directly influenced by the strength of sexually-antagonistic selection. Under this premise, species with few genes under sexually antagonistic selection on their nascent sex chromosomes could evade the selective pressure for reduced recombination, which would slow down the progress of sex chromosome differentiation. Alternatively, sexually-antagonistic selection may be resolved by mechanisms other than suppressed recombination. In particular, the evolution of sex-specific gene expression could eliminate the deleterious effects of sexually-antagonistic alleles in the sex to which they are harmful, as recently suggested for the emu (Vicoso et al. 2013b)

2.2 *Sex chromosomes in reptiles*

Reptiles with GSD possess remarkable variability in sex chromosomes systems. These include simple male and female heterogamety, but also multiple sex chromosome systems, such as $X_1X_1X_2X_2$ females:X_1X_2Y males, $Z_1Z_2Z_2$ males:Z_1Z_2W females and ZZ males:ZW_1W_2 females (Olmo et al. 1986; Solari 1993; Olmo and Signorino 2005). Chromosomal sex determination is present in birds, snakes, most lizards and a few turtles. However, this variability of sex chromosomes is not equally distributed across the major reptilian lineages (see Fig. 2, page 15).

Female heterogamety is present in all bird species so far analyzed (Ezaz et al. 2006a). The bird Z is extremely conserved: it represents either the fourth or fifth largest chromosome pair (Suzuki et al. 1930; Ohno et al. 1964; Solari et al. 1993), and homology and gene content is conserved even between the most distantly-related species (e.g., Shetty et al. 1999; Nanda et al. 2008). The W chromosome, on the contrary, varies from virtualy homomorphic to highly

heteromorphic in different bird lineages (see section 2.2.1). In each lineage, the W chromosome shows homology to the Z, but is degraded to different extents (reviewed in Mank and Ellegren 2007; Stiglec et al. 2007). Snakes also have a conserved ZW system. Chromosome 4 is the Z chromosome in all snakes studied to date (Beçak et al. 1964; Ohno 1967; Solari 1993), and its gene content is conserved between distantly related species (Matsubara et al. 2006; Vicoso et al. 2013a). As mentioned before (section 2.2.1), snakes, like birds, exhibit substantial variation in the degree of W chromosome degeneration among taxa (Ohno 1967; Beçak and Beçak 1969; Matsubara et al. 2006; O'Meally et al. 2010). However, whole-genome analysis of a colubrid snake has shown that a low degree of sex chromosome heteromorphism—as inferred cytologically—may conceal the true extent of divergence between the Z and the W at the DNA sequence level (Vicoso et al. 2013a).

On the other hand, turtles and lizards exhibit considerable variation in their sex chromosomes, with both XY and ZW sex chromosomes systems (Fig. 2). Differentiated sex chromosomes have been identified for only nine out of the approximately 18 turtle species known to possess GSD, with male heterogamety in six species and female reported for three species (see Kawagoshi et al. 2014 and references therein). Most of these species have large or middle-sized sex macrochromosomes, but recent research revealed the occurence of both XY and ZW sex microchromosomes in at least three species (Ezaz et al. 2006b; Kawai et al. 2007; Badenhorst et al. 2013). Phylogenetic reconstruction indicates that TSD is the ancestral state in turtles, while GSD arose multiple times independently (Janzen and Krenz 2004). The haphazard distribution of sex determining systems, and the coexistence of TSD, XY and ZW systems within a single family (e.g., Geomydidae) are consistent with multiple origins of GSD, particularly in the suborder Cryptodira (Fig. 2).

In lizards, sex chromosomes have been identified in 181 of 953 karyotyped species, with 115 species showing male heterogamety and 66 species showing female heterogamety (Ezaz et al. 2009a). Male heterogamety is present in seven families (Iguanidae, Scincidae, Sphaerodactylidae, Pygopodidae, Dibamidae, Teiidae and Gymnophathalmidae), while female heterogamety has been found in six other families (Bipedidae, Lacertidae, Varanidae, Chameleonidae, Agamidae and Phyllodactylidae) (Fig. 2). Both sex chromosomes systems, as well as TSD, are found in the family Gekkonidae (Gamble 2010). Multiple sex chromosomes (XXY) are common in male heterogamety systems, especially in the family Iguanidae, but not in female heterogamety species (see Ezaz et al. 2009a). Morphology and the degree of sex chromosome degeneration varies widely among taxa, ranging from homomorphic to fully

differentiated (Ezaz et al. 2009a). Sex chromosomes may also involve microchromosomes, such as the XY system in *Anolis carolinensis* (Iguanidae) or the ZW systems of Australian agamids (Agamidae) (Ezaz et al. 2005, 2009b; Alföldi et al. 2011). Variation in the sex chromosome pair is often observed among closely related species, or even populations of the same species (Ezaz et al. 2009a). For instance, the morphology of Z and W chromosomes differs within and among populations of *Gehyra purpurascens* (Gekkonidae) (Moritz 1984). Intra-specific variability may also involve the coexistence of simple and multiple sex chromosome systems, as in *Sceloporus clarkii* (XY/XXY; Leaché and Sites 2009) or in *Zootoca vivipara* (ZW/ZZW; Odierna et al. 2001). Despite this overall varibility in sex chromosome constitutions, the levels of sex chromosomes diversification vary considerably between different lizard groups. Sex determination appears to be particularly dynamic in geckos, despite the low number of species for which the sex-determining system is confidently known (Gamble et al. 2015). A good example is found in the genus *Hemidactylus* (Gekkonidae), where the closely related species *H. turcicus* and *H. mabouia* have male heterogamety whereas *H. frenatus* has female heterogamety (Gamble et al. 2015). Yet, other lineages, such as lacertids (only female heterogamety) may possess conserved sex chromosomes (Pokorná and Kratochvíl 2009).

The diversity of sex-determining systems makes reptiles a particularly interesting group to study sex determination and test long-standing hypothesis about the evolution of sex chromosomes (Bull 1983; Sarre et al. 2004; Janzen and Phillips 2006; Organ and Janes 2008; Bachtrog et al. 2011, 2014;). However, comparative analyses of reptilian sex chromosomes have been largely constrained by limited knowledge of sex-determining systems in several important lineages, and by failure to identify sex chromosomes in many of the species investigated (Janzen and Krenz 2004; Sarre et al. 2004; Pokorná and Kratochvíl 2009). Because of widespread occurrence of sex microchromosomes, and the lack of visibly heteromorphic sex chromosomes in many taxa with GSD, sex chromosomes may have been overlooked in some species in which the karyotype has been examined using only standard cytogenetic techniques, such as chromosome staining and banding. The identification of homomorphic or poorly differentiated sex chromosomes may thus require the application of high-resolution cytogenetic techniques, such as comparative genomic hybridization (CGH). Indeed, CGH has successfully detected cryptic, but molecular differentiated, sex chromosomes in several reptiles (Ezaz et al. 2005, 2006b; Kawai et al. 2007; Martinez et al. 2008; Badenhorst et al. 2013). Next-generation sequencing technologies also offer promising

alternatives for sex chromosome discovery. For instance, restriction site-associated DNA sequencing (RAD-seq) has been recently used to discover sex-linked markers and sex-determining regions and subsequently infer the occurrence of male or female heterogamety in gecko species (Gamble et al. 2015).

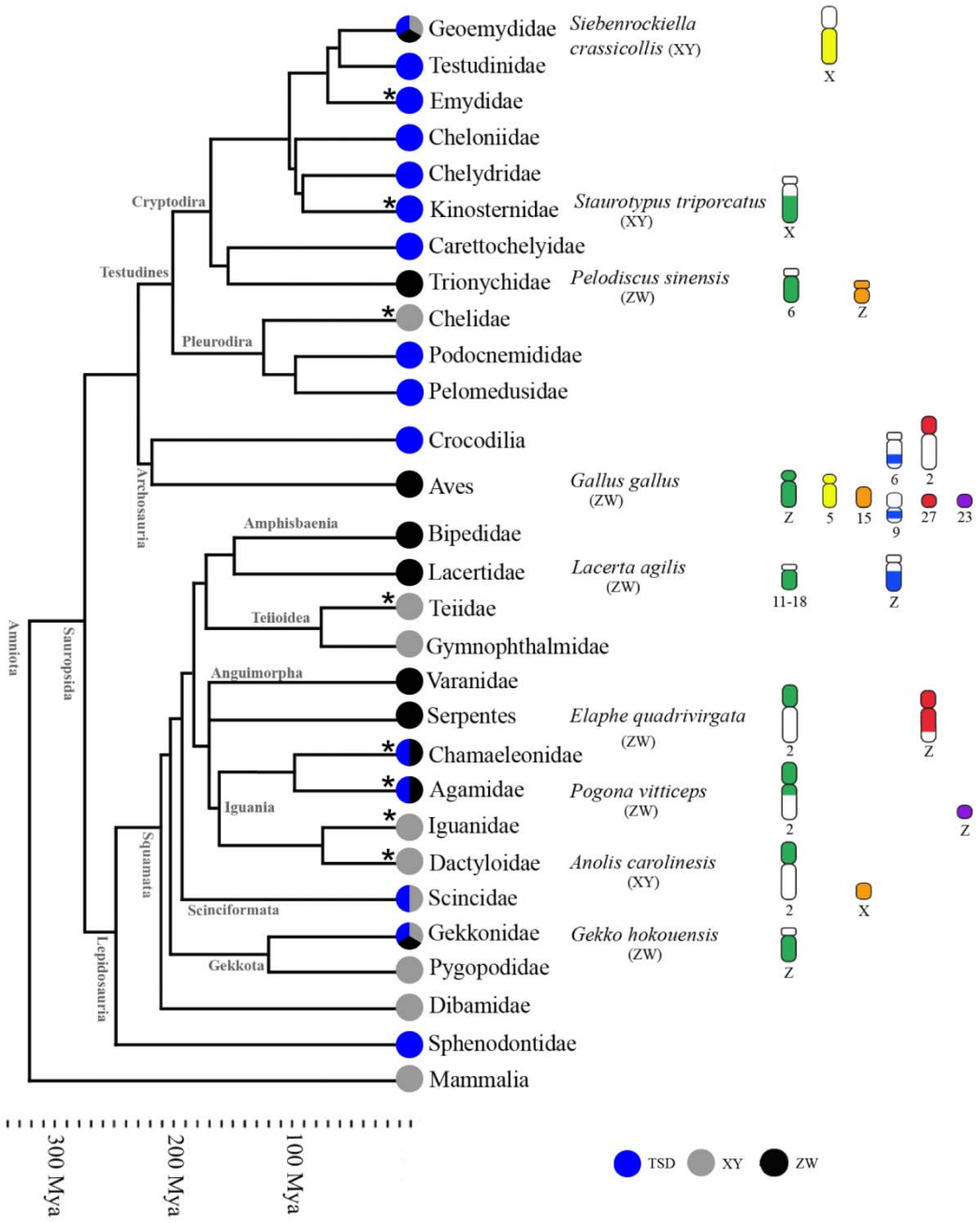


Figure 2. Pruned phylogenetic tree showing the distribution of sex determining systems among amniote lineages with known sex-determining mechanisms (left), and non-homology of sex chromosomes in reptiles (right). The phylogenetic reconstruction follows Organ and Janes (2008), Pokorná and Kratochvíl (2009), and Valenzuela and Adams (2011). Divergence time estimates according to Shedlock and Edwards (2009), Hedges et al. (2015). The schematic representation of non-homologous sex chromosomes is modified from Ezaz et al. (2009a). Asterisks indicate the occurrence of species with GSD without identified sex chromosomes.

2.3 *Multiple origins of reptilian sex chromosomes*

On the whole, the high variability of sex chromosomes and the haphazard distribution of sex determining systems across the reptile phylogeny (Sarre et al. 2004, 2011; Janzen and Phillips 2006; Organ and Janes 2008; Pokorná and Kratochvíl 2009) suggest that transitions between sex-determining systems have occurred in many lineages (Sarre et al. 2011; Johnson Pokorná and Kratochvíl 2014), and that novel sex chromosomes will have arisen also multiple times. Indeed, data obtained from chromosome painting, comparative gene mapping and in silico analysis of whole genomes confirmed the independent origin of sex chromosomes in several reptile lineages. Comparisons with the chicken Z chromosome revealed conserved synteny in the majority of reptiles (Pokorná et al. 2011), but non-homology between avian and reptile sex chromosomes, implying that they evolved from different pairs of autosomes (Ezaz et al. 2009c; Alföldi et al. 2011; Pokorná et al. 2011).

In particular, the chicken Z chromosome is homologous to the short arm of snake chromosome 2, and the snake Z corresponds to chicken chromosomes 2 and 27 (Matsuda et al. 2005; Matsubara et al. 2006, 2012; Kawai et al. 2007; Pokorná et al. 2011) (Fig. 2). Chicken Z chromosome showed homology to chromosome 6 in two turtle species (*Trachemys scripta*, Emydidae and *Pelodiscus sinensis*, Trionychidae) (Kasai et al. 2003; Matsuda et al. 2005; Kawai et al. 2007; Pokorná et al. 2011). Conversely, the ZW sex chromosomes of *P. sinensis* have conserved linkage homology with chicken chromosome 15 (Kawagoshi et al. 2009), whereas the XY sex chromosomes of the black marsh turtle (*Siebenrockiella crassicollis*, Geoemydidae) share linkage homology with chicken chromosome 5 (Kawagoshi et al. 2012). In lizards, physical mapping of protein-coding genes identified regions orthologous to chicken Z on chromosome 2 in *Pogona vitticeps* (Agamidae) chromosome 2 and *Anolis carolinensis* (Dactyloidae) (Ezaz et al. 2009c; Alföldi et al. 2011; Young et al. 2013), and on a small acrocentric autosome in *Lacerta agilis* (Lacertidae) (Srikulnath et al. 2014). On the other hand, the Z chromosome of *P. vitticeps* showed homology with chicken chromosome 23; the X microchromosome of *A. carolinensis* has homology with chicken chromosome 15; and the Z chromosome of *L. agilis* is homologous to chicken chromosomes 6 and 9 (summarized in Fig. 2). At a finer taxonomic scale, the ZW sex microchromosomes of three Australian dragon lizards (*P. vitticeps*, *P. barbata* and *Amphibolurus nobbi*) are presumably homologous, but those of a fourth species (*Ctenophorus fordi)* are not (Ezaz et al. 2009b). In geckos, the ZW sex chromosomes of *Gekko hokouensis* are homologous neither with the ZW chromosomes of *Christinus marmoratus* (Matsubara et al. 2014) nor with the with XXY systems of *Coleonyx*

elegans and *Lialis burtonis* (Pokorná et al. 2011).

Collectively, these data support the notion that sex chromosomes have evolved independently multiple times in birds, turtles and squamate reptiles. In this context, it is surprising that the Z chromosome of the lizard *Gekko hokouensis* (Kawai et al. 2009) and the XY chromosomes of giant musk turtles (*Staurotypus triporcatus* and *S. salvinii*; Kawagoshi et al. 2014) have conserved linkage homology with avian ZW sex chromosomes. This suggests that the three sex chromosome systems share the same origin but, nonetheless, turtles and birds acquired different systems of heterogametic sex determination during their evolution. Hence, the remaining reptilian clades would have more recent, and independently derived, sex chromosomes. Moreover, the discovery of partial synteny of sex chromosomes in birds and monotremes, the sister group to therian mammals (Rens et al. 2007; Veyrunes et al. 2008), led to the hypothesis that the common amniote ancestor had a birdlike ZW system (e.g., O'Meally et al. 2012).

However, given the general lack of homology of reptilian sex chromosomes described above, *G. hokouensis* and *Staurotypus* turtles seem to be exceptions among reptiles. Also, recent phylogenetic analyses that argue for TSD as the ancestral sex-determining mechanism for amniotes (Pokorná and Kratochvil 2009; Johnson Pokorná and Kratochvíl 2014). Therefore, the observed synteny of sex chromosomes in monotremes, birds, gekko and *Staurotypus* turtles might not reflect homology but convergent evolution; that is, the same genomic regions have been co-opted as sex chromosomes independently several times, perhaps because it contains genes (such as *DMRT1*) that are particularly suitable for a role in sex determination (Graves and Peichel 2010; O'Meally et al. 2012).

2.4 *Turnover of sex chromosomes vs. the evolutionary trap hypothesis*

The identification of independently derived sex chromosomes in reptiles create exciting new opportunities to gain a deeper insight into the general processes involved in sex chromosome evolution, to investigate why sex determination is labile in some taxa and not in others, and to test hypothesis related to transitions among sex-determining systems. One of such hypothesis posits that sex chromosomes can act as an evolutionary trap (Bull and Charnov 1977; Bull 1983; Pokorna and Kratochvıl 2009; Bachtrog et al. 2014). According to it, differentiated, nonrecombining sex chromosomes preclude transitions to other sex-determining systems. Transitions are prevented mainly due to the accumulation of sexually antagonistic and sex-

essential genes on differentiated sex chromosomes, and/or the loss of functional genes from the Y (or W) chromosome. This would cause a lower fitness of sexually reverted individuals (e.g., XX males, XY females), or YY (WW) individuals, which arise when sex determination is hijacked by another chromosome pair, inhibiting the spread of new sex-determining genes (see Bachtrog et al. 2014; van Doorn 2014).

Under the evolutionary trap hypothesis, transitions are only possible from young or otherwise poorly differentiated sex chromosomes, or else from TSD (lacking sex chromosomes) to XY or ZW systems. Indeed, homomorphic sex chromosomes in fish and amphibians often exhibit high rates of turnover between species (e.g., Miura 2007; Takehana et al. 2007; Ross et al. 2009; Kikuchi and Hamaguchi 2013). Conversely, the evolutionary stability of sex chromosomes in birds or mammals is consistent with the idea that heteromorphic sex chromosomes constrain shifts in sex determination. Currently available data for reptiles also support the trap-like behaviour of sex chromosomes, which might explain the unequal distribution of variability in sex determination across reptilian lineages. If transitions from TSD to GSD are much easier and frequent than transitions in the opposite direction, the diversity of sex-determining systems in certain amniote lineages could be explained by their ancestral TSD and several independent transitions to GSD (Pokorná and Kratochvíl 2009; Johnson Pokorná and Kratochvíl 2014). In this regard, it is noteworthy that non-homologous sex chromosomes have been only identified so far within reptilian clades where at least some members possess TSD: geckos, dragon lizards, and turtles (see section 2.3; Johnson Pokorná and Kratochvíl 2014). Also, sex chromosomes show no homology between birds and snakes, separated by TSD crocodiles, and sex chromosome in a lacertid lizard evolved independently from those of snakes, iguanas and the bearded dragon, separate by ESD agamids. By contrast, clades with GSD as the ancestral state are predicted to have homologous sex chromosomes. In agreement, recent molecular analyses demonstrated a high conservation of sex chromosomes in colubroid snakes (Matsubara et al. 2006; Vicoso et al. 2013), *Anolis* lizards (Gamble et al. 2014; Rovatsos et al. 2014a), and across most other lineages of iguanas (Rovatsos et al. 2014b). Karyotype data also suggest long-term conservation of sex chromosomes in teiids and gymnophthalmids (male heterogamety), in lacertids (female heterogamety), and Anguimorpha (anguids, helodermatids, and varanids; female heterogamety) (Pokorná and Kratochvíl 2009; Johnson Pokorná and Kratochvíl 2014); unfortunately, molecular or molecular-cytogenetic data testing the homology of sex chromosomes are still lacking for these lineages, and so transitions that do not involve a

change in heterogamety may have beeen overlooked.

A good example of "hidden" turnover of sex chromosomes has been recently reported in dipteran insects, generally considered to show a stable XY system (Vicoso and Bachtrog 2013, 2015). Whole-genome analysis of 37 fly species revealed numerous transitions of sex chromosomes over the course of Diptera evolution, where sex chromosomes have been lost, gained, rearranged or replaced by a new chromosomal pair. For instance, all *Drosopila* species have newly evolved XY sex chromosomes, while the ancestral sex chromosome pair (the dot chromosome or Muller element F) is now autosomal (Vicoso and Bachtrog 2013).

Another recent work in the dragon lizard *P. vitticeps* challenges the prediction that GSD systems should be stable with respect to replacement by TSD. As mentioned before, this species has recognizable sex chromosomes with female heterogamety; however, sex reversed females (ZZ females) were observed in the wild at the warmer ends of the animals geographic range (Holleley et al. 2015). Mating of normal mates to sex-reversal females produced viable and fertile—and exclusively ZZ—offspring, whose phenotypic sex was basically determined by the temperature at which eggs are incubated. This illustrates how the rapid transition from GSD to TSD may occur in the wild in response to extreme environmental conditions (high temperatures). Importantly, ZZ females had markedly higher fecundity that ZW females, which raises interesting questions about the relative advantages of the two sex determining systems and the possible cost of sex chromosome degeneration (Bull 2015).

In light of these recent findings, it seems clear that our understanding of the evolution of sex determination is far from complete, and will undoubtedly benefit from comparative studies of sex chromosomes in diverse non-model taxa. For example, evaluating the homology of sex chromosomes in those lizard groups with presumably conserved sex chromosomes (e.g., lacertids) is an important first step to assess whether the trap-like behaviour is a general consequence of sex chromosome evolution or, instead, the stability observed in mammals, birds or snakes is somehow exceptional. In addition, this will help clarify if the high frequency of homomorphic sex chromosomes in lizards are related to a rapid turnover of sex chromosomes or, on the contrary, to slow rates of degeneration of the heterogametic sex chromosome, as explained in section 2.1.1.

3. Satellite DNA: features and evolution

Eukaryotic genomes contain a large proportion of repetitive DNA sequences which, according to an organizational criterion, can be classified into two main categories: (1) dispersed repeats—mostly transposable elements—scattered thoughout the genome, and (2) tandem repeats, restricted at specific locations and organized in consecutive or nearly consecutive copies copies along a DNA strand (López-Flores and Garrido-Ramos 2012). Among the latter, there are moderately repetitve elements—including gene families such as globins, histones and ribosomal RNA genes (rDNA)—as well as highly repetitive non-coding microsatellite and satellite DNAs (Richard et al. 2008; López-Flores and Garrido-Ramos 2012).

Satellite DNAs (satDNAs) constitute one of the most abundant fractions of repetitive sequences in almost all eukaryotic species, representing in some cases over 50% of genomic DNA (Elder and Turner 1995; Plohl et al. 2008). They are organized as long arrays of head-to-tail linked repeats (monomers), typically spanning up to several megabases. As the main components of constitutive heterochromatin, they are usually located in the (peri)centromeric and/or telomeric regions of chromosomes, but may be also found at interstitial chromosomal locations (Plohl et al. 2012), or even with a chromosome-specific distribution [e.g. The RAYSI satellite family amplified in the Y sex chromosome of the plant *Rumex acetosa* and relatives (Navajas-Pérez et al. 2005)]. The basic repeting units (monomers) of satDNAs are usually AT-rich and range in length from only a few base pairs (bp) to more than 1 kb (Plohl et al. 2008). The preferential monomer length of 150-180 bp and 300-360 bp observed in many in many satellites in both plants and animals is often considered to reflect requirements of DNA length wrapped around one or two nucleosomes, to facilitate regular phasing of nucleosomes in the heterochromatin (Schmidt and Heslop-Harrison 1998; Henikoff et al. 2001).

Several different satDNA families can be present in a species, and they differ not only in monomer length, but also in nucleotide composition, sequence complexity, genomic abundance and in evolutionary history (Plohl 2010). SatDNAs represent fast-evolving components of genomes, undergoing rapid changes in array size and sequence composition (Plohl et al. 2012; Garrido-Ramos 2015). Yet, contrary to what may be expected, monomers of a satDNA family maintain a high degree of intraspecific sequence conservation. Sequence homogeneity is a result of non-independent evolution of repetitive units. That is, mutations do not accumulate in a single monomer sequence, but they either spread among repetitve units they become eliminated (Plohl et al. 2012). This evolutionary model, known as concerted

evolution, is achieved through a process of molecular drive, consisting of sequence homogenization within a genome and fixation of sequence variants among reproductively linked individuals (Dover 1986, 2002). The final outcome of concerted evolution is higher repeat homogeneity within lineages (strains, populations, subspecies, species, etc.) than between them (Dover, 1982; Rudd et al 2006; Plohl et al. 2008).

Sequence homogenization in the genomic level is due to diverse molecular mechanisms of nonreciprocal transfer, such as unequal crossover, gene conversion, rolling circle replication and reinsertion, and transposon-mediated exchange (Stephan, 1986; Dover, 1986, 2002). All these mechanisms, excluding gene conversion, may induce extensive variations in copy number of satDNA repeats, resulting in array length polymorphism and, potentially, in the rapid amplification/contraction of satDNA arrays in short evolutionary periods (e.g., Cheng et al. 2002; Nijman and Lenstra 2001; Plohl et al. 2012). In some cases, this dynamic behaviour of satDNAs has been associated with chromosomal rearrangements,and even with reproductive isolation and speciation (Wichman et al. 1991; Bradley and Wichman 1994; Slamovits et al. 2001; Slamovits and Rossi 2002; Ferree et al. 2012)

Another important by-product of the mechanisms of sequence homogenization is a higher degree of sequence similarity among adjacent repeats than among repeats retrieved at random (Dover 1986). Thus, monomers of a satDNA family can often be grouped into subsets or subfamilies, defined by diagnostic mutations, which are usually chromosome-specific (Willard and Waye 1987; Hall et al. 2005). Adjacent monomer variants can be sometimes homogenized together and form a new, composite higher-order repeat (HOR) unit in which former monomers now constitute subunits (Willard and Waye, 1987; Warburton and Willard, 1990). This complex organization is typical, for example, of the centromeric alpha satellite of primates (e.g. Alexandrov et al. 2001), but seems to be a common trend in other satDNA families and organisms, includin bovids and beetles (Modi et al. 2004; Mravinac et al. 2005; Palomeque et al. 2005).

According to the model of satDNA evolution depicted above, molecular drive is an essentially stochastic process, during which mutations rapidly accumulate in a gradual manner, leading to divergent evolution of satellite repeats in reproductively separated groups of organisms (Bachmann and Sperlich 1993). However, high rates of sequence change are not characteristic of all satDNAs; instead, some satellite families seem to be rather ancient and are widely distributed among higher taxa (Abad et al. 1992; Arnason et al. 1992; Robles et al.

2004). Indeed, the nucleotide sequence of some satellite families have remained "frozen" over long evolutionary periods (see, for example, Mravinac et al. 2002; Plohl et al. 2010). Long-term sequence conservation, and irregular distribution of sequence variability along the monomer sequence, has been commonly interpreted as an indicative of functional constraints on part of repeat monomers or motifs (Meštrović et al. 2006; Plohl et al. 2008) . The best-known example is the 17-bp motif, known as the CENP-B, of alpha satDNA in human and other primates, which is proposed to act as a centromere protein binding site (Masumoto et al. 2004). The functionality of other conserved sequence segments detected in satDNA monomers remains obscure, but they could be related to any of the diverse roles ascribed to satDNAs. In fact, and in contrast with earlier ideas of satDNA being "junk" (Ohno 1972) or "selfish" (Orgel and Crick 1980), there is growing evidence showing that it may have important functional roles in a genome, being involved in centromere formation and function, heterochromatin assembly, regulation of gene expression and in epigenetic regulatory processes (reviewed in Ugarković 2009; Pezer et al. 2012).

Notwithstanding the possible selective constraints on monomer sequences, the overall turnover rate (i.e., homogenization and fixation) of a satDNA families is a complex feature that depends on many factors, which will be later explored in Chapter III. Even despite decades of intensive research, satDNA and heterochromatin are still the most enigmatic genomic compartments, and many questions about their origin and evolution remain open. In addition, studies focused on specific evolutionary questions are still scarce for several major taxonomic groups, including reptiles. New data from these poorly studied groups could add valuable information on the general principles and mechanisms that govern satDNA evolution, as well as on specificities of particular systems.

4. The study species

4.1 *The family Lacertidae*

The lizard family Lacertidae Oppel, 1811 consists of about 321 species in 42 genera (Uetz and Hošek 2015), and is found widely in Eurasia and Africa (Arnold et al. 2007). The Lacertidae is the most species-rich family of lizards focused on Europe, where it also presents a high number of endemisms [48 endemisms out of 65 European species (73.8%); Cox and Temple 2009]. Recent molecular analyses strongly support the monophyly of lacertids, and suggest that they may be the sister-group of the Amphisbaenia, the worm lizards (Townsend et al.

2004; Vidal and Hedges 2005; Pyron et al. 2013).

The family is divided into two subfamilies, the Gallotiinae and Lacertinae, with the latter group composed of two monophyletic tribes: the Eremiadini of Africa and arid southwest and central Asia, and the Lacertini of Europe, northwest Africa and southwest and east Asia (see Arnold et al. 2007 for a review of lacertid systematics). The Lacertidae probably arose in the European area, with the Gallotiinae later reaching Northwest Africa and the Canary Islands, and the ancestor of the Eremiadini invading Africa in the mid-Miocene. A molecular clock based on mitochondrial DNA sequences suggests that the separation of the Gallotinae and Lacertinae occurred around 20 mya, while the separation of the Eremiadini from the Lacertini may have been around 16 mya (Arnold et al. 2007). The Lacertini spread through much of their present European range and split into most of its component living genera about 12-16 mya, so they underwent quite rapid speciation at this time (but see Hipsley et al. 2009 for older divergence time estimates, around 43-46 mya). Most genera in the Lacertini have largely allopatric and often disjunct ranges, which may mean that initial spread of the groups was followed or accompanied by multiple vicariance (Arnold et al. 2007).

Until relatively recently many European lizards were included within the widespread, highly polyphyletic genus *Lacerta;* however sucessive taxonomic studies have now allocated a number of these species to endemic European genera (including *Dalmatolacerta*, *Dinarolacerta*, *Hellenolacerta* and *Iberolacerta*) (e.g., Arnold 1973; Olmo et al. 1991; Arribas 1999; Fu et al. 1997; Carranza et al. 2004; Arnold et al. 2007). In the most recent revision, the approximately 100 species of Lacertini have been classified into 19 genera (Arnold et al. 2007; see also Speybroek et al. 2010 for an updated species list of the European herpetofauna). However, the systematics of the tribe remains complex and phylogenetic relationships among and within many genera are unresolved (Arnold et al. 2007; Pavlicev and Mayer 2009; Kapli et al. 2011).

4.2 *The genus Iberolacerta*

West European Rock lizards, *Iberolacerta* Arribas, 1997 comprise a group of closely related species of medim-sized lacertine lizards, formerly included in the genus *Lacerta*. It is almost entirely confined to small widely separated mountain areas in the Iberian Peninsula and in the Balkan Peninsula (*I. horvathi*) (Fig. 3; Mayer and Arribas 2003; Carranza et al. 2004).

Figure 3. (Top) Distribution map for the genus *Iberolacerta.* 1: *I. horvathi;* 2: *Pyrenenan group;* 3: *I. cyreni;* 4: *I. martinezricai;* 5: *I. galani;* 6: *I. monticola.* (Bottom) Adult male of *I. monticola* from the Natural Park of Fragas do Eume (A Coruña, Spain).

Until very recently, (Salvador 1974, 1984; Arnold & Burton 1987; Barbadillo 1987; Pérez-Mellado 2002) all populations of the genus *Iberolacerta* were considered to belong to

Iberolacerta monticola (Boulenger, 1905). Extensive taxonomic revision—using morphological and osteological data (Arribas 1996 and 1998), karyotypes (Odierna et al. 1996; Arribas and Odierna 2005; Arribas et al. 2006), allozyme-electrophoresis studies (Mayer & Arribas 1996; Almeida et al. 2002), and phylogenetic analyses including DNA sequences (Mayer & Arribas 2003; Crochet et al. 2004; Carranza et al. 2004; Arribas et al. 2006; Galán et al. 2007; Arribas et al. 2014)—indicated that *I. monticola* was, in fact, a species complex. As a result of all these analyses *Iberolacerta cyreni* (Müller & Hellmich, 1937) and *I. bonnali* (Lantz, 1927) were upgraded to the species level (Arribas 1993a, 1996; Perez-Mellado et al. 1993), and four new species were described: *I. aranica* (Arribas, 1993), *I. aurelioi* (Arribas, 1994), *I. martinezricai* (Arribas, 1996), and *I. galani* Arribas, Carranza and Odierna, 2006 (Arribas 1993b, 1994, 1996; Mayer & Arribas 1996; Arribas & Carranza 2004; Arribas & Odierna 2005; Arribas et al. 2006).

So, there are currently eight recognized species in *Iberolacerta*, which can be classified into three main units (Fig. 3): 1) the Horvath's Rock lizard *I. horvathi* (Méhely, 1904), with a patchy distribution across the Eastern Alpine and North Dinaric mountain ranges; 2) Pyrenean Rock lizards, also known as the "Pyrenean group" or "bonnali-group", which belong to the subgenus *Pyrenesaura* Arribas, 1999 and include three allopatric species present at high altitudes (usually above 2000 m) in the Pyrenees: Iberolacerta aranica, I. aurelioi and I. bonnali; and 3) Iberian Rock lizards, also known as the "Iberian group" or "monticola-group", which includes I. cyreni, I. martinezricai, I. galani and I. monticola. The first taxon comprises *I. cyreni cyreni* (Müller & Hellmich, 1937) from the Sierra de Guadarrama, and *I. c. castiliana* (Arribas, 1996) from the Sierra de Gredos. *Iberolacerta martinezricai* is mainly found in Peña de Francia (Salamanca), while *I. galani* inhabits the southern part of the Montes de León. Finally, *I. monticola* is nominally divided into *I. monticola monticola* (Boulenger, 1905), restricted to the Serra da Estrela in Portugal, and *I. m. cantabrica* (Mertens, 1929), distributed across a wide area in northwest Spain. In addition, a recent study described a new subspecies of I. monticola, *I. m. astur* Arribas, Galán, Remón and Naveira, 2014, which inhabits the Northern Montes de León (Arribas et al. 2014). The restricted and generally fragmented distributions of these endemic species render them particularly vulnerable to extinction. In fact, four of the eight species are defined as "endangered" (*I. cyreni*, *I. aranica*, *I. aurelioi*), and "critically endangered" (*I. martinezricai*) in the IUCN Red List of Threatened Species (2015), according to its extent of occurrence, its distribution (severely fragmented), and the quality and extent of its habitat (in continuing decline).

Cytogenetic surveys based on conventional staining and banding techniques demonstrated that *Iberolacerta* is a karyologically heterogeneous group, with interspecific variability both in chromosome number and karyotype macrostructure, as well as in the degree of sex chromosome differentiation (Odierna et al. 1996; Arribas and Odierna 2005; Arribas et al. 2006). Indeed, while some species (*I. aranica*, *I. monticola,* and *I. martinezricai*) appear to lack differentiated sex chromosomes, others (*I. horvathi*, *I. cyreni*, and *I. galani*) show a highly heteromorphic ZW pair, and yet other species (*I. aurelioi* and *I. bonnali*) have multiple Z_1Z_2W chromosome systems. Interestingly, this diversity of sex chromosomes shows no clear phylogenetic segregation (see Chapter I). The phylogeny and evolutionary history of Iberolacerta are relatively well known, and support the monophyly of the genus (Mayer and Arribas 1996; Almeida et al. 2002; Mayer and Arribas 2003; Carranza et al. 2004; Crochet et al. 2004; Arribas et al. 2006, 2014; Arnold et al. 2007).

However, the phylogenetic relationships among some taxa are still controversial, due to the low support or contradictory results produced by different molecular markers and different methods of phylogenetic reconstruction. Main discrepancies concern the position of I. horvathi (i.e., sister to all the remaining species or grouped either with the Pyrenean or with the Iberian groups), as well as the relative order of speciation events within Pyrenesaura and the clade formed by monticola-galani-martinezricai. Failure to resolve the phylogenetic relationships and track lineage splitting has been generally attributed to a rapid succession of speciation events within this group (see Mayer and Arribas 2003; Carranza et al. 2004; Crochet et al. 2004; Arribas et al. 2006, 2014). The phylogenetic affinities and estimates of divergence times used in this thesis report will follow the most recently published phylogeny (Arribas et al. 2014), and they will be detailed later throughout the next sections, since they provide a framework to trace not only the differentiation process of their sex chromosomes (Chapters I and II), but also the evolutionary dynamics of the two satDNA families analyzed (Chapter III).

References

Abad JP, Carmena M, Baars S, Saunders RD, Glover DM, Ludeña P, Sentis C, Tyler-Smith C, Villasante A (1992) Dodeca satellite: a conserved G+ C-rich satellite from the centromeric heterochromatin of *Drosophila melanogaster*. PNAS 89: 4663-4667.

Adolfsson S, Ellegren H (2013) Lack of Dosage Compensation Accompanies the Arrested Stage of Sex Chromosome Evolution in Ostriches. Mol Biol Evol 30: 806-810.

Alexandrov I, Kazakov A, Tumeneva I, Shepelev V, Yurov Y (2001) Alpha-satellite DNA of primates: Old and new families. Chromosoma 110: 253-266.

Alföldi J, Di Palma F, Grabherr M, Williams C, Kong L, Russell EP, Lowe CB, Glor RE, Jaffe JD, Ray DA, Boissinot S, Shedlock AM, Botka C, Castoe TA, Colbourne JK, Fujita MK, Godinez- Moreno R, Ten-Hallers BF, Haussler D, Heger A, Heiman D, Janes DE, Johnson J, De Jong PJ, Koriabine MY, Lara M, Novick PA, Organ CL, Peach SE, Poe S, Pollock DD, De Queiroz K, Sanger T, Searle S, Smith JD, Smith Z, Swofford R, Turner-Maier J, Young JS, Zadissa A, Edwards SV, Glenn TC, Schneider CJ, Losos JB, Lander ES, Breen M, Ponting CP, Lindblad-Toh K (2011) The genome of the green anole lizard and a comparative analysis with birds and mammals. Nature 477: 587-591.

Almeida A, Rosa H, Paulo O, Crespo E (2002) Genetic differentiation of populations of Iberian rock˜lizards *Iberolacerta* (*Iberolacerta*) *sensu* Arribas (1999). J Zool Syst Evol Research 40: 57-64.

Arnason U, Gretarsdottir S, Widegren B (1992) *Mysticete* (baleen whale) relationships based upon the sequence of the common cetacean DNA satellite. Mol Biol Evol 9: 1018-1028.

Arnold EN (1973). Relationships of the Palearctic lizards assigned to the genera *Lacerta*, *Podarcis*, *Algyroides* and *Psammodromus* (Reptilia, Lacertidae). Bull Br Mus (Nat Hist) Zool 25: 291-366.

Arnold EN, Burton JA (1978) A Field Guide to the Reptiles and Amphibians of Britain and Europe. London: Collins.

Arnold EN, Arribas Ó, Carranza S (2007) Systematics of the Palaearctic and Oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa 1430: 1-86.

Arribas Ó (1993a) Estatus especifico para *Lacerta* (*Archaeolacerta*) *monticola bonnali* Lantz, 1927 (Reptilia, Lacertidae). Bol R Soc Esp Hist Nat Secc Biol 90: 101-112.

Arribas O (1993b) Intraspecific variability of *Lacerta* (*Archaeolacerta*) *bonnali* Lantz 1927. (Squamata: Sauria: Lacertidae). Herpetozoa 6: 129-140.

Arribas Ó (1994) Una nueva especie de lagartija de los Pirineos Orientales: *Lacerta* (*Archaeolacerta*) *aurelioi* sp. nov. (Reptilia: Lacertidae). Mus Regionale Sci Nat 12: 327-351.

Arribas Ó (1996) Taxonomic revision of the Iberian 'Archaeolacertae' 1.: a new interpretation of the geographical variation of '*Lacerta*' monticola Boulenger, 1905 and '*Lacerta*' *cyreni* Muller & Hellmich, 1937 (Squamata: Sauria: Lacertidae). Herpetozoa 9: 31-56.

Arribas O (1997) Morfología, filogenia y biogeografía de las lagartijas de alta montaña de los Pirineos. PhD Thesis. Universidad Autónoma de Barcelona.

Arribas Ó (1998) Osteology of the Pyrenean mountain lizards and comparison with other species of the collective genus *Archaeolacerta* Mertens, 1921 s. l. from Europe and Asia Minor. (Squamata: Sauria: Lacertidae.). Herpetozoa 11: 47-70.

Arribas Ó (1999a) Taxonomic revision of the Iberian "Archaeolacertae" II: Diagnosis, morphology and geographic variation of '*Lacerta*' *aurelioi* Arribas, 1994. Herpetozoa 11: 155-180.

Arribas O, Carranza S (2004) Morphological and genetic evidence of the full species status of *Iberolacerta martinezricai* (Arribas, 1996). Zootaxa 634: 1-24.

Arribas O, Odierna G (2005) Karyological and and osteological data supporting the specific status of *Iberolacerta* (*cyreni*) *martinezricai* (Arribas, 1996). Amphibia-Reptilia 25: 359-367.

Arribas O, Carranza S, Odierna G (2006) Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240:1–55.

Arribas OJ, Galán P, Remón N, Naveira H (2014) A new mountain lizard from Montes de León (NW Iberian Peninsula): *Iberolacerta monticola astur* ssp. nov. (Squamata: Lacertidae). Zootaxa 3796: 201-236.

Bachmann L, Sperlich D (1993) Gradual evolution of a specific satellite DNA family in

Drosophila ambigua, *D. tristis*, and *D. obscura*. Mol Biol Evol 10: 647-659.

Bachtrog D, Kirkpatrick M, Mank JE, McDaniel SF, Pires JC, Rice W, Valenzuela N. Are all sex chromosomes created equal? Trends Genet 27: 350-357.

Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Kirkpatrick M, Otto SP, Ashman TL, Hahn MW, Kitano J, Mayrose I, Ming R, Perrin N, Ross L, Valenzuela N, Vamosi JC; Tree of Sex Consortium (2014) Sex Determination: Why So Many Ways of Doing It? PLoS Biol 12: e1001899.

Badenhorst D, Stanyon R, Engstrom T, Valenzuela N (2013) A ZZ/ZW microchromosome system in the spiny softshell turtle, *Apalone spinifera*, reveals an intriguing sex chromosome conservation in Trionychidae. Chromosome Res 21: 137-147.

Barbadillo LJ (1987) La guia de INCAFO de los Anfibios y Reptiles de la Península Ibérica, Islas Baleares y Canarias. Madrid: INCAFO.

Beçak W, Beçak M, Nazareth HRS, Ohno S (1964) Close karyological kinship between the reptilian suborder serpentes and the class aves. Chromosoma 15: 606-617.

Beçak W, Beçak ML (1969) Cytotaxonomy and chromosomal evolution in Serpentes. Cytogenet Genome Res 8: 247-262.

Bergero R, Charlesworth D The evolution of restricted recombination in sex chromosomes. Trends Ecol Evol 24: 94-102.

Boulenger GA (1905) A contribution to our knowledge of the varieties of the wall lizard (*Lacerta muralis*). Trans Zool Soc London, 17: 351-437. Plates XXII–XXIX

Bradley R, Wichman H (1994) Rapidly evolving repetitive DNAs in a conservative genome: A test of factors that affect chromosomal evolution. Chromosome Res 2: 354-360.

Bull JJ (1983) Evolution of sex determining mechanisms. Menlo Park: The Benjamin/Cummings Publishing Company, Inc.

Bull JJ (2015) Evolution: Reptile sex determination goes wild. Nature 523: 43-44.

Bull JJ, Charnov EL (1977) Changes in the heterogametic mechanism of sex determination. Heredity 39: 1-14.

Burt DW (2002) Origin and evolution of avian microchromosomes. Cytogen Genome Res 96: 97-112.

Carranza S, Arnold EN, Amat F (2004) DNA phylogeny of *Lacerta* (*Iberolacerta*) and other lacertine lizards (Reptilia: Lacertidae): did competition cause long-term mountain restriction? Syst Biodivers 2: 57-77.

Charlesworth B (1991) The evolution of sex chromosomes. Science 251: 1030-1033.

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371: 215-220.

Charlesworth B, Charlesworth D (2000) The degeneration of Y chromosomes. Phil Trans R Soc B 355: 1563-1572.

Charlesworth D, Charlesworth B, Marais G (2005) Steps in the evolution of heteromorphic sex chromosomes. Heredity 95: 118-128.

Cheng Z, Dong F, Langdon T, Ouyang S, Buell CR, Gu M, Blattner FR, Jiang J (2002) Functional Rice Centromeres Are Marked by a Satellite Repeat and a Centromere-Specific Retrotransposon. Plant Cell 14: 1691-1704.

Chiari Y, Cahais V, Galtier N, Delsuc F (2012) Phylogenomic analyses support the position of turtles as the sister group of birds and crocodiles (Archosauria). BMC Biology 10: 65.

Cohen MM, Gans C (1970) The chromosomes of the order Crocodilia. Cytogen Genome Res 9: 81-105.

Cox NA, Temple HJ (2009) European Red List of Reptiles. Luxembourg: Office for Official Publications of the European Communities.

Crawford NG, Faircloth BC, McCormack JE, Brumfield RT, Winker K, Glenn TC (2012) More than 1000 ultraconserved elements provide evidence that turtles are the sister group of archosaurs. Biology Letters 8: 783-786.

Crochet PA, Chaline O, Surget-Groba Y, Debain C, Cheylan M (2004) Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae). Mol Phylogenet Evol 30: 860-866.

Deakin JE, Ezaz T (2014) Tracing the evolution of amniote chromosomes. Chromosoma 123: 201-216.

Dover GA (1982) Molecular drive: a cohesive mode of species evolution. Nature 299: 111-117.

Dover G (1986) Molecular drive in multigene families: how biological novelties arise spread and are assimilated. Trends Genet 2: 159-165.

Dover GA (2002) Molecular drive. Trends Genet 18: 587–589.

van Doorn GS (2014) Patterns and mechanisms of evolutionary transitions between genetic sex-determining systems. Cold Spring Harb Perspect Biol 3: 6.

Elder Jr JF, Turner BJ (1995). Concerted evolution of repetitive DNA sequences in eukaryotes. Q Rev Biol 70: 297-320.

Ellegren H (2010) Evolutionary stasis: the stable chromosomes of birds. Trends Ecol Evol 25: 283-291.

Ellegren H, Hultin-Rosenberg L, Brunström B, Dencker L, Kultima K Scholz B (2007) Faced with inequality: chicken do not have a general dosage compensation of sex-linked genes. BMC biology 5: 40.

Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A, Marshall Graves JAM (2005) The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. Chromosome Res 13: 763-776.

Ezaz T, Stiglec R, Veyrunes F, Marshall Graves JAM (2006a) Relationships between vertebrate ZW and XY sex chromosome systems. Curr Biol 16: R736-743.

Ezaz T, Valenzuela N, Grützner F, Miura I, Georges A, Burke RL, Graves JAM (2006b) An XX/XY sex microchromosome system in a freshwater turtle, *Chelodina longicollis* (Testudines: Chelidae) with genetic sex determination. Chromosome Res 14:139-150.

Ezaz T, Sarre SD, O'Meally D, Graves JAM, Georges A (2009a) Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet Genome Res 127: 249-260.

Ezaz T, Quinn AE, Sarre SD, O'Meally D, Georges A, Graves JAM (2009b) Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards. Chromosome Res 17: 91-98.

Ezaz T, Moritz B, Waters P, Graves JAM, Georges A, Sarre SD (2009c) The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. Chromosome Res 17: 965-973.

Ferguson-Smith MA, Trifonov V (2007) Mammalian karyotype evolution. Nat Rev Genet 8:

950-962.

Ferree PM, Prasad S, Ferree PM, Prasad S (2012) How Can Satellite DNA Divergence Cause Reproductive Isolation? Let Us Count the Chromosomal Ways. Genet Res Int 2012: e430136.

Fu J, Murphy RW, Darevsky IS (1997) Toward the phylogeny of Caucasian rock lizards: implications from mitochondrial DNA gene sequences (Reptilia: Lacertidae). Zool J Linnean Soc 120: 463-477.

Galán P, Vila M, Remón N, Naveira H (2007) Caracterización de las poblaciones de *Iberolacerta monticola* en el Noroeste ibérico mediante la combinación de datos morfológicos, ecológicos y genéticos. Munibe (Suplemento) 25: 34-43.

Gamble T (2010) A Review of Sex Determining Mechanisms in Geckos (Gekkota: Squamata). Sex Dev 4: 88-103.

Gamble T, Geneva AJ, Glor RE, Zarkower D (2014). *Anolis* sex chromosomes are derived from a single ancestral pair. Evolution 68: 1027-1041.

Gamble T, Coryell J, Ezaz T, Lynch J, Scantlebury DP, Zarkower D (2015) Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. Mol Biol Evol 32: 12961309.

Garrido-Ramos MA (2015) Satellite DNA in Plants: More than Just Rubbish. Cytogenet Genome Res. In press.

Giovannotti M, Caputo V, O'Brien PCM, Lovell FL, Trifonov V, Nisi Cerioni P, Olmo E, Ferguson-Smith MA, Rens W (2009) Skinks (Reptilia: Scincidae) Have Highly Conserved Karyotypes as Revealed by Chromosome Painting. Cytogenet Genome Res 127: 224-231.

Graves JAM (2008) Weird Animal Genomes and the Evolution of Vertebrate Sex and Sex Chromosomes. Annu Rev Genet 42: 565-586.

Graves JAM, Shetty S (2000) The evolution of sex chromosomes in higher vertebrates. In Clark M, de. Comparative Genomics. Boston: Kluwer Academic Publishers, pp 153-205.

Graves JAM, Peichel CL (2010) Are homologies in vertebrate sex determination due to shared ancestry or to limited options? Genome Biol 11: 205.

Griffin DK, Robertson LBW, Tempest HG, Skinner BM (2007) The evolution of the avian genome as revealed by comparative molecular cytogenetics. Cytogen Genome Res 117: 64-77.

Hall SE, Luo S, Hall AE, Preuss D (2005) Differential rates of local and global homogenization in centromere satellites from Arabidopsis relatives. Genetics 170:1913–1927.

Handley L-JL, Ceplitis H, Ellegren H (2004) Evolutionary strata on the chicken Z chromosome: implications for sex chromosome evolution. Genetics 167: 367-376.

Harrison PW, Mank JE, Wedell N (2012) Incomplete sex chromosome dosage compensation in the Indian meal moth, *Plodia interpunctella*, based on de novo transcriptome assembly. Genome biol evol 4: 1118.1126.

Hedges SB, Marin J, Suleski M, Paymer M, Kumar S (2015) Tree of life reveals clock-like speciation and diversification. Mol Biol Evol. In press.

Henking H (1891) Über Patagonian pejerrey (*Odontesthes hatcheri*) spermatogenese und deren Beziehung zur Entwicklung bei Pyrrhocoris apterus L. Zeitschrift für wissenschaftliche Zoologie 51: 685-736.

Henikoff S, Ahmad K, Malik HS (2001) The Centromere Paradox: Stable Inheritance with Rapidly Evolving DNA. Science 293: 1098-1102.

Hipsley CA, Lin Himmelmann, Dirk Metzler, and Johannes Müller (2009) Integration of Bayesian Molecular Clock Methods and Fossil-Based Soft Bounds Reveals Early Cenozoic Origin of African Lacertid Lizards. BMC Evol Biol 9: 151.

Holleley CE, O'Meally D, Sarre SD, Jennifer A. Marshall Graves, Ezaz T, Matsubara K, Azad B, Zhang X, Georges A (2015) Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523: 79-82.

Itoh Y, Melamed E, Yang X, Kampf K, Wang S, Yehya N, Van Nas A, Replogle K, Band MR, Clayton DF, Schadt EE, Lusis AJ, Arnold AP (2007) Dosage compensation is less effective in birds than in mammals. J Biol 6: 2.

IUCN 2015. The IUCN Red List of Threatened Species. Version 2015.1. http://www.iucnredlist.org. Accessed June 30, 2015.

Janzen FJ, Paukstis GL (1991) Environmental sex determination in reptiles: ecology,

evolution, and experimental design. Q Rev Biol 149-179.

Janzen FJ, Krenz JG (2004) Which was first, TSD or GSD. In: Valenzuela N, Lance VA, eds. Temperature-dependent sex determination in vertebrates. Washington: Smithsonian Books, pp 121-130.

Janzen FJ, Phillips PC (2006) Exploring the evolution of environmental sex determination, especially in reptiles. J Evol Biol 19: 1775-1784.

Johnson Pokorná M, Kratochvíl L (2014) What was the ancestral sex˜de termining mechanism in amniote vertebrates? Biol Rev Camb Philos Soc.

Johnson Pokorná M, Trifonov V, Rens W, Ferguson-Smith MA, Kratochvíl L (2015) Low rate of interchromosomal rearrangements during old radiation of gekkotan lizards (Squamata: Gekkota). Chromosome Res 23: 299-309.

Kapli P, Poulakakis N, Lymberakis P, Mylonas M (2011) A re-analysis of the molecular phylogeny of Lacertidae with currently available data. Basic and Applied Herpetology 25: 97104.

Kasai F, O'Brien PTM, Martin S, Ferguson-Smith MA (2003) Identification of the homologue of chicken Z chromosome in turtle and crocodile. Ann Génét 46: 89.

Kawagoshi T, Uno Y, Matsubara K, Matsuda Y, Nishida C (2009) The ZW micro-sex chromosomes of the Chinese soft-shelled turtle (*Pelodiscus sinensis*, Trionychidae, Testudines) have the same origin as chicken chromosome 15. Cytogenet Genome Res 125: 125-131.

Kawagoshi T, Nishida C, Matsuda Y (2012) The origin and differentiation process of X and Y chromosomes of the black marsh turtle (*Siebenrockiella crassicollis*, Geoemydidae, Testudines). Chromosome Res 20: 95-110.

Kawagoshi T, Uno Y, Nishida C, Matsuda Y (2014) The Staurotypus Turtles and Aves Share the Same Origin of Sex Chromosomes but Evolved Different Types of Heterogametic Sex Determination. PLoS ONE 9: e105315.

Kawai A, Nishida-Umehara C, Ishijima J, Tsuda Y, Ota H, Matsuda Y (2007) Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. Cytogenet Genome Res 117: 92-102.

Kawai A, Ishijima J, Nishida C, Kosaka A, Ota H, Kohno S, Matsuda Y (2009) The ZW sex

chromosomes of *Gekko hokouensis* (Gekkonidae, Squamata) represent highly conserved homology with those of avian species. Chromosoma 118: 43-51.

Kikuchi K, Hamaguchi S (2013) Novel sex-determining genes in fish and sex chromosome evolution. Dev Dyn 242: 339-353.

King M (1977) The Evolution of Sex Chromosomes in Lizards. In Calaly J, Tyndale-Biscoe H, eds. Evolution and Reproduction. Canberra: Australian Academy of Science, pp 55–60.

Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for Sry. Nature 351: 117-121.

Lahn BT, Page DC (1999) Four evolutionary strata on the human X chromosome. Science 286: 964-967.

Leaché AD, Sites J JW (2009) Chromosome Evolution and Diversification in North American Spiny Lizards (Genus *Sceloporus*). Cytogenet Genome Res 127: 166-181.

López-Flores I, Garrido-Ramos M (2012) The repetitive DNA content of eukaryotic genomes. In: Garrido-Ramos MA, ed. Repetitive DNA. Genome dynamics vol 7. Basel: Karger, pp 1-28.

Mank JE (2013) Sex chromosome dosage compensation: definitely not for everyone. Trends Genet 29: 677-683.

Mank JE, Ellegren H (2007) Parallel divergence and degradation of the avian W sex chromosome. Trends Ecol Evol 22: 389-391.

Mank JE, Hosken DJ, Wedell N (2011) Some inconvenient truths about sex chromosome dosage compensation and the potential role of sexual conflict. Evolution 65: 2133-2144.

Martinez PA, Ezaz T, Valenzuela N, Georges A, Graves JAm. (2008) An XX/XY heteromorphic sex chromosome system in the Australian chelid turtle *Emydura macquarii*: a new piece in the puzzle of sex chromosome evolution in turtles. Chromosome Res 16: 815-825.

Masumoto H, Nakano M, Ohzeki J (2004) The role of CENP-B and α-satellite DNA: de novo assembly and epigenetic maintenance of human centromeres. Chromosome Res 12: 543-556.

Matsubara K, Tarui H, Toriba M, Yamada K, Nishida-Umehara C, Agata K, Matsuda Y (2006)

Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. PNAS 103: 18190-18195.

Matsubara K, Kuraku S, Tarui H, Nishimura O, Nishida C, Agata K, Kumazawa Y, Matsuda Y (2012) Intra-genomic GC heterogeneity in sauropsids: evolutionary insights from cDNA mapping and GC3 profiling in snake. BMC genomics 13: 604.

Matsubara K, Gamble T, Matsuda Y, Zarkower D, Sarre SD, Georges A, Graves JAM, Ezaz T (2014) Non-Homologous Sex Chromosomes in Two Geckos (Gekkonidae: Gekkota) with Female Heterogamety. Cytogen Genome Res 143: 251-258.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417: 559-563.

Matsuda Y, Nishida-Umehara C, Tarui H, Kuroiwa A, Yamada,K, Isobe T, Ando J, Fujiwara A, Hirao Y, Nishimura O, Ishijima J. Hayashi A, Saito T, Murakami T, Murakami Y, Kuratani S, Agata K (2005) Highly conserved linkage homology between birds and turtles: Bird and turtle chromosomes are precise counterparts of each other. Chromosome Res 13: 601-615.

Mayer W, Arribas O (1996) Allozyme differentiation and relationships among the Iberian-Pyrenean Mountain lizards (Squamata: Sauria: Lacertidae). Herpetozoa 9: 57-61.

Mayer W, Arribas O (2003) Phylogenetic relationships of the European lacertid genera *Archaeolacerta* and *Iberolacerta* and their relationships to some other "*Archaeolacertae*" (sensu lato) from Near East, derived from mitochondrial DNA sequences. J Zool Syst Evol Res 41: 157-161.

Mengden G, Stock AD (1980) Chromosomal evolution in serpentes; a comparison of G and C chromosome banding patterns of some colubrid and boid genera. Chromosoma 79: 53-64.

Mertens R (1929) Zur Kenntnis der Eidechsenfauna Nordwest-Spaniens. Senckenbergische Naturforschende Gesellschaft 39: 282-289.

Meštrović N, Castagnone-Sereno P, Plohl M (2006) Interplay of Selective Pressure and Stochastic Events Directs Evolution of the MEL172 Satellite DNA Library in Root-

Knot Nematodes. Mol Biol Evolution 23: 2316-2325.

Miura I (2007) An Evolutionary Witness: the Frog *Rana rugosa* Underwent Change of Heterogametic Sex from XY Male to ZW Female. Sex Dev 1: 323-331.

Modi WS, Ivanov S, Gallagher DS (2004) Concerted evolution and higher-order repeat structure of the 1.709 (satellite IV) family in bovids. J Molecular Evol 58: 460-465.

Moritz C (1984) The evolution of a highly variable sex chromosome in *Gehyra purpurascens* (Gekkonidae). Chromosoma 90: 111-119.

Mravinac B, Plohl M, Mestrović N, Ugarković D (2002) Sequence of PRAT satellite DNA "frozen" in some Coleopteran species. J Mol Evol 54: 774-783.

Mravinac B, Ugarković Đ, Franjević D, Plohl M (2005) Long Inversely Oriented Subunits Form a Complex Monomer of *Tribolium brevicornis* Satellite DNA. J Mol Evol 60: 513-525.

Muller HJ (1914) A gene for the fourth chromosome of *Drosophila*. J Exp Zool 17: 325-336.

Müller L, Hellmich W (1936) Mitteilungen über die Herpetofauna der Iberischen Halbinsel II: Zur kenntnis der *Lacerta monticola*. Zoologischer Anzeiger 117: 65-73.

Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Schartl M (2002) A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. PNAS 99: 11778-11783.

Nanda I, Schlegelmilch K, Haaf T, Schartl M, Schmid M. (2008) Synteny conservation of the Z chromosome in 14 avian species (11 families) supports a role for Z dosage in avian sex determination. Cytogenet Genome Res 122: 150-156.

Navajas-Pérez R, de la Herrán R, Jamilena M Lozano R, Rejón CR, Rejón MR, Garrido-Ramos MA (2005) Reduced rates of sequence evolution of Y-linked satellite DNA in *Rumex* (Polygonaceae). J Mol Evol 60: 391-399.

Nijman IJ, Lenstra JA (2001) Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite DNA repeats. J Mol Evol 52: 361-371.

O'Meally D, Patel HR, Stiglec R, Sarre, SD, Geordes A, Graves, JAM, Ezaz T (2010) Non-homologous sex chromosomes of birds and snakes share repetitive sequences. Chromosome Res 18: 787-800.

O'Meally D, Ezaz T, Georges A, Sarre SD, Graves JA (2012) Are some chromosomes particularly good at sex? Insights from amniotes. Chromosome Res 20: 7-19.

Odierna G, Aprea G, Arribas OJ, Capriglione T, Caputo V, Olmo E (1996) The karyology of the Iberian rock lizards. Herpetologica 52: 542-550.

Odierna G, Heulin B, Guillaume C, Vogrin N, Aprea G, Capriglione T, Surget-Groba Y, Kupriyanova L (2001) Evolutionary and biogeographical implications of the karyological variations in the oviparous and viviparous forms of the lizard *Lacerta* (*Zootoca*) *vivipara*. Ecography 24: 332-340.

Odierna G, Arribas O (2004) Karyological and osteological data supporting the specific status of Iberolacerta (cyreni) martinezricai (Arribas, 1996). Amphibia-Reptilia 25:359–367.

Ohno S (1967) Sex Chromosomes and Sex-Linked Genes. Heidelberg, Germany: Springer-Verlag.

Ohno S (1972) So much "junk" DNA in our genome. Brookhaven Symp Biol 23: 366-370.

Ohno S, Stenius C, Christian LC, Becak W, Becak ML (1964) Chromosomal uniformity in the avian subclass Carinatae. Chromosoma 15: 280-288.

Olmo E (2008) Trends in the evolution of reptilian chromosomes. Integr Comp Biol 48: 486-493.

Olmo E, Odierna G, Cobror O (1986) C-band variability and phylogeny of Lacertidae. Genetica 71: 63-74.

Olmo, E., Odierna, G., Capriglione, T. (1991). A Karyological approach to the systematics of Lacertidae (Reptilia, Sauria). Rev Esp Herpetol 6: 81-90.

Olmo E, Signorino G (2005) Chromorep: a reptiles chromosomes database. http://chromorep.univpm.it/. Accessed June 20, 2015.

Organ CL, Janes DE (2008) Evolution of sex chromosomes in Sauropsida. Integr Comp Biol 48: 512-519.

Orgel LE, Crick FHC (1980) Selfish DNA: the ultimate parasite. Nature 284: 604-607.

Palomeque T, Muñoz-López M, Carrillo J, Lorite P (2005) Characterization and evolutionary dynamics of a complex family of satellite DNA in the leaf beetle *Chrysolina carnifex* (Coleoptera, Chrysomelidae). Chromosome Res 13: 795-807.

Pavlicev M, Mayer W (2009) Fast radiation of the subfamily Lacertinae (Reptilia: Lacertidae): history or methodical artefact? Mol Phylogenet Evol 52: 727-734.

Pérez-Mellado V (2002) *Lacerta monticola* Boulenger, 1905. Lagartija serrana. In: Pleguezuelos JM, Márquez R, Lizana M, eds. Atlas y libro rojo de los anfibios y reptiles de España. Madrid: Dirección General de Conservación de la Naturaleza-A.H.E., pp. 227-229.

Pérez-Mellado V, Barbadillo LJ, Barahona F, Brown RP, Corti C, Guerrero F, Lanza, B (1993) A systematic survey of the Iberian Rock lizard *Lacerta* (*Archaeolacerta*) *monticola*. In: Valakos E, Bohme W, Pérez-Mellado V, Maragou P, eds. Lacertids of the Mediterranean region. Athens: Hellenic Zoological Society, pp 85-105.

Pezer Ž, Brajković J, Feliciello I, Ugarković D. Satellite DNA-mediated effects on genome regulation. In: Garrido-Ramos MA, ed. Repetitive DNA. Genome dynamics vol 7. Basel: Karger, pp 153–169.

Plohl M, Luchetti A, Meštrović N, Mantovani B (2008) Satellite DNAs between selfishness and functionality: Structure, genomics and evolution of tandem repeats in centromeric (hetero)chromatin. Gene 409: 72-82.

Plohl M, Petrovic V, Luchetti A, Ricci A, Satović E, Passamonti M, Mantovani B (2010) Long-term conservation vs high sequence divergence: the case of an extraordinarily old satellite DNA in bivalve mollusks. Heredity 104: 543-551.

Plohl M, Meštrović N, Mravinac B (2012) Satellite DNA evolution. In: Garrido-Ramos MA, ed. Repetitive DNA. Genome dynamics vol 7. Basel: Karger, pp 126–152.

Pokorná M, Kratochvíl L (2009) Phylogeny of sex-dermining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? Zool J Linn Soc 156: 168–183.

Pokorná M, Giovannotti M, Kratochvíl L, Kasai F, Trifonov VA, O'Brien PC, Caputo V, Olmo E, Ferguson-Smith MA, Rens W (2011) Strong conservation of the bird Z chromosome in reptilian genomes is revealed by comparative painting despite 275 million years divergence. Chromosoma 120: 455-468.

Pokorná M, Giovannotti M, Kratochvíl L, Caputo V, Olmo E, Ferguson-Smith MA, Rens W (2012) Conservation of chromosomes syntenic with avian autosomes in squamate reptiles revealed by comparative chromosome painting. Chromosoma 121: 409-418.

Pyron RA, Burbrink FT, Wiens JJ (2013) A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol Biol 13: 93.

Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves J AM (2007) Temperature sex reversal implies sex gene dosage in a reptile. Science 316: 411.

Radder RS, Quinn AE, Georges A, Sarre SD, Shine R (2008) Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. Biol Lett 4: 176-178.

Rens W, O'Brien PC, Grützner F, Clarke O, Graphodatskaya D, Tsend-Ayush E, Trifonov VA, Skelton H, Wallis MC, Johnston S, Veyrunes F, Graves JAM, Ferguson-Smith MA (2007) The multiple sex chromosomes of platypus and echidna are not completely identical and several share homology with the avian Z. Genome Biol 8: R243-R243.

Rhen T, Schroeder A (2010) Molecular mechanisms of sex determination in reptiles. Sex Dev 4: 16-28.

Rice WR (1987) The accumulation of sexually antagonistic genes as a selective agent promoting the evolution of reduced recombination between primitive sex chromosomes. Evolution 911-914.

Richard GF, Kerrest A, Dujon B (2008) Comparative Genomics and Molecular Dynamics of DNA Repeats in Eukaryotes. Microbiol Mol Biol Rev 72: 686-727.

Robles F, de la Herrán R, Ludwig A, Ruiz Rejón C, Ruiz Rejón M, Garrido-Ramos MA (2004) Evolution of ancient satellite DNAs in sturgeon genomes. Gene 338: 133-142.

Rodionov, A.V. (1997) Evolution of avian chromosomes and linkage groups. Russ J Genet 33: 605-617.

Ross JA, Urton JR, Boland J, Shapiro MD, Peichel CL, Mank JE (2009) Turnover of Sex Chromosomes in the Stickleback Fishes (Gasterosteidae). PLoS Genet 5: e1000391.

Rovatsos M, Altmanová M, Pokorná M, Kratochvíl L (2014a) Conserved Sex Chromosomes Across Adaptively Radiated *Anolis* Lizards. Evolution 68: 2079-2085.

Rovatsos M, Pokorná M, Altmanová M, Kratochvíl L (2014b) Cretaceous park of sex determination: sex chromosomes are conserved across iguanas. Biol Letters. 10: 20131093.

Rudd M K, Wray GA, Willard HF (2006) The evolutionary dynamics of α-satellite. Genome

Res 16: 88-96.

Salvador A (1974) Guía de los Anfibios y Reptiles españoles. Madrid: ICONA.

Salvador A (1984) *Lacerta monticola* Boulenger, 1905. Iberische Gebirgseidechse. In: Böhme W, ed. Handbuch der Reptilien und Amphibien Europas. Wiesbaden: Aula Verlag, pp 276-289.

Sarre SD, Georges A, Quinn A (2004) The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26: 639-645.

Sarre SD, Ezaz T, Georges A (2011) Transitions between sex-determining systems in reptiles and amphibians. Annu Rev Genomics Hum Genet 12: 391-406.

Schmidt T, Heslop-Harrison JS (1998). Genomes, genes, and junk: The large-scale organization of plant chromosomes. Trends Plant Sci. 3**:** 195–199.

Shedlock AM, Edwards SV (2009) Amniota. In: Hedges SB, Kumar S, eds. The timetree of life. New York: Oxford University Press, pp 375-379.

Shetty S, Griffin DK, Graves JAM (1999) Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. Chromosome Res 7: 289-295.

Shine R, Elphick MJ, Donnellan S (2002) Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecol Lett 5: 486-489.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346: 240-244.

Slamovits CH, Cook JA, Lessa EP, Susana Rossi M (2001) Recurrent Amplifications and Deletions of Satellite DNA Accompanied Chromosomal Diversification in South American Tuco-tucos (Genus *Ctenomys*, Rodentia: Octodontidae): A Phylogenetic Approach. Mol Biol Evol 18: 1708-1719.

Slamovits CH, Rossi MS (2002) Satellite DNA: agent of chromosomal evolution in mammals. A review. Mastozool Neotrop 9: 297-308.

Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH (2009) The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. Nature 461: 267-271.

Solari AJ (1993) Sex Chromosomes and Sex Determination in Vertebrates. Boca Raton, FL: CRC Press.

Srikulnath K, Matsubara K, Uno Y, Thongpan A, Suputtitada S, Apisitwanich S, Matsuda Y, Nishida C (2009) Karyological characterization of the butterfly lizard (*Leiolepis reevesii rubritaeniata*, Agamidae, Squamata) by molecular cytogenetic approach. Cytogenet Genome Res 125: 213-223.

Srikulnath K, Uno Y, Nishida C, Matsuda Y (2013) Karyotype evolution in monitor lizards: cross-species chromosome mapping of cDNA reveals highly conserved synteny and gene order in the Toxicofera clade. Chromosome Res 21: 805-819.

Srikulnath K, Matsubara K, Uno Y, Nishida C, Olsson M, Matsuda Y (2014) Identification of the linkage group of the Z sex chromosomes of the sand lizard (*Lacerta agilis*, Lacertidae) and elucidation of karyotype evolution in lacertid lizards. Chromosoma 123: 563-575.

Steinemann S, Steinemann M (2005) Y chromosomes: born to be destroyed. Bioessays 27: 1076-1083.

Stephan W (1986) Recombination and the evolution of satellite DNA. Genet Res 47: 167-174.

Stevens NM (1905) Studies in Spermatogenesis with Especial Reference to the "Accessory Chromosome" vol. 36. Carnegie Institution of Washington Publication.

Stiglec R, Ezaz T, Graves JAM (2007) A new look at the evolution of avian sex chromosomes. Cytogenet Genome Res 117: 103-109.

Suzuki K (1930) On the chromosomes of the domestic fowl. Zool Mag (Japan) 42: 358-359.

Takehana Y, Naruse K, Hamaguchi S, Sakaizumi M (2007) Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, *Oryzias hubbsi* and *O. dancena*. Chromosoma 116: 463-470.

Townsend TM, Larson A, Louis E, Macey JR (2004) Molecular Phylogenetics of Squamata: The Position of Snakes, Amphisbaenians, and Dibamids, and the Root of the Squamate Tree. Syst Biol 53: 735-757.

Trifonov V, Giovannotti M, O'Brien PM, Wallduck M, Lovell F, Rens W, Parise-Maltempi PP, Caputo V, Ferguson-Smith MA (2011) Chromosomal evolution in Gekkonidae. I. Chromosome painting between *Gekko* and *Hemidactylus* species reveals phylogenetic

relationships within the group. Chromosome Res 19: 843-855.

Tzika AC, Ullate-Agote A, Grbic D, Milinkovitch MC (2015) Reptilian Transcriptomes v2.0: An Extensive Resource for Sauropsida Genomics and Transcriptomics. Genome Biology and Evolution 7: 1827-1841.

Ugarković D (2009) Centromere-competent DNA: structure and evolution. Prog Mol Subcell Biol 48: 53–76.

Uno Y, Nishida C, Tarui H, Ishishita S, Takagi C, Nishimura O, Ishijima J, Ota H, Kosaka A, Matsubara K, Murakami Y, Kuratani S, Ueno N, Agata K, Matsuda Y (2012) Inference of the protokaryotypes of amniotes and tetrapods and the evolutionary processes of microchromosomes from comparative gene mapping. PLoS ONE 7: e53027.

Valenzuela N, Adams DC, Janzen FJ (2003) Pattern does not equal process: exactly when is sex environmentally determined? Amer Nat 161: 676-683.

Valenzuela N, Lance V (2004) Temperature-dependent sex determination in vertebrates. Washington: Smithsonian Books.

Valenzuela N, Adams DC (2011) Chromosome number and sex determination coevolve in turtles. Evolution 65: 1808-1813.

Veyrunes F, Waters PD, Miethke P, Rens W, McMillan D, Alsop AE, Grützner F, Deakin JE, Whittington CM, Schatzkamer K, Kremitzki CL, Graves T, Ferguson-Smith MA, Warren W, Graves JAM. (2008) Bird-like sex chromosomes of platypus imply recent origin of mammal sex chromosomes. Genome Res 18: 965-973.

Vicoso B, Bachtrog D (2013) Reversal of an ancient sex chromosome to an autosome in *Drosophila*. Nature 499: 332-335.

Vicoso B, Emerson JJ, Zektser Y, Bachtrog, D (2013a) Comparative Sex Chromosome Genomics in Snakes: Differentiation, Evolutionary Strata, and Lack of Global Dosage Compensation. PLoS Biology 11: e1001643.

Vicoso B, Kaiser VB, Bachtrog D (2013b) Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. PNAS 110: 6453-6458.

Vicoso B, Bachtrog D (2015) Numerous Transitions of Sex Chromosomes in Diptera. PLoS Biol 13: e1002078.

Vidal N, Hedges SB (2005) The phylogeny of squamate reptiles (lizards, snakes, and amphisbaenians) inferred from nine nuclear protein-coding genes. C R Biol 328: 1000-1008.

Wang J, Na J-K, Yu Q, Gschwend AR, Han J, Zeng F, Aryal R, VanBuren R, Murray JE, Zhang W, Navajas-Pérez R, Feltus FA, Lemke C, Tong EJ, Chen C, Wai CM, Singh R, Wang ML, Min XJ, Alam M, Charlesworth D, Moore PH, Jiang J, Paterson AH, Ming R (2012) Sequencing papaya X and Yh chromosomes reveals molecular basis of incipient sex chromosome evolution. PNAS 109: 13710-13715.

Warburton PE, Waye JS, Willard HF (1993) Nonrandom localization of recombination events in human alpha satellite repeat unit variants: implications for higher-order structural characteristics within centromeric heterochromatin. Mol Cell Biol 13: 6520-6529.

White MJD (1973) Animal cytology and evolution. Cambridge: Cambridge University Press.

Wichman HA, Payne CT, Ryder OA, Hamilton MJ, Maltbie M, Baker RJ (1991) Genomic Distribution of Heterochromatic Sequences in Equids: Implications to Rapid Chromosomal Evolution. J Hered 82: 369-377.

Willard HF, Waye JS (1987) Chromosome-specific subsets of human alpha satellite DNA: analysis of sequence divergence within and between chromosomal subsets and evidence for an ancestral pentameric repeat. J Mol Evol 25: 207-214.

Wilson EB (1905) The chromosomes in relation to the determination of sex in insects. Science 22: 500-502.

Yoshimoto S, Okada E, Umemoto H, Tamura K, Uno Y, Nishida-Umehara C, Matsuda Y, Takamatsu N, Shiba T, Ito M. (2008) A W-linked DM-domain gene, *DM-W*, participates in primary ovary development in *Xenopus laevis*. PNAS 105: 2469-2474.

Young MJ, O'Meally D, Sarre SD, Georges A, Ezaz T (2013) Molecular cytogenetic map of the central bearded dragon, *Pogona vitticeps* (Squamata: Agamidae). Chromosome Res 21: 361-374.

Zhou Q, Ellison CE, Kaiser VB, Alekseyenko AA, Gorchakov AA, Bachtrog D (2013) The Epigenome of Evolving *Drosophila* Neo-Sex Chromosomes: Dosage Compensation and Heterochromatin Formation. PLoS Biol 11: e1001711.

AIMS AND OBJECTIVES

Aims and objectives

Within the broad aim of investigating sex chromosome and karyotype evolution in lacertid lizards, the particular objectives of this thesis are:

- To better characterize the karyotype of *Iberolacerta monticola* and potentially identify cryptic sex chromosomes; and to compare these new data with the cytogenetic information available for other *Iberolacerta* species, in order to clarify chromosome evolution within this genus.

- To carry out a comparative analysis of sex chromosomes and evaluate the homology of ZW systems among five lacertid species using molecular cytogenetic techniques; to perform a genome-wide comparison and detect chromosomal rearrangements between *I. monticola*, *Timon lepidus* and *Lacerta schreiberi*; and to investigate chromosome homology between *I. monticola* and more distantly related reptilian species through PCR-assisted gene mapping.

- To analyze the patterns of sequence variability, genomic organization, and chromosomal distribution of two satellite DNA families in all eight *Iberolacerta* species, in order to understand the processes that determine the structure and evolutionary dynamics of these repetitive elements, and their possible role in chromosomal evolution.

CHAPTER I

Karyological characterization of the endemic Iberian rock lizard, *Iberolacerta monticola* (Squamata, Lacertidae): insights into sex chromosome evolution

Abstract

Rock lizards of the genus *Iberolacerta* constitute a promising model to examine the process of sex chromosome evolution, as these closely related taxa exhibit remarkable diversity in the degree of sex chromosome differentiation with no clear phylogenetic segregation, ranging from cryptic to highly heteromorphic ZW chromosomes and even multiple chromosome systems ($Z_1Z_1Z_2Z_2/Z_1Z_2W$). To gain a deeper insight into the patterns of karyotype and sex chromosomes evolution, we performed a cytogenetic analysis based on conventional and differential staining, fluorescence in situ hybridization and comparative genomic hybridization (CGH) in the species *Iberolacerta monticola*, for which previous cytogenetic investigations did not detect differentiated sex chromosomes. The karyotype is composed of 2n=36 acrocentric chromosomes. NORs and the major ribosomal genes were located in the subtelomeric region of chromosome pair 6. Hybridization signals of the telomeric sequences $(TTAGGG)_n$ were visualized at the telomeres of all chromosomes and interstitially in five chromosome pairs. C-banding showed constitutive heterochromatin at the centromeres of all chromosomes, as well as clear pericentromeric and light telomeric C-bands in several chromosome pairs. These results highlight some chromosomal markers which can be useful to identify species diagnostic characters, although may not accurately reflect the phylogenetic relationships among taxa. In addition, C-banding and CGH revealed the presence of a heteromorphic ZW sex chromosome pair, where W is smaller than Z and almost completely heterochromatic, showing a massive accumulation of female-specific sequences. This finding sheds light on sex chromosome evolution in the genus *Iberolacerta* and suggests that further comparative cytogenetic analyses are needed to understand the processes underlying the origin, differentiation and plasticity of sex chromosome systems in lacertid lizards.

Key Words: Rock lizards · Comparative cytogenetics · Chromosome banding · FISH · Sex chromosomes

Introduction

The genus *Iberolacerta* is a group of rock lizards (family Lacertidae) mainly distributed in highland areas of Western Europe. According to recent taxonomic revisions (Mayer and Arribas 2003; Arribas and Carranza 2004; Carranza et al. 2004; Crochet et al. 2004; Arribas and Odierna 2005; Arribas et al. 2006), the genus *Iberolacerta* comprises eight species, which can be subdivided into three main units: 1) *I. horvathi*, occurring in the Eastern Alps and the north of the Dinaric Chains; 2) the subgenus *Pyrenesaura*, which includes the three species found in the Pyrenees Mountains, namely *I. aranica*, *I. aurelioi* and *I. bonnali*; and 3) the four species included in the "Iberian group", i.e*., I. cyreni*, *I. martinezricai*, *I. galani* and *I. monticola,* with disjunct distributions in central and northern mountain ranges of the Iberian Peninsula.

The phylogeny of this genus has been under continual revision, but the evolutionary relationships among some taxa still remain unresolved (Mayer and Arribas 2003; Carranza et al. 2004; Crochet et al. 2004; Arribas et al. 2006, 2014). Within the Iberian group, data from mitochondrial genes suggest that *I. cyreni* split earlier, between 7.3 and 8.5 mya (million years ago), while the speciation events within the clade formed by *I. martinezricai*, *I. galani* and *I. monticola* occurred considerably later, at the beginning of the Pleistocene (roughly 2.5 mya). Recent molecular analyses support the hypothesis that *I. monticola* was the first lineage to diverge from the common branch, shortly before the separation of *I. martinezricai* and *I. galani,* approximately 1.8 mya (Remón et al. 2013) (Fig. S1, Supplementary Material).

Karyological studies based on conventional staining and banding techniques have proven useful for establishing phylogenetic relationships and delimiting species and subspecies boundaries in the genus *Iberolacerta*, as well as in several other lacertid groups (e.g., Olmo et al. 1993; Odierna et al. 1996; Bosch et al. 2003; Kupriyanova and Melashchenko 2011). Previous cytogenetic surveys of the *Iberolacerta* species (Capula et al. 1989; Odierna et al. 1996; Arribas and Odierna 2005; Arribas et al. 2006) showed a common diploid number of 2n=36 and a similar karyotypic macrostructure, with all chromosomes acrocentric. Only the karyotypes of the three *Iberolacerta* species from the Pyrenees differ from this formula, with reduced diploid numbers that range from 2n=24 to 26 in males and from 23 to 26 in females and numerous biarmed chromosomes, which probably evolved from the ancestral acrocentric complement through a series of Robertsonian fusions (Odierna et al. 1996) (Fig. S1, Supplementary Material).

Interestingly, C-banding analyses uncovered high levels of diversity regarding the sex chromosome system. A ZW sex chromosome pair, in which the W chromosome is smaller than the Z and highly heterochromatic, has been described in *I. horvathi*, *I. cyreni* and *I. galani* (Capula et al. 1989; Odierna et al. 1996; Arribas et al. 2006). In contrast, the sex chromosomes of *I. aranica*, *I. martinezricai* and *I. monticola* are reported to be homomorphic and indistinguishable by differences in size, morphology or heterochromatinization (Odierna et al. 1996; Arribas and Odierna 2005). More significant differences are present in the Pyrenean species *I. bonnali* and *I. aurelioi*, with multiple $Z_1Z_2W/Z_1Z_2Z_1Z_2$ sex chromosome systems where the W chromosome is biarmed and the Z_1 and Z_2 counterparts are uniarmed (Odierna et al. 1996) (Fig. S1, Supplementary Material). The presence of ZW-derived multiple sex chromosome systems is a particularly uncommon feature within lizards, so far reported for only two other species of lacertids, namely *Zootoca vivipara* and *Podarcis taurica* (Olmo and Signorino 2005).

The heterogeneous situation concerning sex chromosomes in the genus *Iberolacerta* is illustrative of the wide diversity of sex chromosomes found in the family Lacertidae. Female heterogamety is considered to be universal within this family. Even so, sex chromosomes at different stages of differentiation are frequently found between closely related species and even between populations of the same species, suggesting that sex chromosomes can have multiple and independent origins in related lacertid taxa (e.g., Olmo et al. 1987; Odierna et al. 1993, 2001; Bosch et al. 2003).

Typically, sex chromosomes are thought to evolve after suppression of recombination through increasing stages of differentiation, from a primitive form, in which nascent sex chromosomes differ only in a limited region and are otherwise indistinguishable, to an advanced state in which sex chromosomes are highly heteromorphic (Charlesworth et al. 2005; recently reviewed in Charlesworth and Mank 2010). Reports on lacertid karyotypes, mainly accomplished through conventional banding techniques, suggest that lacertid sex chromosomes have evolved primarily via heterochromatinization followed by degeneration of the female-specific W chromosome, although this is probably not the only mechanism operating in this family (Olmo et al. 1986; Olmo et al. 1987; Ezaz et al. 2009). Chromosomal rearrangements, such as inversions or translocations, can be also involved in the primary differentiation of lizard sex chromosomes (for a review see Olmo et al. 1987; Ezaz et al. 2009), implying that even newly evolved sex chromosomes can be heteromorphic

(Charlesworth and Mank 2010). In this regard, comparative cytogenetic analyses within the genus *Iberolacerta* can provide valuable insights into the processes underlying the origin, differentiation and evolutionary transitions of sex chromosomes.

In this study, we focus on one of the *Iberolacerta* species for which previous cytogenetic investigations did not detect differentiated sex chromosomes, *I. monticola*. This species is distributed across a wide area in the north of the Iberian Peninsula, along the Cantabrian mountain range, where it inhabits mainly rocky habitats at middle-high altitudes (Mayer and Arribas 2003; Crochet et al. 2004; Carranza et al. 2004). Apart from this continuous area, there are several other isolated populations in the Serra da Estrela mountains, in Portugal, and in Galicia, at the north-west corner of Spain (fig. 1). Some populations in this last region are found at areas of exceptionally low altitudes, most of them associated to Atlantic forests in shady fluvial gorges (Galán 1999; Galán et al. 2007).

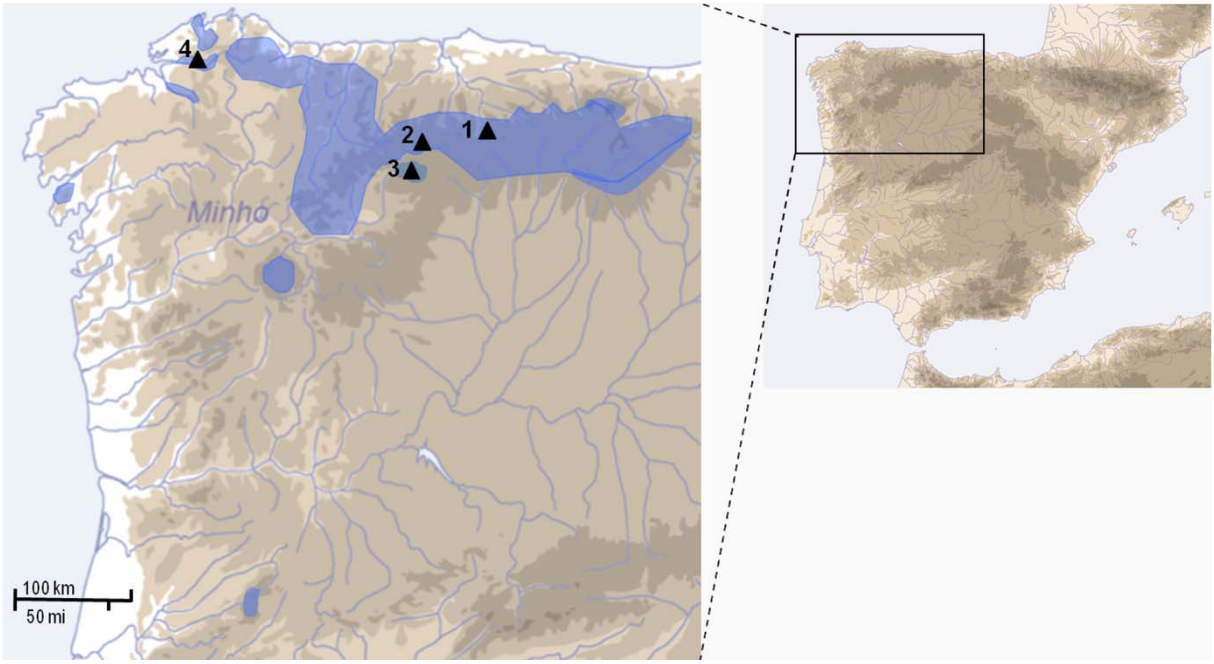


Fig. 1. Map of the Iberian Peninsula showing the current distribution area of *I. monticola* (blue areas). Numbers represent localities sampled in the present study: (1) Puerto de Vegarada, (2) Villabandín, (3) Salientes and (4) Eume. See text for further details.

The karyotype of *I. monticola* has been previously described based on conventional staining and banding techniques (C-banding and silver (Ag)-staining) for the populations of Puerto de Vegarada, in the Cantabrian mountains, and Serra da Estrela (Odierna et al. 1996). Here, we

re-investigate the specimens from the Cantabrian population (locality 1 in Fig. 1) and extend the cytogenetic analysis to two additional isolated populations from the Cantabrian area, Villabandín and Salientes (localities 2 and 3 in Fig. 1, respectively), as well as to the lowland population of Eume, in the northwestern-most edge of the species' range (locality 4 in Fig. 1), with the aim to (1) better characterize the karyotype of *I. monticola*, and perform a comparative cytogenetic analysis within a phylogenetic framework, in order to clarify chromosome evolution within the genus *Iberolacerta* and (2) search for sex-specific differences that enable the identification of cryptic sex chromosomes. This was accomplished by using conventional staining and banding techniques (C-banding and differential fluorochrome staining), as well as comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) with 18S-5.8S-28S rDNA and telomeric $(TTAGGG)_n$ probes.

Material and Methods

Specimens

One adult male and one adult female of *I. monticola* were collected from each of the following localities: 1) Puerto de Vegarada (UTM: 30T TN98), 2) Villabandín (UTM: 29T QH35), 3) Salientes (UTM: 29T QH19) and 4) the fluvial valley of the river Eume (UTM: 29T NJ70) (Fig. 1). Permissions for fieldwork and ethics approval of experimental procedures were issued by the competent authorities, *Xunta de Galicia* and *Junta de Castilla-León*, in Spain, in accordance with the Spanish legislation (Royal Decree 1201/2005 and Law 32/2007, on the protection of animals used for experimentation and other scientific purposes).

Phenotypic sex was determined on the basis of external morphology and then confirmed via visual inspection of gonads upon dissection.

Cell culture and chromosome preparations

Metaphase chromosome spreads were prepared according to previously described protocols (Giovannotti et al. 2009a). Fibroblast cell lines were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco) and 2 mM L-glutamine (Gibco). Cultures were incubated at 30ºC in a humidified atmosphere of 5% CO_2 in air. When exponential cell growth was observed around the primary explants (usually after 2-3 weeks of culture) the cells were trypsinized and

subcultivated at a 1:2 split ratio. Following this first passage, the cell lines were grown until a 70-80% of overall confluence was reached. Six hours prior to harvesting, 0.1 μg/ml colcemid (Roche) was added to the cultures followed by 30 min of hypotonic treatment in 0.075 M KCl at 30ºC and fixation in 3:1 methanol:glacial acetic acid. Fifteen microlitres of cell suspension were dropped onto glass slides and air-dried.

Chromosome staining and banding

Conventional chromosome staining was performed using a 5% Giemsa solution at pH 7. C-banding was carried out according to Sumner (1972). C-banded chromosomes were independently stained with 10% Giemsa solution at pH 7 for 10 minutes, Chromomycin A_3 (CMA_3), 4',6-diamidino-2-phenylindole (DAPI), and sequentially stained with both fluorochromes (Schweizer 1976; Schmid et al. 1983). Ag-staining of nucleolar organizer regions (Ag-NORs) was performed as described by Howell and Black (1980).

Fluorescence in situ hybridization (FISH)

Chromosomal locations of the 18S-5.8S-28S rRNA genes were determined by FISH as described in González-Tizón et al. (2000), with slight modifications, using the DNA probe p*Dm* 238 from *Drosophila melanogaster* (Roiha et al. 1981), labeled by nick translation with digoxigenin-11-dUTP (Roche).

Briefly, the slides were dehydrated by serial ethanol washes (twice for 2 min in 70% (vol/vol) ethanol, twice for 2 min in 90% ethanol and once for 5 min in 100% ethanol), air dried, and aged at 65ºC for 30 min. Subsequently, they were incubated in DNase-free RNase (100 μg/ml in 2x SSC) at 37ºC for 30 min and washed in 2x SSC for 10 min. One hundred ng of labelled probe (2.5 μl) were made up to 30 μl with hybridization buffer (50% formamide, 2xSSC and 10% dextran sulphate), denatured at 75ºC for 15 min, chilled on ice, placed onto each slide, covered with a coverslip, and finally sealed with rubber cement. Chromosome denaturation was performed in a slide-PCR (MJ Research, MJ 100) as follows: 75ºC for 7 min, 55ºC for 2 min, 50ºC for 30 s, 45ºC for 1 min, 42ºC for 2 min, 40ºC for 5 min, 38ºC for 5 min and 37ºC for 5 min. Hybridization took place at 37ºC overnight in a humid chamber. Post-hybridization washes consisted of two 5-min incubations in 2x SSC at 37ºC and at room temperature respectively, followed by a 5-min incubation in 0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20 at room temperature. Signal detection included three consecutive incubation steps, at 37ºC for 30 min each, with: i) mouse anti-digoxigenin antibody (Roche), ii) fluorescein

isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich), and iii) FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich). After each incubation step, slides were washed three times for 5 min with 0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20 at room temperature. Chromosomes were counterstained with 1.5 μg/ml propidium iodide in anti-fade medium Vectashield (Vector Laboratories).

Chromosome mapping of the $(TTAGGG)_n$ sites was carried out with a Cy3-labeled pan-telomeric DNA probe (Cambio) following the manufacturer's instructions. The slides were mounted using the anti-fade medium Vectashield (Vector Laboratories), containing 1.5 μg/ml DAPI.

Comparative genomic hybridization (CGH)

Total genomic DNA was extracted from ethanol preserved tissues of one male and one female of *I. monticola* using a commercial kit (RealPure Genomic DNA Extraction Kit, Durviz), following the manufacturer's instructions. Female genomic DNA was labeled with FITC-dUTP while male genomic DNA was labeled with TRITC-dUTP using the Prime-It Random Priming Labeling Kit (Agilent Technologies), according to the manufacturer's specifications.

CGH was performed following Ezaz et al. (2005) with minor modifications. For each slide that was made, 250 ng of FITC-labeled female and 250 ng of TRITC-labeled male DNA were ethanol-precipitated with 20 μg of glycogen and 4 μg of unlabeled, sheared genomic DNA from the homogametic sex (male). Metaphase chromosome slides were dehydrated through ethanol series; aged at 65°C for 1 h; denatured in 70% formamide/2x SSC at 70ºC for 1-2 min, dehydrated again and air-dried at room temperature until hybridization. Following an overnight co-precipitation at -20ºC, the probe mix was centrifuged at 14,000 x g at 4ºC. The supernant was discarded and the probe DNA pellet was resuspended in 20 μL of 37ºC pre-warmed hybridization buffer (50% formamide, 10% dextran sulfate, 2x SSC and 40 mmol/L sodium phosphate pH7.0) and resuspended at 37ºC for at least 30 min. The hybridization mixture was denatured at 70ºC for 10 min, inmediately chilled on ice for 5 min, and then 15 μL of the probe mixture was placed as a single drop per slide. Slides were covered with a coverslip, sealed with rubber cement and placed inside a humid chamber at 37ºC for 3 days. Post-hybridization washes were performed in 0.4x SSC, 0.3% Tween 20 at 55ºC for 2 min, and in 2x SSC, 0.1% Tween 20 at room temperature for 2 min. Slides were left to air dry at room temperature and mounted with anti-fade medium Vectashield with DAPI (Vector

Laboratories).

Microscopy and data analyses

Images were captured using an epifluorescence microscope Nikon Microphot-FXA equipped with a cooled CCD camera (DS-Qi1Mc, Nikon Instruments). The NIS-Elements D 3.10 software (Nikon Instruments) was used to capture grey-scale images of DAPI, Cy3/TRITC and FITC signals, which were then merged into a color image. Karyotypes were reconstructed from reversed greyscale images of C-banded metaphases with Adobe Photoshop CS4 11.0.1 (Adobe Systems Inc.).

Results

Karyotypes, heterochromatin distribution and fluorochrome staining

All analyzed specimens of *I. monticola* showed a karyotype composed of 2n=36 acrocentric chromosomes of gradually decreasing size (Fig. 2).

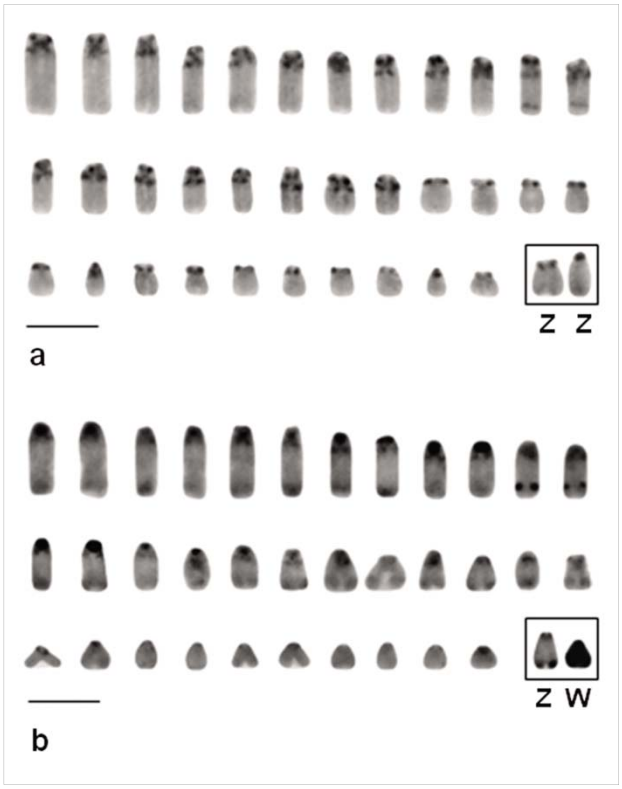


Fig. 2. C-banded karyotypes of *I. monticola* male (**a**) and female (**b**) from the population of Eume. In the inset, sex chromosome pairs ZZ and ZW. Scale bars represent 5 μm.

C-banding evidenced constitutive heterochromatin at the centromeres of all chromosomes, and interstitially at the pericentromeric regions of the ten larger chromosome pairs (Figs. 3 and

4). These conspicuous heterochromatic blocks were uniformly stained with both DAPI and CMA_3 and hence they do not seem to contain particularly AT- or GC- rich repetitive DNA families (Figs. 3c-f and 4). Faint C-positive bands were also found at the ends of several chromosome pairs (tentatively, in the twelve larger chromosome pairs) and resulted only positively stained by CMA_3, indicating that this telomeric heterochromatin was composed of GC-rich sequences. In addition, CMA_3 staining produced an intense fluorescent signal in the subterminal region of a large chromosome pair, probably correlated with NORs-associated heterochromatin (Figs. 3e, f and 4c, d).

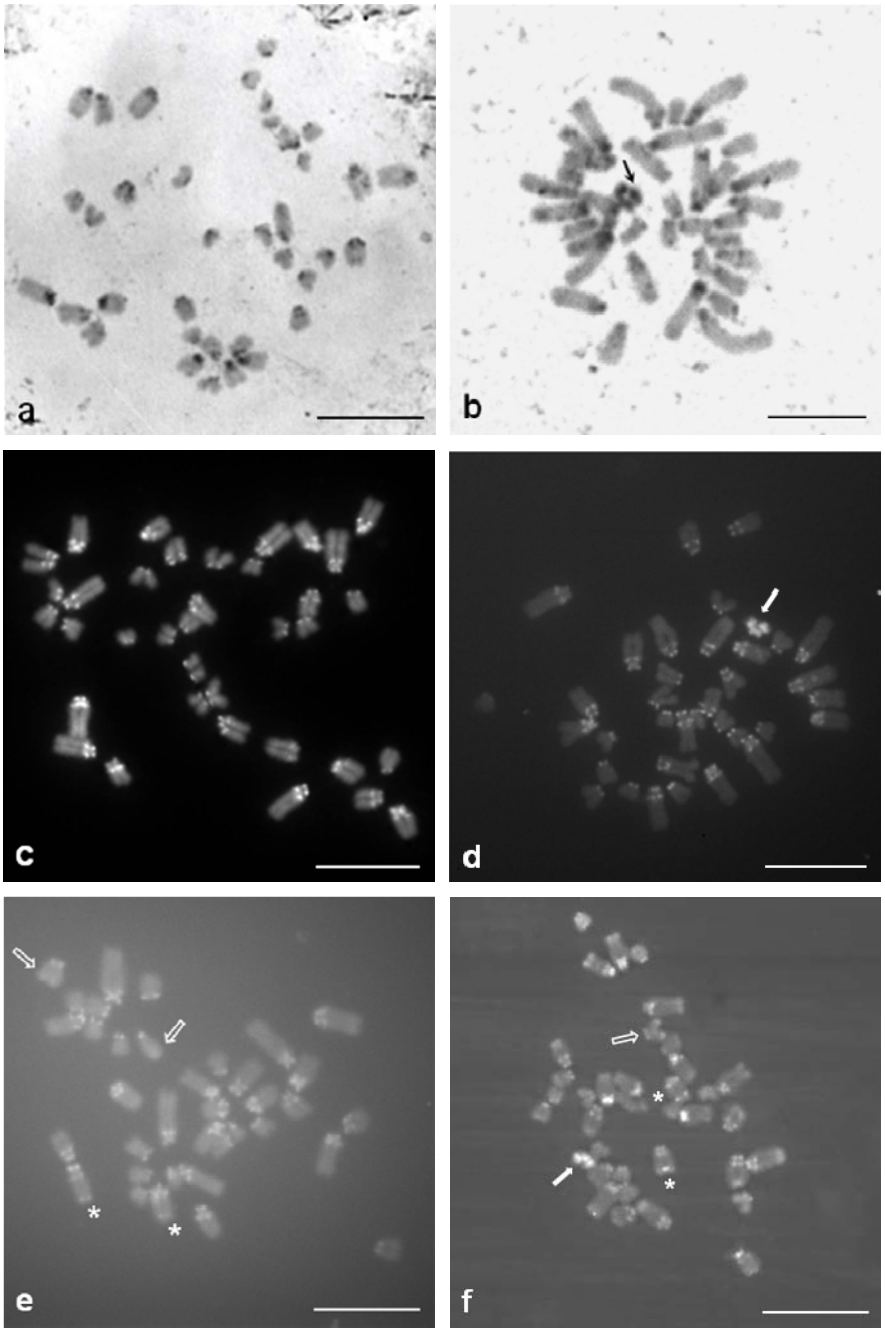


Fig. 3. Metaphase plates of *I. monticola* male (**a**, **c**, **e**) and female from Eume (**b, d, f**) C-banded and stained with Giemsa (**a**, **b**), DAPI (**c, d**) and CMA_3 (**e, f**). Asterisks in **e** and **f** indicate CMA_3 positive signals associated with NORs. Empty and filled arrows point to Z and W sex chromosomes, respectively. Scale bars represent 10 μm.

The differences in the pattern of heterochromatin distribution between sexes clearly revealed the presence of a cytologically differentiated ZW sex chromosome pair. The W chromosome was easily recognizable in female metaphases, being one of the smallest chromosomes of the karyotype (Fig. 2b) and almost completely heterochromatic, with only a small euchromatic region located in an interstitial position (Fig. 3b). The heterochromatin of the W chromosome was intensely stained with both DAPI and CMA_3 (Figs. 3d, f and 4b, d). C-banding also allowed the identification of the Z chromosome, present in two copies in males and in single copy in females. This element was as large as the chromosomes of the 9th or 10th pair and differed only slightly from the autosomes in bearing a brighter, CMA_3-positive, telomeric C-band (Figs. 2a, 3e and 4c).

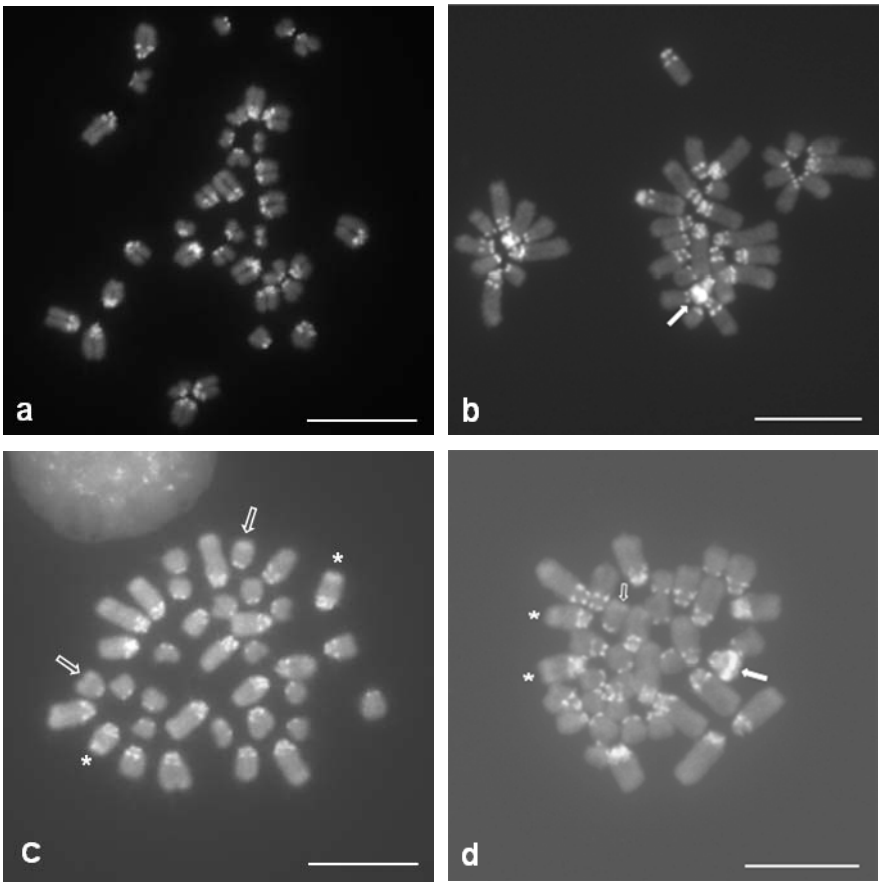


Fig. 4. Metaphase plates of *I. monticola* male (**a**, **c**) and female from Puerto de Vegarada (**b, d**) C-banded and stained with DAPI (**a, b**) and CMA_3 (**c, d**). Asterisks in **c** and **d** indicate CMA_3 positive signals associated with NORs. Empty and solid arrows point to Z and W sex chromosomes, respectively. Scale bars represent 10 μm.

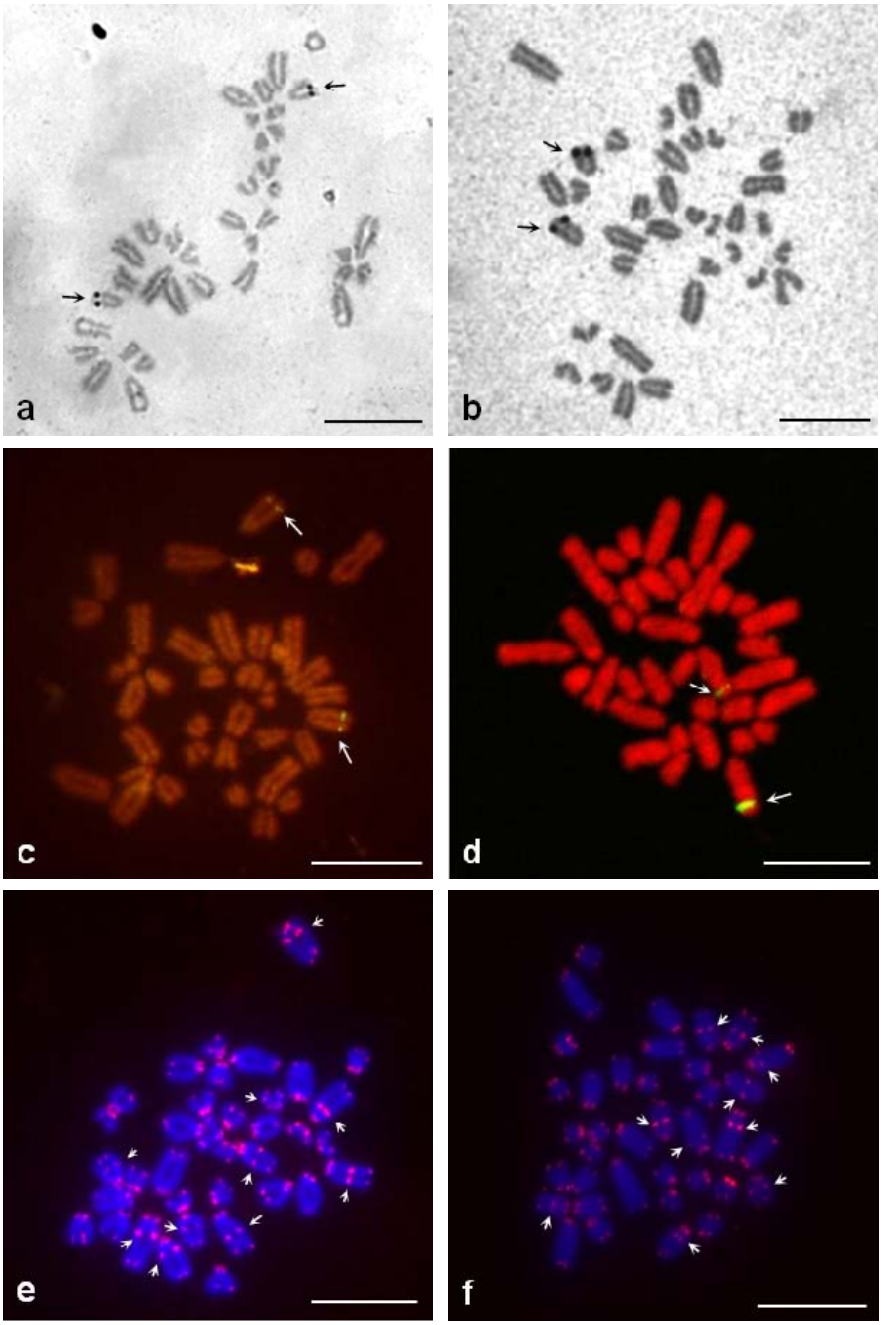


Fig. 5. Chromosomal localization of the 18S-5.8S-28S rRNA genes and $(TTAGGG)_n$ telomeric sequences in male (**a, c, e**) and female (**b, d, f**) I. monticola. (**a, b**) Ag-NOR bands and (**c**, **d**) FISH signals of the 18S-5.8S-28S rRNA genes (arrows). (**e**, **f**) Hybridization patterns of the telomeric probe $(TTAGGG)_n$. Arrows point to interstitial telomeric sites. Scale bars represent 10 μm.

Chromosomal mapping of the 18S-5.8S-28S rRNA genes

Ag-NOR banding agreed with CMA_3 evidence and showed active NORs on the secondary constriction in the subtelomeric regions of chromosome pair 6 (Figs. 2 and 5a, b).

Fluorescent hybridization signals of the 18S-5.8S-28S rRNA genes were also coincident with Ag-NOR bands and did not reveal more inactive loci (Figs. 5c, d).

Chromosomal location of the $(TTAGGG)_n$ sites

FISH with a telomeric probe $(TTAGGG)_n$ produced discrete fluorescent signals at the telomeres of all chromosomes (Fig. 5e, f). Additionally, bright hybridization signals were detected at interstitial sites (so called Interstitial Telomeric Sites, ITSs) in five large chromosome pairs in all the metaphase spreads examined. None of these ITSs were located on either the sex chromosomes or the NOR-bearing pairs.

Comparative genomic hybridization (CGH)

CGH highlighted a strong hybridization signal of the female-derived genomic probe on a single small chromosome in female metaphase spreads (Figs. 6a, b), but not in male metaphases (Figs. 6c, d), as would be expected for the W chromosome. This signal was predominantly located in the distal portion of the W chromosome, indicating that this chromosomal region is enriched in female-specific sequences (Fig. 6b). The Z chromosome could not be distinguished from the autosomes by CGH.

The centromeric and interstitial heterochromatin blocks were labeled evenly with both male and female-derived probes (yellow fluorescence) (Figs. 6b, d).

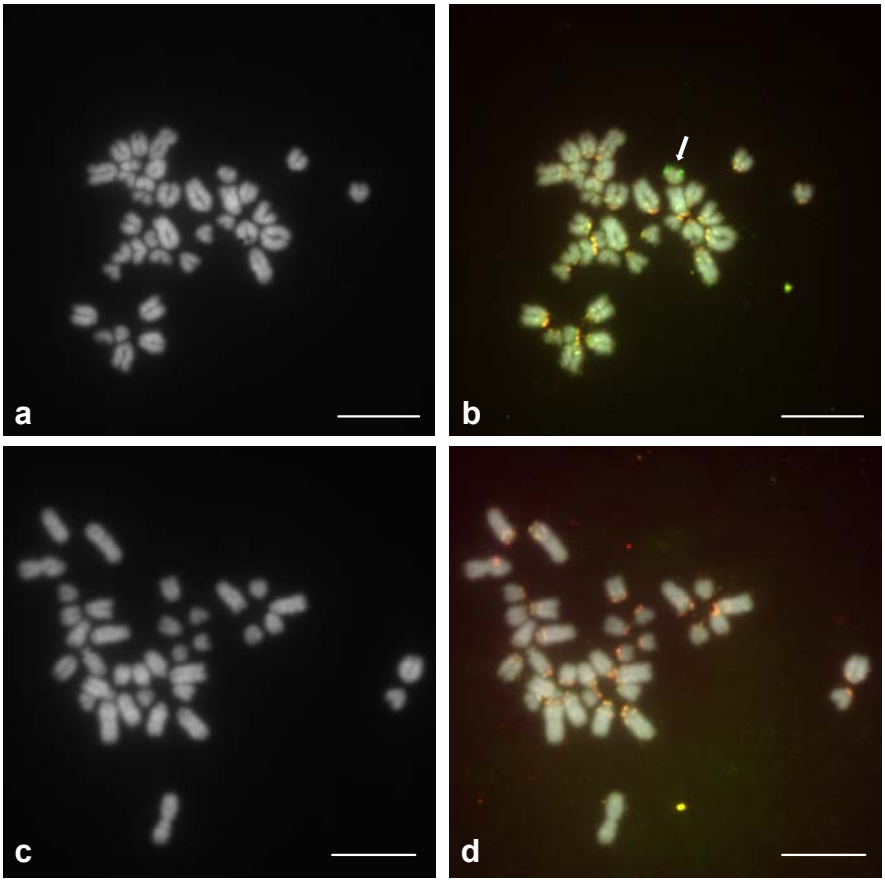


Fig. 6. Comparative genomic hybridization on female (**a**, **b**) and male (**c**, **d**) metaphases of *I. monticola*. Male genomic DNA is stained with FITC (green) and female genomic DNA with TRITC (red). (**a, b**) DAPI-stained metaphases; (**c**, **d**) Merged images. Arrow in **b** points to the W chromosome.

Discussion

Chromosome number and karyotypes

In accordance with previously published results (Odierna et al. 1996), the karyotypes obtained from males and females of *I. monticola* showed a diploid chromosome complement of 2n=36 acrocentric elements, which is common to all the species assigned to the "Iberian group" of the genus *Iberolacerta*, namely *I. galani*, *I. martinezricai*, *I. cyreni* and the said *I. monticola.*

In contrast with chromosome morphology, the pattern of heterochromatin distribution is not so conservative between these taxa (Odierna et al. 1996), and each species displays its own heterochromatin profile. In general, all the *Iberolacerta* species—with the only exception of *I. bonnali*—show prominent C-bands at the centromeres of almost all the acrocentric chromosome pairs. The presence of centromeric heterochromatin is a widespread character in

lacertids (Olmo et al. 1986, 1993; Odierna et al. 1996), and it has been suggested that it may play a role in centromere structure and function (e.g., Capriglione et al. 1998).

However, the composition of the highly repetitive DNA sequences that constitute this centromeric heterochromatin is not necessarily conserved between the different *Iberolacerta* species, as indicated by the fact that these DAPI-positive C-bands are also brightly stained by CMA_3 in *I. monticola* and *I. galani* (Arribas et al. 2006), but are CMA_3-negative in *I. martinezricai* (Arribas and Odierna 2005).

Moreover, the C-banding technique revealed the presence of additional DAPI and CMA_3-positive heterochromatin in the pericentromeric regions of the 10 larger chromosome pairs. These interstitial heterochromatic regions have not been previously detected by C-banding in any of the *Iberolacerta* species, although they are probably correlated with the pericentromeric bands generated on the six larger chromosome pairs of *I. monticola* after the digestion of heterochromatin with the endonuclease *Alu*I (Odierna et al. 1996). This *Alu*I banding pattern shows the variation in sequence composition between the *Alu*I-sensitive heterochromatin located at the centromeres and the pericentromeric *Alu*I-resistant heterochromatin present at least on six chromosome pairs.

Even though satellite DNAs in constitutive heterochromatin are usually composed of AT-rich elements (e.g., King and Cummings 1997; Plohl et al. 2008), the faint C-bands revealed at the telomeres in the 12 larger chromosome pairs of *I. monticola* were only visible after CMA_3 staining and, therefore, a high GC content can be postulated. GC-rich satellites have been reported for some animal species (Meneveri et al. 1995; Malykh et al. 2001; Barragán et al. 2002; Petrović et al. 2009) and, in Squamate reptiles, a telomeric GC-rich satellite has been described for the skink *Eumeces schneideri* (Giovannoti et al. 2009b). The compartmentalization of GC-rich elements in telomeric heterochromatin could be related to the hypothesized role of short guanine stretches in telomere maintenance and stability (Muniyappa et al. 2000), as well as in promoting chromosome rearrangements through recombination between satellite and telomeric sequences (e.g., Hartmann and Scherthan 2004).

The presence of telomeric heterochromatin blocks in some chromosome pairs of *I. monticola* and in all chromosomes of *I. galani* (Arribas et al. 2006) constitutes a cytogenetic marker that further discriminates the karyotypes of both species from *I. martinezricai*, where all chromosomes are devoid of telomeric C-bands (Arribas and Odierna 2005).

On the whole, C-banding data gathered so far in the genus *Iberolacerta* reveal extensive heterogeneity in the amount and distribution of the heterochromatic fraction, even between species so closely related as *I. martinezricai*, *I. monticola* and *I. galani*. However, the karyological affinities unveiled between *I. monticola* and *I. galani* are not consistent with molecular data (Arribas et al. 2006; Remón et al. 2013), which indicate that *I. monticola* is the sister taxon to the clade formed by *I. galani* and *I. martinezricai* (Fig. S1, Supplementary Material). In light of the phylogeny, it seems likely that the C-banding patterns of *I. monticola* and *I. galani* represent the ancestral condition for this lineage; thus the particular differences in heterochromatin distribution and composition reported for *I. martinezricai* constitute a derived character that, similarly to other cytogenetic traits (e.g., NOR location, see below) or osteological autapomorphies distinctive of this taxon (Arribas and Odierna 2005), could have become fixed after the species divergence, due to random genetic drift in small populations. In conclusion, our findings support the idea that, even if C-banding patterns in lacertid lizards can be useful to identify species diagnostic characters, they may not accurately reflect the phylogenetic relationships among taxa (Olmo et al. 1986).

Ribosomal loci

As previously reported in *I. monticola* (Odierna et al. 1996), silver-staining documented a single NOR site in a subtelomeric position of chromosome pair 6. Such NOR location at the telomeres of a large chromosome pair (L-type after Olmo et al. 1993) appears to be ubiquitous among lacertids (Olmo et al. 1993), and it is also the plesiomorphic condition for the genus *Iberolacerta*, where only *I. cyreni* and *I. martinezricai* differ in showing a NOR in an interstitial position on a medium-sized chromosome pair (M-type after Olmo et al. 1993) (Odierna et al. 1996; Arribas and Odierna 2005).

FISH with the 28S-5.8S-18S rDNA probe, carried out for the first time in this genus, confirmed the presence of the ribosomal clusters at the sites identified by silver-staining, and did not show additional transcriptionally inactive loci. In addition, the bright CMA_3 signal associated with the NOR site highlighted the GC richness in rDNA base composition, as reported for a wide variety of organisms (e.g., Sumner 1990 and references therein).

Telomeric repeats

Hybridization signals of the $(TTAGGG)_n$ probe were located at the telomeres of all chromosomes and at interstitial positions on five large chromosome pairs.

ITSs have been observed in many vertebrate species (e.g., Meyne et al. 1990; Lee et al. 1993; Nanda and Schmid 1994; Garagna et al. 1997; Ventura et al. 2006), including several families of Squamate reptiles (Meyne et al. 1990; Schmid et al. 1994; Pellegrino et al. 1999; Bertolotto et al. 2001; Srikulnath et al. 2009). They usually consist of large arrays of telomeric-like repeats commonly located in pericentromeric regions, within or at the margins of constitutive heterochromatin.

A large body of evidence indicates that ITSs may be remnants of chromosomal rearrangements that occurred during chromosome evolution (for a review, see Lin and Yan 2008; Ruiz-Herrera et al. 2008). Likewise, the ITSs detected in *I. monticola* could be the result of chromosome reorganization events, such as tandem fusions of ancestral acrocentric chromosomes, paracentric inversions involving the telomeric sequences or pericentric inversions in ancestral sub-/metacentric chromosomes. The high intensity of the ITS signals, generally larger than those detected at the telomeric ends, suggests that the retained $(TTAGGG)_n$ sequences have also been amplified. In this regard, it is interesting to point out that karyotype evolution in lacertids is thought to be characterized by a progressive translocation of microchromosomes to macrochromosomes (Olmo 1986; Odierna et al. 1987). In fact, the basic diploid number of *Iberolacerta* (2n=36) differs from the common lacertid karyotype in that it lacks a pair of microchromosomes (Olmo et al. 1993). Moreover, ITSs have been associated with fragile sites and recombination hotspots (recently reviewed in Bolzán 2012) that may confer greater flexibility for karyotype change by providing potential new sites for telomere formation (Meyne et al. 1990).

However, the presence of ITSs in the karyotype is not always related to structural chromosome changes. Pre-existing ITSs, including the short stretches of telomeric hexamers that are presumably inserted during the repair of double strand breaks (Nergadze et al. 2004, 2007), could be subsequently spread and expanded at different intrachromosomal regions by common mechanisms of repetitive DNA amplification, such as unequal crossing-over or sequence conversion (Wiley et al. 1992; Vermeesch et al. 1996; Garagna et al. 1997; Nanda et al. 2008). For instance, a process of heterochromatin association and unequal exchange has been proposed to explain the dispersion and amplification of ITSs embedded within heterochromatin to new chromosomal locations in lemur and rodent species (Go et al. 2000; Rovatsos et al. 2011).

Therefore, further studies of the occurrence of ITSs and comparative karyological analyses,

such as chromosome painting, between lacertids and closely related lizard families are required to elucidate the origin of these non-telomeric sites and clarify their association with karyotype evolution in this lineage.

Sex chromosomes

Populations of *I. monticola* from the locality of Puerto de Vegarada, in the Cantabrian mountain range, were first reported to lack differentiated sex chromosomes (Odierna et al. 1996). In the present study, however, a heteromorphic ZW chromosome pair was consistently identified in the female specimen analyzed from this same population. The discrepancy between those observations and our results could be just due to experimental artifacts. For instance, the higher degree of chromosome condensation in metaphase spreads obtained by scraping techniques from tissues (former work) in comparison with chromosomes obtained from cell cultures (present study) could hamper the detection of the small-sized W chromosome by C-banding.

The presence of a cytologically distinguishable ZZ/ZW system was also confirmed in specimens from two other Cantabrian populations, as well as from the population of Eume, at the northwesternmost edge of the species' range. All four studied populations are currently isolated and, according to recent molecular analysis (Remón et al. 2013), their independent evolution began roughly between 1.5 and 0.9 mya, possibly as a consequence of climatic fluctuations during the Pleistocene. Even so, the sex chromosome pairs of any of these populations are highly similar in terms of relative size and in the amount and distribution of heterochromatin, albeit they could exhibit some differentiation at finer scales hardly evidenced by C-banding and fluorochrome staining.

Likewise, the sex chromosome pair detected in *I. monticola* closely resembles that of other *Iberolacerta* species for which sex chromosomes have been described, i.e., *I. horvathi*, *I. cyreni* and *I. galani* (Capula et al. 1989; Odierna et al. 1996; Arribas et al. 2006). All of them possess a highly heteromorphic ZW pair, in which the W chromosome is smaller than the Z and completely or almost completely heterochromatic. Nevertheless, greater similarities are found between *I. monticola* and *I. galani.* In particular, the presence of a bright telomeric heterochromatic block in the Z chromosome is a feature that appears to be exclusive of both species. Even if the nature of the sequences responsible for the heteromorphism in the sex chromosome pair is not known, reverse fluorochrome staining revealed at least certain differences in molecular composition, since heterochromatin in the Z chromosome resulted

only positive after CMA_3 staining (similarly to the weak C-bands at the ends of some autosomal pairs), while W chromosome heterochromatin was completely stained with both CMA_3 and DAPI. Accordingly, CGH results confirmed that the Z and W chromosomes are highly differentiated in sequence content, probably owing to extensive accumulation of female-specific repetitive sequences in the distal region of the W chromosome.

In general, the properties of sex chromosomes in *I. monticola* and the remaining *Iberolacerta* species are concordant with the evolutionary model proposed for other lacertids (Olmo et al. 1987; Odierna et al. 1993), which suggests that the accumulation of repetitive sequences and heterochromatinization is an early change that initiates sex chromosome differentiation. This may subsequently be followed by structural rearrangements, such as deletion of heterochromatic regions not involved in sex determination, originating a heteromorphic sex chromosome pair in which the W is distinctly smaller than the Z. In this context, it would be of interest to verify whether the W chromosome of *I. galani*, reported to be totally imbibed with heterochromatin (Arribas et al. 2006), certainly lacks the intercalary euchromatic region observed in the W chromosome of *I. monticola* and thus represents a more advanced stage of sex chromosome differentiation.

Despite the common features of the ZW pair of these *Iberolacerta* species, it is likely that not all the sex chromosome systems in this genus followed the same evolutionary pathway: multiple sex chromosomes systems ($Z_1Z_1Z_2Z_2$ male and Z_1Z_2W female), with W chromosomes at different degrees of heterochromatinization, have been found in *I. bonnali* and *I. aurelioi* (Odierna et al. 1996). In addition, homomorphic and cytologically undetectable sex chromosomes are presumably present in *I. aranica* and *I. martinezricai* (Odierna et al. 1996; Arribas and Odierna 2005) (Fig. S1, Supplementary Material). Moreover, variation in the degree of sex chromosome differentiation is found among species that diverged no more than 2.5 mya (*I. monticola*, *I. galani* and *I. martinezricai*).

Such interspecific variability in the stage of degeneration of W chromosomes, with no clear phylogenetic correlation, is representative of the remarkable heterogeneity of sex chromosome systems reported for lacertid lizards (Olmo et al. 1986, 1987; Odierna et al. 1993; Olmo and Signorino 2005), which suggests that in this family, as in many reptile lineages, sex chromosomes can have multiple independent origins even in closely related taxa (e.g., Ezaz et al. 2009).

Thus, considering that degradation of W chromosome and dosage compensation would

evolve more slowly in ZW taxa, as compared with XY taxa (Naurin et al. 2010), and bearing in mind the advanced state of degeneration of the W chromosome in the basal *Iberolacerta* species, *I. horvathi* (Capula et al. 1989), it seems probable that the presence of a heteromorphic ZZ/ZW pair is the ancestral condition for this genus. Accordingly, it could be hypothesized that the seemingly undifferentiated sex chromosomes in *I. martinezricai* and *I. aranica* might represent neo-sex chromosomes resulting from recent turnover events (e.g., the appearance of a new sex determining gene on an autosome or the transposition of a sex determining gene to a new chromosomal location), which would have replaced the pre-existing heteromorphic ZW pair. Nonetheless, the putative absence of heteromorphic sex chromosomes in both species should be further investigated in detail.

Future comparative cytogenetic analyses, along with the application of high-resolution molecular cytogenetic techniques such as CGH, will therefore be necessary to deepen the knowledge about the degree and patterns of sex chromosome differentiation and the transitions between simple ZW and multiple Z_1Z_2W systems in the genus *Iberolacerta*, which ultimately would shed light on the mechanisms underlying sex chromosome evolution and the plasticity of sex determination systems in lacertid lizards.

References

Arribas O, Carranza S (2004) Morphological and genetic evidence of the full species status of *Iberolacerta martinezricai* (Arribas, 1996). Zootaxa 634: 1-24.

Arribas O, Odierna G (2005) Karyological and and osteological data supporting the specific status of *Iberolacerta* (*cyreni*) *martinezricai* (Arribas, 1996). Amphibia-Reptilia 25: 359-367.

Arribas O, Carranza S, Odierna G (2006) Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240: 1-55.

Arribas O, Galán P, Remón, Naveira H (2014) A new mountain lizard from Montes de León (NW Iberian Peninsula): *Iberolacerta monticola astur* ssp. nov. (Squamata: Lacertidae). Zootaxa 3796: 201–236.

Barragán MJL, Martínez S, Marchal JA, Bullejos M, Díaz de la Guardia R, Sánchez A (2002) Highly repeated DNA sequences in three species of the genus *Pteropus* (Megachiroptera, Mammalia). Heredity 88: 366-370.

Bertolotto CEV, Rodrigues MT, Yonenaga-Yassuda Y (2001) Banding patterns, multiple sex chromosomes system and localization of telomeric $(TTAGGG)_n$ sequences by FISH on two species of *Polychrus* (Squamata, Polychrotidae). Caryologia 54: 217-226.

Bolzán AD (2012) Chromosomal aberrations involving telomeres and interstitial telomeric sequences. Mutagenesis 27: 1-15.

Bosch HAJ in den, Odierna G, Aprea G, Barucca M, Canapa A, Capriglione T, Olmo E (2003) Karyological and genetic variation in Middle Eastern lacertid lizards *Lacerta laevis* and *Lacerta kulzeri*-complex: a case of chromosomal allopatric speciation. Chromosome Res 11: 165-178.

Capula M, Lapini L, Capanna E (1989) The karyotype of *Lacerta horváthi* (Reptilia, Sauria, Lacertidae) Genetica 79: 11-16.

Capula M, Lapini L, Capanna E (1991) The karyotype of *Lacerta mosorensis* (Reptilia: Lacertidae): Evidence for a new case of female heterogamety in a lacertid lizard. Rend Fis Acc Lincei 9: 51–57.

Capriglione T, De Santo MG, Odierna G, Olmo E (1998) An alphoid-like satellite DNA sequences is present in the genome of a lacertid lizard. J Mol Evol 46: 240-244.

Carranza S, Arnold EN, Amat F (2004) DNA phylogeny of *Lacerta* (*Iberolacerta*) and other lacertine lizards (Reptilia: Lacertidae): did competition cause long-term mountain restriction? Syst Biodivers 2: 57-77.

Charlesworth D, Charlesworth B, Marais G (2005) Steps in the evolution of heteromorphic sex chromosomes. Heredity 95: 118-128.

Charlesworth D, Mank JE (2010) The birds and the bees and the flowers and the trees: lessons from genetic mapping of sex determination in plants and animals. Genetics 186: 9-31.

Crochet PA, Chaline O, Surget-Groba Y, Debain C, Cheylan M (2004) Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae). Mol Phylogenet Evol 30: 860-866.

Ezaz T, Sarre SD, O'Meally D, Marshall Graves JA, Georges A (2009) Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet Genome Res 127: 249-260.

Galán P (1999) Declive y extinciones puntuales en poblaciones de baja altitud de *Lacerta monticola cantabrica*. Bol Asoc Herpetol Esp 10: 47-51.

Galán P, Vila M, Remón N, Naveira H (2007) Caracterización de las poblaciones de *Iberolacerta monticola* en el Noroeste ibérico mediante la combinación de datos morfológicos, ecológicos y genéticos. Munibe (Suplemento) 25: 34-43.

Garagna S, Ronchetti E, Mascheretti S, Crovella S, Formenti D, Rumpler Y, Manfredi Romanini MG: (1997) Non-telomeric chromosome localization of $(TTAGGG)_n$ repeats in the genus *Eulemur*. Chromosome Res 5: 587-591.

Giovannotti M, Caputo V, O'Brien PC, Lovell FL, Trifonov V, Cerioni PN, Olmo E, Ferguson-Smith MA, Rens W (2009a) Skinks (Reptilia: Scincidae) have highly conserved karyotypes as revealed by chromosome painting. Cytogenet Genome Res 127: 224-31.

Giovannotti M, Nisi Cerioni P, Caputo V, Olmo E (2009b) Characterisation of a GC-rich telomeric satelliteDNA in *Eumeces schneideri* Daudin (Reptilia, Scincidae). Cytogenet Genome Res 125: 272-278.

Go Y, Rakotoarisoa G, Kawamoto Y, Randrianjafy A, Koyama N, Hirai H (2000) PRINS analysis of the telomeric sequence in seven lemurs. Chromosome Res 8: 57-65.

González-Tizón A, Martıíne-Lage A, Rego I, Ausió J, Méndez J (2000) DNA content, karyotype and chromosomal location of 18S-5.8S-28S ribosomal loci in some species of bivalve molluscs from the Pacific Canadian coast. Genome 43: 409-411.

Hartmann N, Scherthan H (2004) Characterization of ancestral chromosome fusion points in the Indian muntjac deer. Chromosoma 112: 213-220.

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 3: 1014-1015.

King LM, Cummings MP (1997) Satellite DNA repeat sequence variation is low in three species of burying beetles in the genus *Nicrophorus* (Coleoptera: Silphidae). Mol Biol Evol 14: 1088-1095.

Kupriyanova LA, Melashchenko O (2011) The common Eurasian lizard *Zootoca vivipara* (Jacquin, 1787) from Russia: sex chromosomes, subspeciation, and colonization. Russ J Herpetol 18: 99-104.

Lee C, Sasi R, Lin CC (1993) Interstitial localization of telomeric DNA sequences in the Indian muntjac chromosomes: further evidence for tandem chromosome fusions in the karyotypic evolution of the Asian muntjacs. Cytogenet Cell Genet 63: 156-159.

Lin KW, Yan J (2008) Endings in the middle: current knowledge of interstitial telomeric sequences. Mutat Res 658: 95–110.

Malykh AG, Zhurov VG, Saifitdinova AF, Deryusheva SE, Gaginskaya ER (2001) Structural and Functional Characterization of a Centromeric Highly Repetitive DNA Sequence of *Fringilla coelebs* L. (Aves: Passeriformes). Mol Biol 40: 331-335.

Mayer W, Arribas O (2003): Phylogenetic relationships of the European lacertid genera *Archaeolacerta* and *Iberolacerta* and their relationships to some other 'Archaeolacertae' (sensu lato) from Near East, derived from mitochondrial DNA sequences. J Zool Syst Evol Res 41: 157-161.

Meneveri R, Agresti A, Rocchi M, Marozzi A, Ginelli E (1995) Analysis of GC-rich repetitive nucleotide sequences in great apes. J Mol Evol 40: 405-412.

Meyne J, Baker RJ, Hobart HH T. Hsu C, Ryder OA, Ward OG, Wiley JE, Wurster-Hill DH, Yates TL, Moyzis RK (1990) Distribution of non-telomeric sites of the $(TTAGGG)_n$ telomeric sequence in vertebrate chromosomes. Chromosoma 99: 3-10.

Muniyappa K, Anuradha S, Byers B (2000) Yeast meiosis-specific protein Hop1 binds to G4 DNA and promotes its formation. Mol Cell Biol 20: 1361-1369.

Nanda I, Schmid M (1994) Localization of the telomeric $(TTAGGG)_n$ sequence in chicken (*Gallus domesticus*) chromosomes. Cytogenet Cell Genet 65: 190-193.

Nanda I, Fugate M, Steinlein C, Schmid M (2008) Distribution of $(TTAGGG)_n$ telomeric sequences in karyotypes of the *Xenopus* species complex. Cytogenet Genome Res 122: 396-400.

Naurin S, Hansson B, Bensch S, Hasselquist D (2010) Why does dosage compensation differ between XY and ZW taxa? Trends Genet 26: 15-20.

Nergadze SG, Rocchi M, Azzalin CM, Mondello C, Giulotto E (2004) Insertion of telomeric repeats at intrachromosomal break sites during primate evolution. Genome Res 14: 1704–1710.

Nergadze SG, Santagostino M, Salzano A, Mondello C, Giulotto E (2007) Contribution of telomerase RNA retrotranscription to DNA double-strand break repair during mammalian genome evolution. Genome Biol 8: R260.

Odierna G, Olmo E, Cobror O (1987) Taxonomic implications of NOR-localisation in lacertid lizards. Amphibia-Reptilia 8: 373-382.

Odierna G, Kupriyanova L, Capriglione T, Olmo E (1993) Evolution of sex chromosomes in lacertid lizards. Amphibia-Reptilia 14: 1-11.

Odierna G, Aprea G, Arribas O, Capriglione T, Caputo V, Olmo E (1996) The karyology of the Iberian rock lizards. Herpetologica 52: 542-550.

Odierna G, Heulin B, Guillaume CP, Vogrin N, Aprea G, Capriglione T, Surget-Groba Y, Kupriyanova LMS (2001) Further analysis of the karyological variations existing within and between oviparous and viviparous forms of *Lacerta* (*Zootoca) vivipara:* evolutionary and biogeographic implications. Ecography 24: 332-340.

Odierna G, Arribas OJ (2005) The karyology of '*Lacerta*' *mosorensis* Kolombatovic, 1886, and its bearing on phylogenetic relationships to other European Mountain lizards. Ital J Zool 72: 93-96.

Olmo E, Odierna G, Cobror O (1986) C-band variability and phylogeny of Lacertidae. Genetica 71: 63-74.

Olmo E, Odierna G, Capriglione T (1987) Evolution of sex-chromosomes in lacertid lizards. Chromosoma 96: 33-38.

Olmo E, Odierna G, Capriglione T (1993) The karyology of Mediterranean lacertid lizards, in Valakos ED, Boehme W, Pérez-Mellado V, Maragou P (eds): Lacertids of the Mediterranean region, pp 61-84 Hellenic Zoological Society, Athens, Bonn, Alicante.

Olmo E, Signorino G: Chromorep (2005) A reptile chromosomes database http://chromorep.univpm.it/. (Accessed February 15, 2013).

Pellegrino KCM, Rodrigues MT, Yonenaga-Yassuda Y (1999) Chromosomal evolution in the Brazilian lizards of genus *Leposoma* (Squamata, Gymnophthalmidae) from Amazon

and Atlantic rain forests: banding patterns and FISH of telomeric sequences. Hereditas 131: 15-21.

Petrović V, Pérez-García C, Pasantes JJ, Šatović E, Prats E, Plohl M (2009) A GC-rich satellite DNA and karyology of the bivalve mollusc *Donax trunculus*: a dominance of GC-rich heterochromatin. Cytogenet Genome Res 124: 63-71.

Plohl M, Luchetti A, Meštrović N, Mantovani B (2008) Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. Gene 409: 72–82.

Remón N, Galán P, Vila M, Arribas O, Naveira H (2013) Causes and evolutionary consequences of population subdivision of an Iberian mountain lizard *Iberolacerta monticola*. PLoS ONE 8: e66034.

Roiha H, Miller JR, Woods LC, Glower DM (1981) Arrangements and rearrangements of sequences flanking the two types of rDNA insertion in *D. melanogaster*. Nature 290: 749-753.

Rovatsos MTh, Marchal JA, Romero-Fernández I, Fernández FJ, Giagia-Athanosopoulou EB, Sánchez A (2011) Rapid, independent, and extensive amplification of telomeric repeats in pericentromeric regions in karyotypes of arvicoline rodents. Chromosome Res 19: 869–882.

Ruiz-Herrera A, Nergadze SG, Santagostino M, Giulotto E (2008) Telomeric repeats far from the ends: mechanisms of origin and role in evolution. Cytogenet Genome Res 122: 219–228.

Schmid M, Haaf T, Geile B, Sims S (1983) Chromosome banding in Amphibia. VIII. An unusual XY/XX sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). Chromosoma 88: 69-82.

Schmid M, Feichtinger W, Nanda I, Schakowski R, Garcia RV, Puppo JM, Badillo AF (1994) An extraordinary low diploid chromosome number in the reptile *Gonatodes taniae* (Squamata, Gekkonidae). J Heredity 85: 255-260.

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58: 307-324.

Srikulnath K, Matsubara K, Uno Y, Thongpan A, Suputtitada S, Apisitwanich S, Matsuda Y, Nishida C. (2009) Karyological characterization of the butterfly lizard (*Leiolepis reevesii rubritaeniata*, Agamidae, Squamata) by molecular cytogenetic approach. Cytogenet Genome Res 125: 213-223.

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75: 304-306.

Sumner AT (1990) Chromosome Banding Unwin Hyman Ltd., London.

Ventura K, Silva MJJ, Fagundes V, Christoff AU, Yonenaga-Yassuda Y (2006) Non-telomeric sites as evidence of chromosomal rearrangement and repetitive $(TTAGGG)_n$ arrays in heterochromatic and euchromatic regions in four species of *Akodon* (Rodentia, Muridae). Cytogenet Cell Genet 115: 169-175.

Vermeesch JR, De Meurichy W, Van Den Berghe H, Marynen P, Petit P (1996) Differences in the distribution and nature of the interstitial telomeric $(TTAGGG)_n$ sequences in the chromosomes of the Giraffidae, okapai (*Okapia johnstoni*), and giraffe (*Giraffa camelopardalis*): evidence for ancestral telomeres at the okapi polymorphic rob(5;26) fusion site. Cytogenet Cell Genet 72: 310-315.

Wiley JE, Meyne J, Little ML, Stout JC (1992) Interstitial hybridization sites of the $(TTAGGG)_n$ telomeric sequence on the chromosomes of some North American hylid frogs. Cytogenet Cell Genet 61: 55–57.

Supplementary Material

Fig. S1. Cladogram of the genus Iberolacerta based on the molecular phylogeny reported by Arribas et al. (2014), with information on chromosome numbers and sex chromosome systems. Estimated divergence times in millions of years (myr) are indicated at each node. Cytogenetic data for the outgroup species, Dinarolacerta mosorensis, were obtained from Capula et al. (1991) and Odierna and Arribas (2005). M, male; F, female.

Chromosome number
Sex chromosomes
Dinarolacerta mosorensis
2n=38
ZZ/ZW
I. bonnali
2n=24 (M)
2n=23 (F)
Z1Z1Z2Z2/
Z1Z2W
I. aurelioi
2n=26 (M)
2n=25 (F)
Z1Z1Z2Z2/
Z1Z2W
I. aranica
2n=26
Homomorphic (?)
I. horvathi
2n=36
ZZ/ZW
I. cyreni
2n=36
ZZ/ZW
I. monticola
2n=36
ZZ/ZW
I. martinezricai
2n=36
Homomorphic (?)
I. galani
2n=36
ZZ/ZW
13.5 Myr
11.5 Myr
8 Myr
3.8 Myr
2.5 Myr
1.8 Myr

CHAPTER II

Comparative chromosome painting in lacertid lizards: highly conserved karyotypes but independent origins of sex chromosomes?

Abstract

Lizards of the family Lacertidae exhibit remarkable diversity in the degree of sex chromosome differentiation, even between closely related taxa. However, comparative data on lacertid sex chromosomes are scarce, and have been mainly gathered using standard banding techniques. Here, we applied high-resolution molecular cytogenetic techniques [chromosome painting and comparative genomic hybridization (CGH)] to evaluate the homology between the sex chromosomes of five lacertid species, namely *Iberolacerta monticola*, *I. galani*, *I. bonnali*, *Lacerta schreiberi*, and *Timon lepidus*. Chromosome painting with the probe derived from the W chromosome of *I. monticola* (IMOW) showed that the euchromatic region of the female-specific sex chromosome is conserved in the other two species of *Iberolacerta*. The biarmed W chromosome of *I. bonnali* originated from a centric fusion involving *I. monticola* chromosome 15 or 16. Conversely, the W chromosomes of *I. monticola*, *T. lepidus* and *L. schreiberi* are highly differentiated from each other, and probably evolved independently through rapid accumulation of female-specific sequences characteristic of each lineage. Moreover, our preliminary data with a Z chromosome paint suggest at least two independent origins of sex chromosomes in lacertids. A genome-wide comparison of chromosome synteny between these three species revealed a high degree of karyotype conservation but some characteristic rearrangements, including a translocation of microchromosomes to macrochromosomes in *Iberolacerta*. Finally, we carried out PCR-assisted gene mapping on flow-sorted chromosome libraries of *I. monticola* to investigate chromosome homology with other reptilian species. Although inconclusive, the results of this approach support lack of homology between the sex chromosomes of lacertids and *A. carolinensis*, and suggest that the loss of microchromosomes in Lacertidae was due to repeated fusions between microchromosomes that existed in the ancestral karyotype of squamate reptiles.

Key Words: Lacertids · Chromosome painting · Karyotype evolution · Sex chromosomes · Chromosome synteny

Introduction

The evolution of sex determination in squamate reptiles has attracted much interest over the past years, since this group exhibits an astonishing diversity of sex-determining systems, which range from environmental sex determination to genotypic sex determination, including male heterogamety (XX/XY), female heterogamety (ZZ/ZW) and multiple sex chromosomes (Sarre et al. 2004; Ezaz et al. 2009a; Pokorná and Kratochvíl 2009). Lack of clear phylogenetic segregation of sex- determining mechanisms suggests multiple transitions between systems and independent origins of sex chromosomes in many squamate lineages (Ezaz et al. 2009a; Sarre et al. 2011).

However, well-supported variability in the mode of sex determination has been documented so far only in dragon lizards (Agamidae) (Ezaz et al. 2009b) and geckos (Gekkota) (Gamble 2010; Pokorná et al. 2010, 2014; Koubová et al. 2014; Gamble et al. 2015). On the other hand, phylogenetic reconstruction of the evolution of sex determination has shown that other squamate clades might posses relatively conserved systems and sex chromosomes (Pokorná and Kratochvíl 2009). Indeed, molecular-cytogenetic studies confirming such conservation of sex chromosomes have been recently published for colubroid snakes (Matsubara et al. 2006; Vicoso et al. 2013) and iguanas (Gamble et al. 2014; Rovatsos et al. 2014a, b).

Nonetheless, the portrait of the evolution of sex determination is largely incomplete, and information on the sex-determining mechanisms and sex chromosomes is still lacking for many phylogenetically important groups (e.g., Pokorná and Kratochvíl 2009). In addition, chromosome homology has been traditionally established using standard banding and staining techniques, which may actually undercount the real number of transitions among sex-determining systems. For instance, recent work using chromosome painting revealed that the morphologically similar ZW sex chromosomes of two gecko species are not homologous and represent independent origins of female heterogamety within the Gekkonidae (Matsubara et al. 2014). Molecular cytogenetic analyses evaluating the homology of sex chromosomes at the sequence level are thus required to investigate the evolutionary transitions of sex chromosomes within and among multiple squamate lineages.

One of these lineages is the Old World Lizard family Lacertidae. With about 321 species in 42 genera (Uetz and Hošek 2015), it is the predominant lizard group in Europe and a substantial component of the squamate reptile diversity in Africa (Arnold et al. 2007; Hipsley et al. 2009). Genetic analyses suggest a fast diversification and radiation of lacertids (Harris et al. 1998; Fu 2000; Arnold et al. 2007), even though divergence time estimates for these events are controversial, with some authors indicating the mid-Miocene (12-16 My ago) (Arnold et al.

2007; Pavlicev and Mayer 2009) and other the mid-Eocene (43-46 My ago) (Hipsley et al. 2009). Perhaps due to this rapid diversification, lacertids are characterized by the presence of relatively conserved karyotypes. Most species possess a diplod number of 2n=38, with 36 acrocentric macrochromosomes and 2 microchromosomes (Gorman 1973; Olmo et al. 1986; Olmo and Signorino 2005). As previously reported in Chapter I, there are some exceptions to this pattern. For instance, the karyotypes of some species of the genus *Iberolacerta* consist of 36 acrocentric macrochromosomes and no microchromosomes. Greater differences are found in the Pyrenean *Iberolacerta*, with reduced diplod numbers that range from 2n=24 to 26 in males and from 23 to 26 in females (Gaetano Odierna et al. 1996) and numerous biarmed chromosomes.

The phylogenetic distribution of species with known sex chromosomes suggests that female heterogamety is ancestral for the family (Pokorná and Kratochvíl 2009). ZW or ZW-derived sex chromosomes have been described so far for approximately 40% (43 species) of the 104 species karyotyped (Olmo and Signorino 2005; Ezaz et al. 2009). Cytogenetic analyses, mainly accomplished through Giemsa staining, C-banding and G-banding (Olmo et al. 1986; Olmo et al. 1987; Odierna et al. 1993) revealed extensive variability in morphology and in the degree of W chromosome differentiation, which includes at least the following situations: (1) sex chromosomes that are homomorphic and completely euchromatic (e.g., *Podarcis tiliguerta* and *P. wagleriana*); (2) homomorphic sex chromosomes in which the Z is euchromatic and the W is heterochromatic (e.g., *Takydromus sexlineatus* or *Eremias velox*) ; (3) heteromorphic sex chromosomes in which the W is heterochromatic and distinctly smaller than the Z (e.g., *Eremias arguta* or *Lacerta graeca*) (Ivanov and Fedorova 1973; Olmo et al. 1986, 1987; Odierna et al. 1993; Pokorná et al. 2011). Based on these observations, it has been proposed that the main evolutionary pathway of lacertid sex chromosomes involves heterochromatinization followed by progressive deletion of the heterochromatic areas (Olmo et al. 1987; Odierna et al. 1993). Moreover, variability in the extent of sex chromosome differentiation has been found not only among closely related species, but also among populations of the same species (see, for example, Odierna et al. 2001; Bosch et al. 2003), which suggests that sex chromosome differentiation in this family took place repeatedly and independently in the different taxa (Odierna et al. 1993). To our knowledge, molecular cytogenetic techniques have been only applied recently to characterize the sex chromosomes of *Iberolacerta monticola* (this thesis) and *Lacerta agilis* (Srikulnath et al. 2014), but they are a valuable tool to investigate the evolution of lacertid sex chromosomes.

Here, we describe the preparation of flow-sorted chromosome paints from the Iberian Rock lizard *I. monticola*, and their subsequent use in cross-species chromosome painting to carry out a comparative analyses of sex chromosomes between the following lacertid species: the congeneric *I. galani* (2n = 36), with ZW sex chromosomes (Arribas et al. 2006); *I. bonnali* (2n = 24 in males, 2n =23 in females), with a multiple Z_1Z_2W chromosome system (Odierna et al. 1996); *Lacerta schreiberi* (2n = 38), for which no sex chromosomes have been described yet (Mateo and Cano 1991); and *Timon lepidus* (2n = 36), with a W sex microchromosome (De Smet 1981; Olmo et al. 1987). Comparison of sex chromosomes at the molecular level was further extended through comparative genomic hybridization (CGH) between *I. monticola*, *L. schreiberi* and *T. lepidus*. In addition, the whole set of *I. monticola* chromosome paints was used in genome-wide comparisons with the chromosomal complements of *L. schreiberi* and *T. lepidus*, in order to detect chromosomal rearrangement and syntenies between the three different genera.

Finally, we also used the flow-sorted chromosomes of *I. monticola* to investigate chromosomal homology at a broader taxonomic scale, in comparison with the genome map of the green anole, *Anolis carolinensis* (Iguania) (Alföldi et al. 2011). This species possess a chromosome number of 2n=36, which is widely distributed among squamate reptiles (Gorman 1973; Alföldi et al. 2011; Young et al. 2013). However, this putative ancestral karyotype consists of 12 large metacentric chromosomes and 24 microchromosomes and, hence, differs markedly from the typical lacertid karyotype. Despite considerable chromosomal variation, comparative genome studies through gene mapping, chromosome painting and in silico analysis of genome assemblies revealed extensive chromosomal synteny among the members of Aves, Testudines, Crocodylia and Squamata (Matsuda et al. 2005; Matsubara et al. 2006, 2012; Srikulnath et al. 2009, 2013; Alföldi et al. 2011; Pokorná et al. 2011, 2012; Uno et al. 2012; Young et al. 2013) after 275 millions years of divergence (Shedlock and Edwards 2009). Based on these observations, it can be speculated that the chromosomes of *I. monticola* will be largely syntenic with chromosomes of *A. carolinensis*, and that the acrocentric elements in the lacertid karyotype derived from the common karyotype of squamates by frequent fissions of the metacentric macrochromosomes and fusions of the microchromomes. To test this hypothesis, we used information from the draft genome assembly of *A. carolinensis* (Alföldi et al. 2011) to select at least one gene anchored to each chromosomal arm of the metacentric chromosomes and to each microchromosome. The location of the target genes on the chromosomes of *I. monticola* was then examined by PCR-assisted mapping on the flow-sorted chromosome libraries. Interestingly, a cytogenetic map of the sand lizard (*Lacerta agilis*,

Lacertidae), has been recently published before completion of this work (Srikulnath et al. 2014). This data offer and excellent framework to check the results of gene mapping retrieved by a different experimental approach, and to obtain a more complete view of karyotype evolution between lacertids and other reptilian lineages.

Material and Methods

Animals

Two adult females and one adult male of *I. monticola* were collected from the population of the fluvial valley of the river Eume (A Coruña, Spain). The tail tips of one adult female of *L. schreiberi* and *T. lepidus* were collected from the Natural Park Montes do Invernadeiro (Ourense, Spain). In addition, two adult females of *I. galani* and the tail tip of one adult female of *I. bonnali* were collected from the localities of A Ponte, Pena Trevinca (Ourense, Spain) and Pico de Urdiceto, Pirineos (Huesca, Spain), respectively. The sex of each animal was determined by examination of sexually dimorphic external morphology. All these samples were used to make metaphase chromosomes. Permissions for fielwork and ethics approval of experimental procedures were issued by the competent authorities (Xunta de Galicia, Junta de Castilla-León and Gobierno de Aragón, in Spain), in accordance with the Spanish legislation (Royal Decree 1201/2005 and Law 32/2007, on the protection of animals used for experimentation and other scientific purposes).

Metaphase chromosome preparation

The tail tip collected from each specimen (approximately 10 mm) was pre-treated before setting up the cell cultures as described in Ezaz et al. (2008), with slight modifications. Briefly, the surfaces of the tail tips were sterilized by wiping with gauze soaked in 70% ethanol, clipped and incubated at 30ºC for 24 h in Collection Medium [RPMI 1640 Medium containing 25 mM HEPES (Sigma) with 1 mg/mL kanamycin (Sigma) and 1% antibiotic-antimycotic (Life Technologies-Gibco)].

Fibroblast cell lines and metaphase chromosome spreads were prepared following the protocol described in Chapter I. Cultures for flow-sorting were split up to 4 passages before the chromosomes were harvested.

Probe preparation

Chromosome paints from a female *I. monticola* were prepared from chromosomes sorted with a dual laser cell sorter (Mo-Flo, Dako) at the Cambridge Resource Centre for

Comparative Genomics, Department of Veterinary Medicine, University of Cambridge, Cambridge, UK, as previously described (Yang et al. 1995). Sorted chromosomes were used as templates for DNA amplification by DOP-PCR (Telenius et al. 1992). Primary DOP-PCR products were used as templates in a secondary DOP-PCR to incorporate biotin-16-dUTP (Roche).

Karyotyping and C-banding

For karyotypying, the slides were stained with DAPI (1.5 μg/mL) in anti-fade medium Vectashield (Vector Laboratories). C-banding and sequential staning with DAPI and CMA_3 was performed as described in Chapter I.

Fluorescence in situ hybridization and signal detection

The chromosome content and purity of flow-sorted fractions was first determined by fluorescence in situ hybridization (FISH) onto metaphase spreads of female *I. monticola*. Unidirectional chromosome painting with the probe containing the W sex chromosome of *I. monticola* was performed on *I. galani*, *I. bonnali*, *L. schreiberi* and *T. lepidus*. The full set of chromosome-specific probes of *I. monticola* was used in cross-species hybridization to metaphase spreads of *I. bonnali*, *L. schreiberi* and *T. lepidus*.

FISH was performed using the protocols described in Yang et al. (1995) and Rens et al. (2006) with several modifications. Briefly, slides were dehydrated through ethanol series; aged at 65°C for 1 h; denatured in 70% formamide/2x saline-sodium citrate (SSC) at 70°C for 1 up to 3 min (time depending on species and metaphase preparation) and dehydrated again. One microlitre of biotinylated probe was made up to 12 μL with hybridization buffer (50% deionized formamide (v/v), 10% dextran sulfate, 2x SSC, 0.05 M phosphate buffer, pH 7.3). This mixture was denatured at 75°C for 10 min, preannealed at 37°C for 30 min and applied to each slide. Hybridization was carried out at 37 °C overnight, for the same species, and over 48h and 72h, for congeneric and more distantly related species, respectively. Posthybridization washes were performed in 50% formamide/2x SSC twice for 5 min each, followed by 2x SSC twice for 5 min each and 4x SSC with 0.05% Tween-20 (4xT) once for 4 min. Washes were carried out at 42 °C. Probe detection was performed using 200 μL of diluted (1:500) Cy3-Streptavidin antibody (Amersham) per slide at 37°C for 30 min. After detection, slides were washed in 4xT three times for 3 min each at 42°C and mounted in with anti-fade medium Vectashield (Vector Laboratories) containing 1.5 μg/mL DAPI.

Interspecies comparative genomic hybridization (iCGH)

Total genomic DNA was extracted from ethanol preserved tissues of *I. monticola*, *L. schreiberi* and *T. lepidus* females using a commercial kit (RealPure Genomic DNA Extraction Kit, Durviz), following the manufacturer's instructions. Total genomic DNA was labeled by random priming with the Prime-It Random Priming Labeling Kit (Agilent Technologies), according to the manufacturer's specifications. Genomic DNA of *I. monticola* and *T. lepidus* was labeled with TRITC-dUTP, while genomic DNA of *L. schreiberi* and *T. lepidus* was labeled with FITC-dUTP.

iCGH was performed as described in Chapter I. Reciprocal iCGH experiments were done between each pair of species. For each slide that was made, 250 ng of TRITC-labeled and 250 ng of FITC-labeled DNA were ethanol-precipitated with 20 μg of glycogen and 4 μg of unlabeled, sheared genomic DNA (as competitor) derived from a male of the same species as the target metaphases. In situ hybridization was performed as described in Chapter I.

Microscopy and data analyses

Images were captured using the epifluorescence microscopes Leica DMRXA and Nikon Microphot-FXA, equipped with cooled CCD cameras [Photometrics Sensys and DS-Qi1Mc (Nikon Instruments), respectively]. The Leica CW4000 FISH and the NIS-Elements D 3.10 (Nikon Instruments) softwares were used to capture 16-bit grey-scale images of DAPI, Cy3/TRITC and FITC signals, which were then normalized and merged to a 24-bit colour image. For karyotyping, the DAPI images were displayed in contrast-adjusted reversed greyscale images. The final composition of the images was performed with Adobe Photoshop CS4 11.0.1 (Adobe Systems Inc.).

PCR-assisted gene mapping

Chromosome-specific DNA from flow-sorted chromosomes of *I. monticola* was used as template for PCR mapping of *Anolis carolinensis* genes using conserved gene-specific primers. Primers designed by Brunner et al. (2001) and Pokorná et al. (2011) were used to amplify the conserved B region of *DMRT1* gene and a region of *ATP5A1* gene, respectively. Primers for amplification of all the other genes were newly designed according to *A. carolinensis* DNA sequence available as AnoCar2.0 assembly (the Ensembl Anole Lizard Genome Browser, http://www.ensembl.org/Anolis_carolinensis). Genomic regions of *A. carolinensis* comprising genes of interested were aligned in Muscle (Edgar 2004) with the orthologous regions of *G.*

gallus (the Ensembl Chicken Genome Browser, http://www.ensembl.org/Gallus_gallus), *Pelodiscus sinensis* (the Ensembl Chinese Softshell Turtle Genome Browser http://www.ensembl.org/Pelodiscus_sinensis) and, when available, sequences of other squamate species (*Lacerta agilis*, *Leiolepis reevesii rubritaeniata*, *G. hokouensis, Python bivittatus* and *Alligator mississippiensis*) retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Degenerate primers were designed with Primaclade (Gadberry et al. 2005). Once forward and reverse PCR primers were successfully designed, a BLAST search (BLASTn) for the primer sequences was performed against the *A. carolinensis* genome sequence, and the results were checked to ensure that there were no homologous regions in the genomes, except for the primer sequences themselves (E-value <0.1).

Primer sequences, annealing temperatures and DDBJ/EMBL/GenBank or Ensembl accession numbers of the sequences used for primer design are listed in Table S1a (Supplementary Material). Each PCR reaction was conducted in a final volume of 25 μL and contained ~25 ng of genomic DNA, 1x NZYTaq Green Master Mix (NZYTech) including 2.5 mM $MgCl_2$, 0.5 μM of each primer and 400 ng/μL bovine serum albumin (Sigma). The general reaction conditions were as follows: initial denaturation at 94ºC for 5 min; 35 cycles of denaturation at 94ºC for 30 s, annealing at the corresponding temperature (see Table 1a) for 30 s, extension at 72ºC for 30-60 s; and a final extension at 72ºC for 7 min. A sample of genomic DNA of *I. monticola* was also tested for each primer pair, as positive control. The obtained PCR products were run on 2% agarose gels, stained with Real Safe (Real) and imaged under UV light. PCR products with the expected size and single-band patterns were directly sequenced. Bidirectional sequencing with the PCR primers was performed on an ABI PRISM 3730XL (Applied Biosystems) automatic sequencer. When the PCR product had more than one band, amplicons of interest were excised from the agarose gel and eluted using Pure Link Quick Gel Extraction Kit (Invitrogen).

Some PCRs yielded a product of expected size on the sample of genomic DNA, but not on chromosome templates. In those cases, sequences obtained from genomic DNA were used to design specific internal primers (Table S1b, Supplementary Material), which were then employed in PCR-assisted gene mapping as described above.

Results

Karyotyping and C-banding

DAPI-stained karyotypes of all the analyzed species are shown in Fig. 1. The karyotypes of *I. monticola* and *I. galani* (2n=36) consisted exlusively of acrocentric chromosomes of gradually

decreasing size. A similar heteromorphic sex chromosome pair was found in female specimens of both species, in which the W chromosome (chromosome 15) is distinctly smaller than the Z counterpart (tentatively, chromosome 9 or 10), and showed an intense fluorescent signal after DAPI staining.

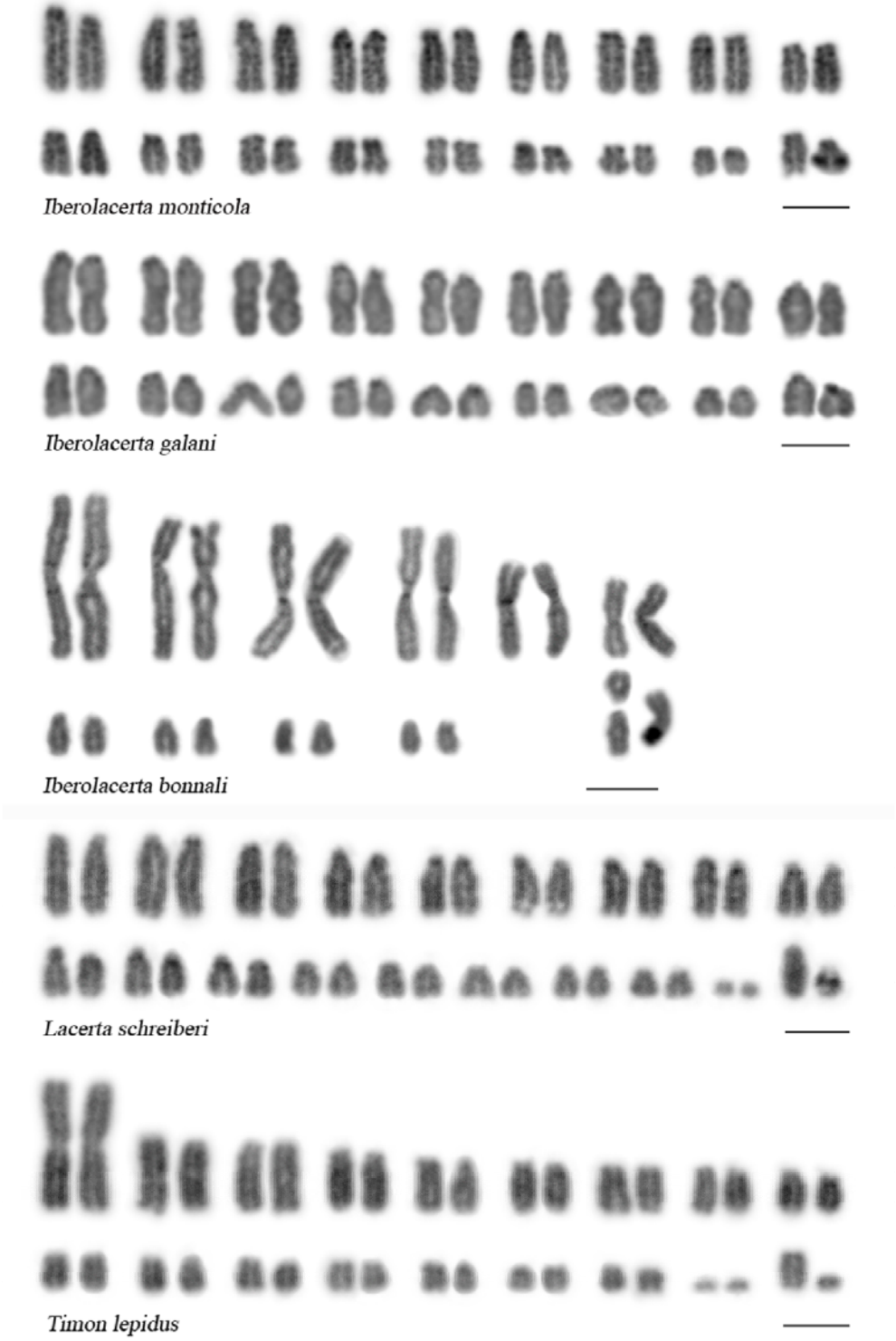


Fig. 1. Karyotypes of female specimens of each of the species studied arranged from DAPI stained metaphases. Scale bars represent 5 μm.

The diplod chromosome number in female *I. bonnali* was 2n=23, and the karyotype comprised 13 biarmed and 10 acrocentric chromosomes. In this species, the W chromosome is a metacentric element (chromosome 7) and its homologues—Z_1 and Z_2—are two smaller acrocentric elements (chromosomes 8 and 12, respectively). A bright DAPI-positive region was observed in the q arm of the W chromosome.

The karyotype of *L. schreiberi* (2n=38) was composed 36 acrocentric chromosomes, gradually decreasing in size, and a pair of microchromosomes. The female specimen analyzed showed a heteromorphic pair formed by a small, DAPI-positive element (chromosome 14 or 15), and a medium-sized counterpart, which was as large as the chromosomes of pair 9, suggesting a possible ZZ/ZW sex chromosome system.

The karyotype of female *T. lepidus* (2n=36) contained one large metacentric chromosome pair, 31 acrocentric chromosomes and three microchromosomes. One of the microchromomes, distinctively stained by DAPI, was recognized as the W sex chromosome, while the putative Z was identified as a medium-sized acrocentric element (chromosome 10).

C-banding revealed similarities in the abundance and distribution of constitutive heterochromatin in the karyotypes of these species, such as the presence of DAPI- and CMA_3-positive centromeric and interstitial/pericentromeric blocks, and the occurrence of GC-rich, faint telomeric C-bands in at least the largest chromosomes of the karyotypes (Fig 2). Differences in the C-banding patterns of these species were mainly associated to the sex chromosomes. The W chromosomes of *I. monticola* and *I. galani* are almost completely heterochromatic, with only a small euchromatic region located in an interstitial position (Figs. 2a, b). The submetacentric W chromosome of *I. bonnali* shows a prominent C-band in the distal region of the q-arm (Fig. 2c). In *L. schreiberi*, the smaller chromosome of the heteromorphic pair (the putative W chromosome) is also easily recognizable after C-banding by bearing a prominent heterochromatin block in interstitial position (Fig. 2d). This same pattern is found in the W chromosome of *T. lepidus* which, despite its small size, seems to be only partially heterochromatic, with an interstitial C-positive region surrounded by proximal and distal euchromatic areas (Fig. 2e). In all the cases, the heterochromatin of the W chromosomes resulted intensely stained after both DAPI and CMA3 staining. As described previously in Chapter 1, the Z chromosome of *I. monticola* could be distinguished from the autosomes by bearing a brighter, CMA_3-positive, telomeric C-band. Lack of males from all the remaining analyzed species hindered the unequivocal identification of the Z chromosomes by C-banding, so they could be only designated by pairing of chromosomes according to size, as described above.

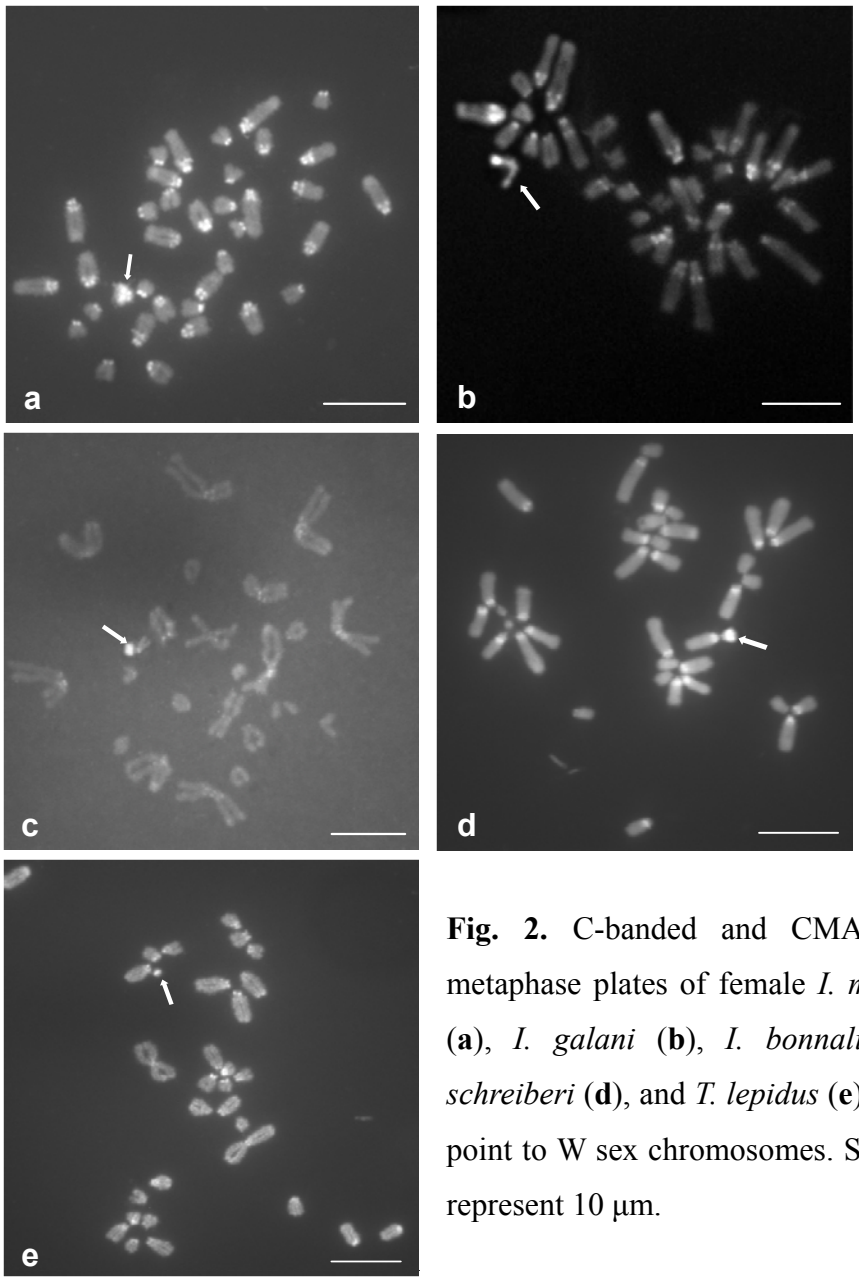


Fig. 2. C-banded and CMA_3-stained metaphase plates of female *I. monticola* (**a**), *I. galani* (**b**), *I. bonnali* (**c**), *L. schreiberi* (**d**), and *T. lepidus* (**e**). Arrows point to W sex chromosomes. Scale bars represent 10 μm.

Flow sorting of I. monticola chromosomes and characterization of painting probes

The 36 chromosomes of the karyotype of *I. monticola* were differentiated into 14 separate flow peaks. Painting probes from each peak were hybridized onto *I. monticola* metaphase chromosomes to determine the chromosome content of these flow peaks (Fig. S1, Supplementary Material). Nine chromosome pairs could be resolved separately, and chromosome-specific painting probes were obtained from them (IMO1-3, 6-10, 17). Three peaks contained two chromosomes each (IMO4,5; IMO5,7 and IMO15,16), and two peaks contained three chromosomes (IMO11,12,Z; IMO13, 14+W).

Cross-species chromosome painting

The probe containing *I. monticola* chromosomes 13, 14 and the W sex chromosome (IMO13,14,W) hybridized to two small, acrocentric chromosome pairs in *I. galani* (IGA13 and 14) and *I. bonnali* (IBN9 and 10) (Figs. 3a, b). It also painted the euchromatin of the W chromosome in *I. galani*, and the euchromatin at the end of the q-arm of the submetacentric W chromosome in *I. bonnali*. A screening with the remaining flow-sorted fractions of *I. monticola* showed that the p-arm of the W chromosome of *I. bonnali* is homologous to *I. monticola* chromosomes 15 or 16 (Fig. 3c). The probe IMO13,14,W also hybridized to a pair of small acrocentric chromosomes in *L. schreiberi* and *T. lepidus* (chromosomes 13 and 14 in both species). However, no signal was detected either on the W chromosome of *L. schreiberi* or on the W microchromosome of *T. lepidus* (Figs. 3d, e).

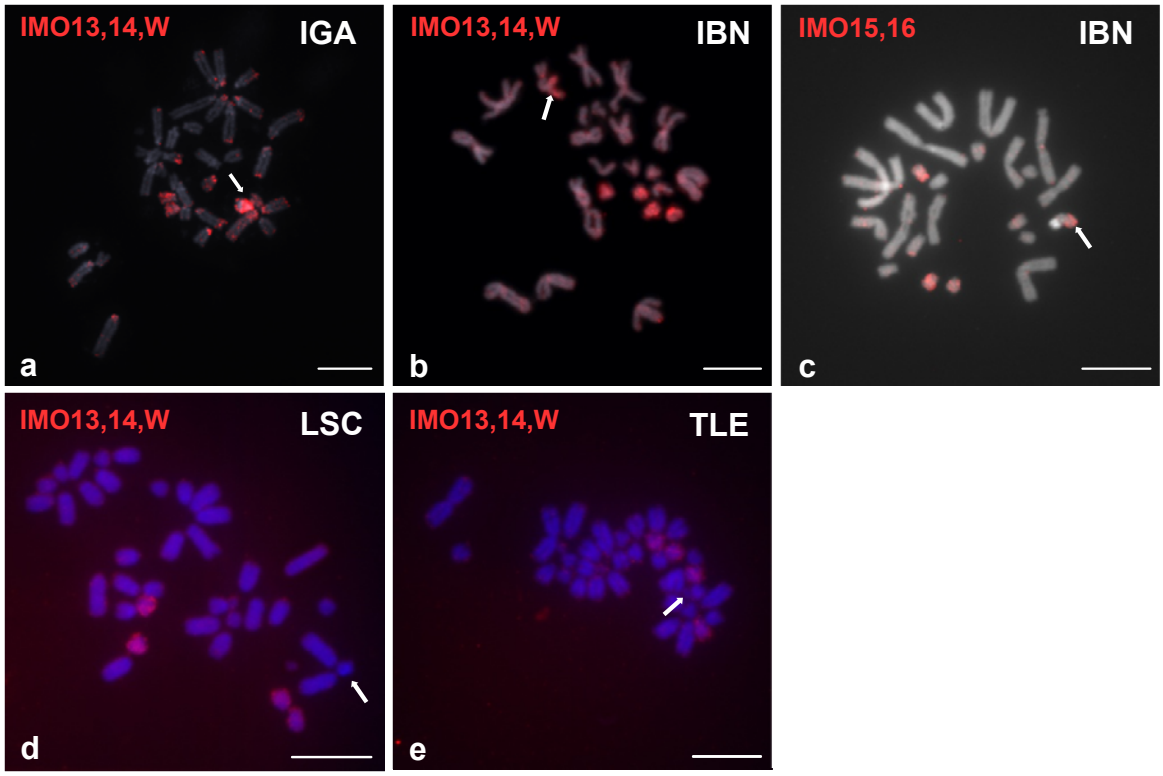


Fig. 3. Cross-species chromosome painting with the IMO13,14,W probe on metaphases of **a** *I. galani* (IGA); **b** *I. bonnali* (IBN); **d** *L. schreiberi* (LSC); **e** *T. lepidus* (TLE). Arrows point to W chromosomes. **c** Chromosome painting with the IMO15,16 probe on *I bonnali*. The arrow indicates the p-arm of the neo-W chromosome. Scale bars represent 10 μm.

The study of chromosome synteny with the whole set of *I. monticola* probes on *L.*

schreiberi and *T. lepidus* revealed a high degree of karyotype conservation between the three species (see Figs. S2 and S3, Supplementary Material, for the complete results of chromosome painting on *L. schreiberi* and *T. lepidus*, respectively). Most *I. monticola* chromosomes were completely preseved—both in DNA content and morphology—in the other lacertids. One of the few rearrangements detected involved *I. monticola* chromosomes 2 and 4, which form the q and p-arms, respectively, of the metacentric chromosome 1 of *T. lepidus* (Figs. 4a, b). In contrast, they are homologous to acrocentric chromosomes 2 and 4 in *L. schreiberi* (Figs. 4c, d).

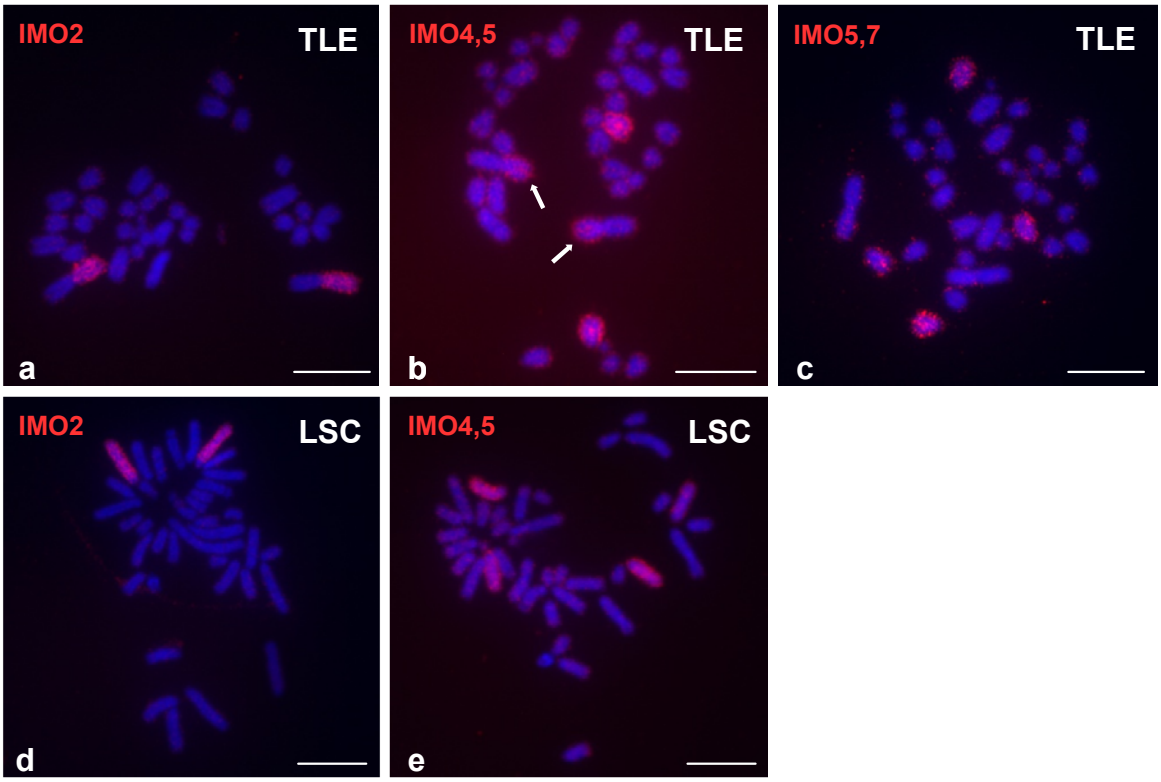


Fig. 4. Cross-species chromosome painting with IMO2 and IMO4,5 probes on metaphases of *T. lepidus* (**a, b)** and *L. schreiberi* (**c**, **d**). Arrows in **b** point to the p-arm of *T. lepidus* chromosome 1, which painted by the IMO4,5 probe, but not by IMO5,7. Scale bars represent 10 μm.

Another discrepancy was found with the paint IMO11,12,Z. This probe painted an odd number of medium-sized chromosomes in *I. monticola* (Fig. 5a). The unpaired chromosome—which, according to its size, could be chromosome 10—is presumably the Z sex chromosome. Chromosome painting with this probe on male *I. monticola* metaphases labeled an even number of chromosomes, thus confirming that this flow peak contains the Z chromosome (Fig.

5b). Similarly, IMO11,12,Z hybridized to five medium-sized acrocentric chromosomes on female *T. lepidus* metaphases. Based on karyotype reconstruction, the Z sex chromosome could also be the tenth largest element (Fig. 5c). In addition, the probe also marked the microchromosome pair of *T. lepidus*. A different painting pattern was found in *L. schreiberi*, which showed signals in three medium-sized chromosome pairs and in the microchromome pair, suggesting that IMOZ is not conserved in this species (Fig. 5d). Among all the paints examined, only IMO15,16 detected an odd number of chromosomes in *L. schreiberi*. This probe labeled the three smallest chromosome pairs in *I. monticola* and *T. lepidus*. However, in *L. schreiberi* it painted two small chromosome pairs and a medium-sized element (chromosome), which would tentatively be the Z (Fig. 5e).

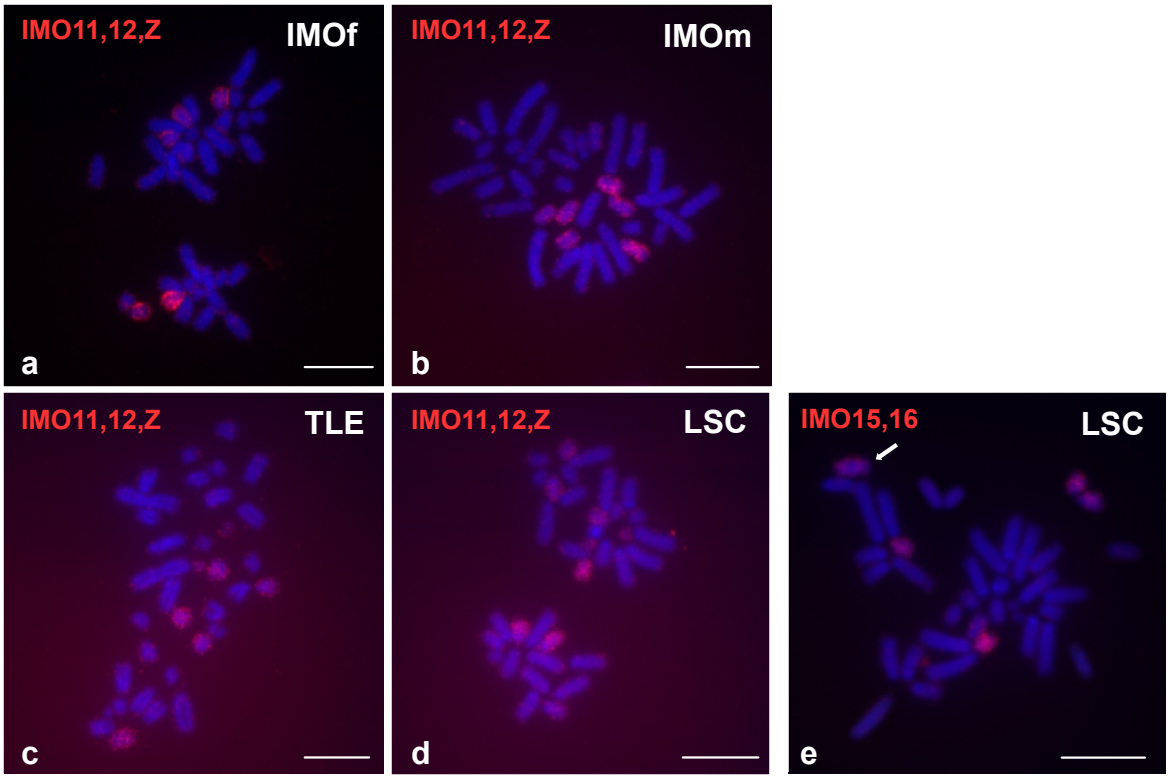


Fig. 5. Cross-species chromosome painting with the IMO11,12,Z probe on metaphases of **a** female *I. monticola* (IMOf); **b** male *I. monticola* (IMOm); **c** *T. lepidus*; **d** *L. schreiberi*. **e** Chromosome painting with the IMO15,16 probe on *L. schreiberi*. The arrow indicates the putative Z chromosome. Scale bars represent 10 μm.

Interspecies comparative genomic hybridization (iCGH)

Absence of hybridization signal with the IMO13,14,W probe on the W chromosomes of *L.*

schreiberi and *T. lepidus* lead us to further investigate the differentation of W chromosomes between the three species by carrying out interspecies CGH. Reciprocal CGH experiments highlighted the accumulation of species-specific sequences in the W chromosomes previously identified as the W (Fig. 6). For instance, the W chromosome of *I. monticola* was predominantly labeled by *I. monticola* genomic DNA when co-hybridized with genomic DNA of either *L. schreiberi* or *T. lepidus* (Figs. 6a, b). The same pattern was observed on metaphases of *L. schreiberi* and *T. lepidus* (Figs. 6c, d and 6e, f, respectively). Due to the bright signals produced by the repetitive content of the W chromosomes, it was not possible to elucidate if the molecular composition of sex chromosomes differed only at the heterochromatic or also at the euchromatic regions.

Moreover, all pairwise comparisons showed differentially labeled regions at the centromeres of *I. monticola* and *T. lepidus* chromosomes, suggesting that centromeric heterochromatin is composed of repetitive elements that are either species-specific or have been differentially amplified DNA in these taxa.

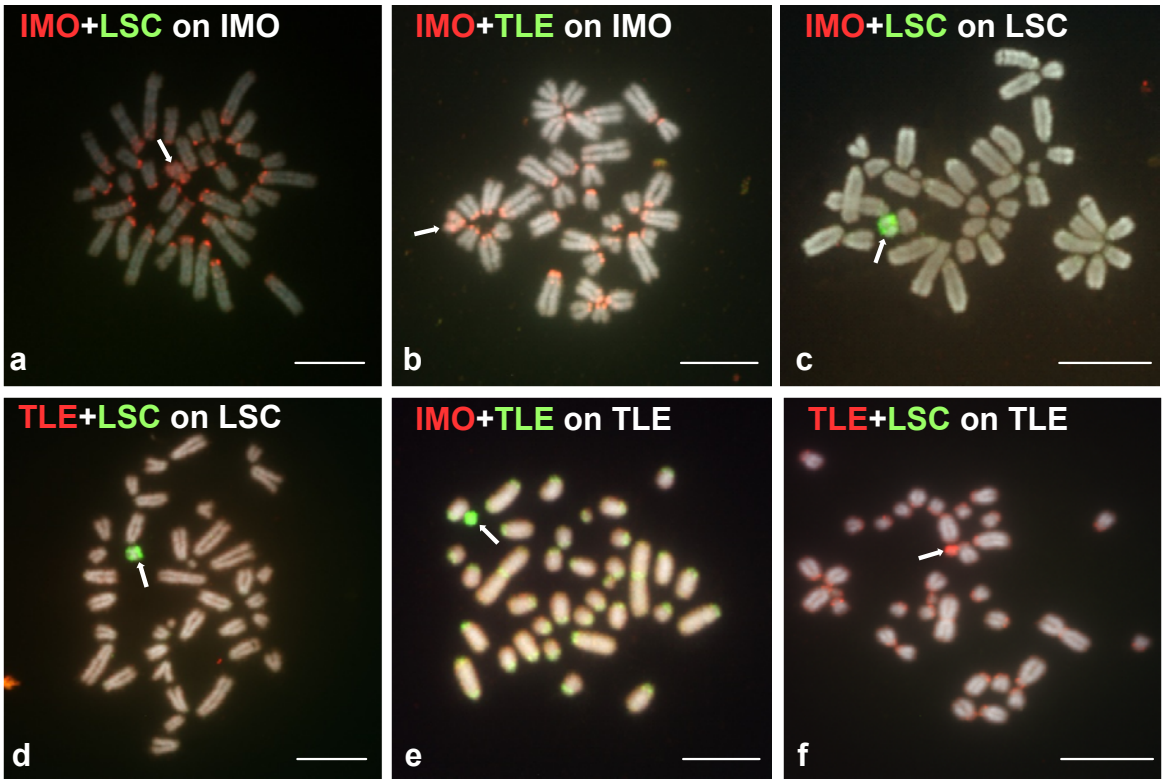


Fig. 6. Interspecies comparative genomic hybridization on female metaphases of *I. monticola* (**a**, **b**), *L. schreiberi* (**c**, **d**), and *T. lepidus* (**e**, **f**). Genomic DNA of *I. monticola* is stained with TRITC (IMO; red), genomic DNA of *L. shcreiberi* is stained with FITC (LSC; green), and genomic DNA of *T. lepidus* with both FITC (**b**, **e**) and TRITC (**d**, **f**). Arrows point to W chromosomes. Scale bars represent 10 μm.

PCR-assisted gene mapping

The results of gene mapping on sorted chromosomes of *I. monticola* and the chromosomal location of the selected markers in other reptilian species are summarized in Table 2 and in Fig 7. Of the 30 primer pairs designed for this analysis, seven yielded the expected products on genomic DNA but failed to amplify in the chromosomal templates. PCR amplification for these markers was unsuccessful or inespecific even after the use of internal, species-specific primers designed from the sequences of the amplicons obtained on genomic DNA.

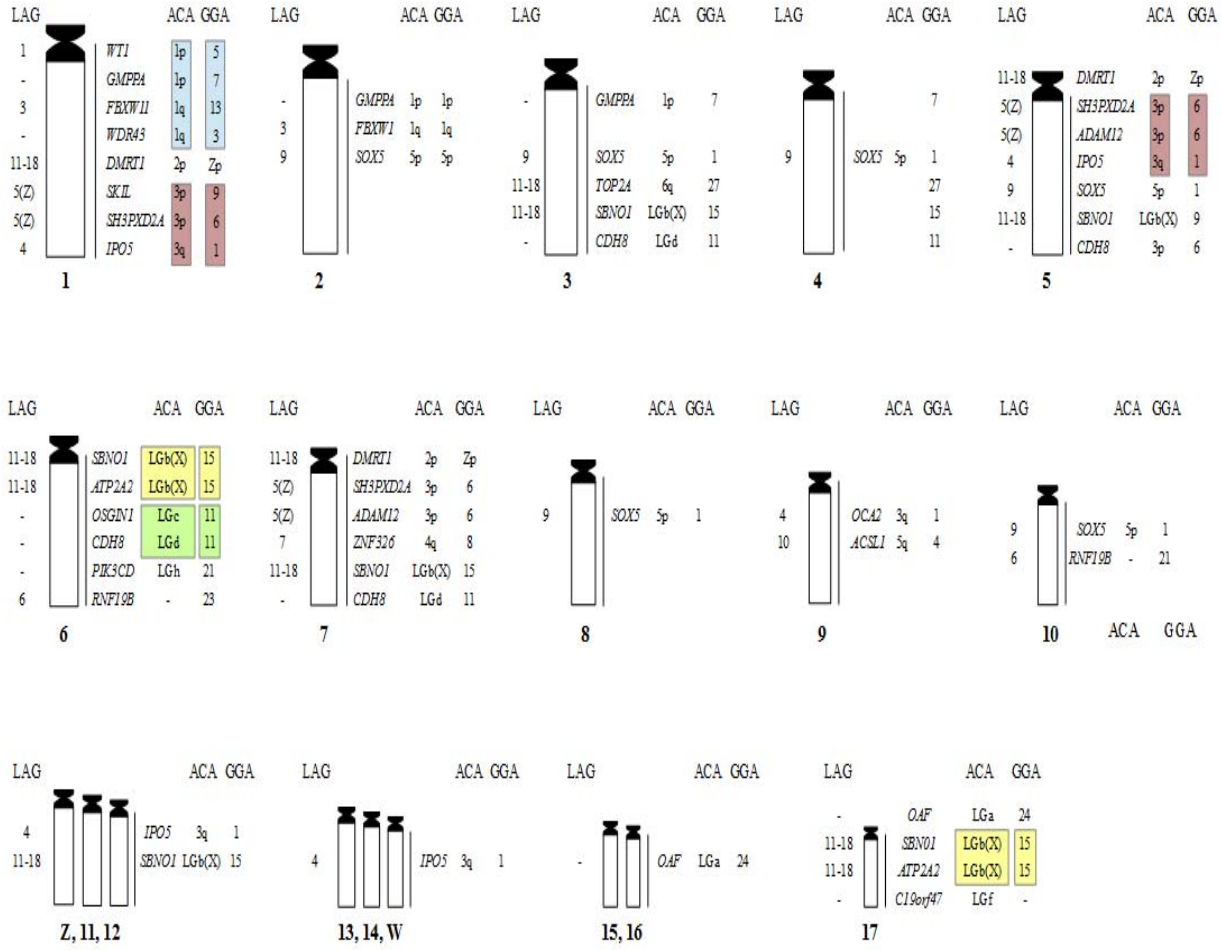
Only 12 out of 30 genes were amplified in a single chromosomal template and hence could be unambiguously assigned to particular chromosomes. The remaining 11 genes yielded PCR amplification in two or more chromosome fractions. Even if the regions for primer design were selected to avoid simultaneous amplification of paralogs in *A. carolinensis*, genomic sequence information was not available for species phylogenetically closer to *I. monticola*. Therefore, it is posible that some of these PCR products are the result of cross-amplification of paralogs of the target genes located on different chromosomes. However, sequences of the amplicons obtained from different chromosome templates were virtually identical and we did not find specific nucleotide differences which enabled the discrimination of paralogs. Hence, it was not possible to solve the correct map position of these markers.

Even so, some interesting results were obtained by comparing our data with the cytogenetic maps available for several sauropsid species [*Gallus gallus, Lacerta agilis*, and five representatives of the so-called Toxicofera clade (i.e., the group encompassing snakes, anguimorphs and iguanians; Vidal and Hedges 2005): *Anolis carolinensis, Varanus salvator macromaculatus*, *Leiolepis reevesii rubritaeniata*, *Pogona vitticeps* and *Elaphe quadrivirgata*]. For instance, four genes that were mapped to *I. monticola* (IMO) chromosome 1 were located on *A. carolinensis* (ACA) chromosome 1 and *G. gallus* (GGA) chromosomes 3, 5, 7 and 13. Three genes on IMO6 were localized to microchromosomes LGc, LGd and LGh in ACA, which are homologous to GGA11 and GGA21. The smallest chromosome in the karyotype of *I. monticola*, IMO17, also seems to have homology with ACA and GGA microchromosomes (LGa and LGf, and GGA24, respectively). In addition, two genes, localized to ACA microchromosome LGb (the XY sex chromosome pair) and GGA15, were mapped to both IMO6 and IMO17. Interestingly, thee genes located on the Z chromosome of *L. agilis* (LAG5) were assigned to IMO1 and/or IMO5-7. They were amplified neither on the chromosomal fraction containing IMO Z chromosome, nor on IMO15-16-17, which painted the putative Z chromosome of *L. schreiberi.*

Table 1. List of genes mapped to *I. monticola* chromosomes and their chromosomal locations in chicken (*Gallus gallus*), the green anole (*Anolis carolinensis*), the sand lizard (*Lacerta agilis*), the water monitor lizard (*Varanus salvator macromaculatus*), the bearded dragon (*Pogona vitticeps*), the butterfly lizard (*Leiolepis reevesii rubritaeniata*), and the Japanese four-striped snake (*Elaphe quadrivirgata*). Lack of data is indicated by a dash.

Gene symbol	Chromosomal location							
	Gallus gallus **(Galliformes, Phasianidae)**	***Anolis carolinensis*** **(Squamata, Dactyloidae)**	***Iberolacerta monticola*** **(Squamata, Lacertidae)**	***Lacerta agilis*** **(Squamata, Lacertidae)**	***Varanus salvator macromaculatus*** **(Squamata, Varanidae)**	***Pogona vitticeps*** **(Squamata, Agamidae)**	***Leiolepis reevesii rubritaeniata*** **(Squamata, Agamidae)**	***Elaphe quadrivirgata*** **(Squamata, Colubridae)**
	(2n=78; 18 biarmed macrochromosomes, 60 microchromosomes	(2n=36; 12 biarmed macrochromosomes, 24 microchromosomes)	(2n=36 uniarmed macrochromosomes)	(2n=38; 36 uniarmed Macrochromosomes, 2 microchromosomes)	(2n=40; 16 biarmed macrochromosomes, 24 microchromosomes)	(2n=36; 12 biarmed macrochromosomes, 24 microchromosomes)	(2n=36; 12 biarmed macrochromosomes, 24 microchromosomes)	(2n=36; 16 biarmed macrochromosomes 10 microchromosomes)
WT1	5	1p	1	1	2q	-	1q	1q
GMPPA	7	1p	1; 2; 3	-	-	1q	-	-
FBXW11	13	1q	1; 2	3	2p	-	-	1p
WDR43	3	1q	1	-	-	-	-	1p
ATP5A1	Zp	1p	-	11-18	1p	2p	2p	2p
DMRT1 **(B region)**	Zp	2p	1; 5-7	11-18	1p	2p	2p	2p
TKT	12	2q	-	2	1q	-	2q	2q
SKIL	9	3p	1	5 (Z)	6q	-	-	-
SH3PXD2A	6	3p	1; 5-7	5 (Z)	6q	-	3p	5q
ADAMI2	6	3p	5–7	5 (Z)	6q	-	-	-
IPO5	1	3q	1; 5; 11-12-Z	4	5q	-	-	-
OCA2	1	3q	9	4	5q	-	3q	-
SS18	2	4p	-	8	7q	-	4p	3p
ZNF326	8	4q	7	7	8p	-	4q	3q
SOX5	1	5p	2; 3; 4-5; 8; 10	9	3p	-	5q	-
ACSL1	4	5q	9	10	3q	-	5p	5p
WAC	2	6p	-	11-18	4q	6p	6q	Zp
TOP2A	27	6q	3	11-18	4p	-	-	-
MYST2	27	6q	3; 9; 13-14-W; 15-16	11-18	4p	6q	6p	Zq
OAF	24	Micro LGa	17	-	-	-	-	-
SBNO1	15	Micro LGb (X)	3; 6; 17	11-18	Micro	-	Micro	-
ATP2A2	15	Micro LGb (X)	6; 17	11-18	Micro	-	Micro	Micro
OSGIN1	11	Micro LGc	6	-	-	-	-	-
CDH8	11	Micro LGd	3; 5-7; 6	-	-	-	-	-
C19orf47	-	Micro LGf	17	-	-	9q	-	-
COL5A1	17	Micro LGg	-	-	-	-	-	-
PIK3CD	21	Micro LGh	6	-	-	-	-	
RNF19B	23	-	6	6	Micro	-	-	-
TRIM37	19	-	-	1	Micro	-	Micro	-
EEF2	28	1p	-	Micro	Micro	-	Micro	1q

Fig. 7. Schematic representation of *I. monticola* karyotype showing the chromosomal locations of mapped genes in comparison with *L. agilis* (LAG), *A. carolinensis* (ACA) and *Gallus gallus* (GGA). The gene order in *I. monticola* is unknown. Colored regions indicate putatively conserved chromosome blocks.



Discussion

Karyotype evolution and sex chromosome differentiation in lacertids

Our results confirm the previously published karyotypes of *I. galani* (Arribas et al. 2006), *I. bonnali* (Odierna et al. 1996) and *T. lepidus* (De Smet 1981; Olmo et al. 1987), in addition to the karyotype of *I. monticola*, previously detailed in Chapter I. Even though only males of *I. monticola* were available to us at the time of the study, the sex chromosomes described for all these species concur with the previous reports.

The karyotype of *L. schreiberi* described by Mateo and Cano (1991) showed a diploid

chromosome number of 2n=38, containing 36 macrochromosomes and 2 microchromosomes, and no heteromorphic sex chromosomes. Our investigation confirmed this diploid complement, but revealed the presence of a heteromorphic chromosome pair in female metaphases of *L. schreiberi*, where one of the homologues had an intertitial C-positive heterochromatic block that might indicate a putative W sex chromosome. Unfortunately, this work could not be extended to males of this species. However, ongoing analyses in a male specimen have already demonstrated that the heterochromatinized chromosome is specific to females, thus supporting the occurrence of a ZZ/ZW sex chromosome system in this species (I. Gómez-Seoane, personnal communication). The identical chromosome number in male and female indicates that the Z chromosome must be present in two copies in males and a single copy in females, and thus rules out a multiple Z_1Z_2/Z_1Z_2W sex chromosome system. Identification of the Z chromosome was not straightforward, but it could tentatively be a medium-sized chromosome, as large as the chromosomes of pair 9 or 10. It is possible that the differences between our observations and those of Mateo and Cano (1991) are due to the different origin of the examined individuals: the specimens studied in that previous work were sampled from the locality of Santa María de Ortigueira (A Coruña, Spain), while the female analyzed in the present work came from a different population (Natural Park Montes do Invernadeiro; Ourense, Spain). Therefore, they could represent different chromosomal races, even though these populations seem to constitute a single genetic unit with extensive gene flow (e.g., Paulo et al. 2001; Godinho et al. 2008). Alternatively, differences in the karyotypes could be related to the experimental approach followed by Mateo and Cano: although both males and females were included in their work, sex chromosome heteromorphism was investigated in male meiosis, which would have hampered the detection of a female-specific sex chromosome.

A comparison among karyotypes of the three genera analyzed in this study concurs with the previously reported stable chromosome morphology in lacertid lizards (Olmo et al. 1986), which typically show a karyotype with 36 acrocentric chromosomes and two microchromosomes (Olmo and Signorino 2005). Among the species analyzed, *L. schreiberi* has retained the ancestral karyotype. The main structural differences among their karyotypes concern the presence of a large metacentric chromosome pair in *T. lepidus* and the lack of microchromosomes in *Iberolacerta*. Reduced diplod numbers of 2n=36 in *T. lepidus* and in most *Iberolacerta* species suggest that the metacentric element in the former species could be the result of a Robertsonian fusion between two acrocentric chromosomes, while the loss of microchromosomes could be a consequence of a translocation of microchromosomes to

macrochromosomes before species radiation within *Iberolacerta* (Cobror 1984; Olmo et al. 1986). Indeed, our results of chromosome painting show that the metacentric element of *T. lepidus* has been formed by a centric fusion involving chromosomes homologous with *I. monticola* chromosomes 2 and 4. On the other hand, the microchromosomes of *T. lepidus* and *L. schreiberi* were painted by the probe IMOZ,11,12, indicating that lack of microchromosomes in *I. monticola* is a result of their translocation to chromosomes 11 or 12. The analysis of homology of sex chromosomes between the three species (discussed below) make the fusion between microchromosomes and the Z chromosome of *I. monticola* less likely than their fussion to autosomes 11 or 12. Interestingly, interstitial telomeric sites (ITSs) were not detected by FISH in any of these three chromosome pairs (see Chapter I), which suggests that telomeric sequences were not retained at the fusion point.

Thus, ancestral syntenies have remained unchanged for at least 12-16 myr (Arnold et al. 2007; Pavlicev and Mayer 2009) in Lacertidae, and the rapid diversification of this family has been accompanied by only a few chromosome rearrangements. The general karyological uniformity of lacertids contrast sharply with the karyotype of *I. bonnali*, that shows a highly derived diplod number (2n=23 in females, 24 in males) and the presence of numerous biarmed elements (Odierna et al. 1996; present work). This condition is not exclusive of *I. bonnali*, but is also shared by the two other species of the Pyrenean clade of *Iberolacerta*, *I. aranica* (2n= 26) and *I. aurelioi* (2n=25-26) (Odierna et al. 1996). As both the ancestral and derived karyotypes possess the same fundamental number (NF=36), the latter probably originated through chromosomal rearrangements involving several Robertsonian fusions, after the separation of the Pyrenean and the *I. horvathi*-Iberian clades, about 11.6-15.6 mya (Arribas et al. 2014). The cause of this increased rate of karyotype evolution in the Pyrenean taxa remains to be investigated.

Homology and evolution of lacertid sex chromosomes

Cytogenetic analyses of lacertid sex chromosomes published so far suggest that female heterogamety is universal within this family, and that the W chromosome exhibits various stages of differentiation in different lineages: from homomorphic and poorly differentiated to heteromorphic and highly heterochromatinized (Olmo et al. 1986, 1987; Odierna et al. 1993; Olmo and Signorino 2005). However, the number of lacertid taxa with differentiated sex chromosomes will probably increase as the karyotypes of more species with cryptic or presumably homomorphic sex chromosomes are subjected to detailed investigations with more sensitive cytogenetic techniques, as found in this thesis for *I. monticola* and *L. schreiberi*.

According to the proposed model of sex chromosome evolution in lacertids (Olmo et al. 1987; Odierna et al. 1993), the W chromosome found in our analysis in *L. schreiberi*—moderately heterochromatic with two broad euchromatic areas—would represent an earlier stage of degeneration than the W chromosomes of *I. monticola* and *I. galani*—comparatively smaller and mostly heterochromatic—while the W microchromosome of *T. lepidus* would show the highest level of differentiation. Besides the well-documented diversity of sex chromosomes in *Iberolacerta*, previously reported in Chapter I, the two other genera investigated in this work are good representatives of the plasticity of sex chromosomes in lacertids. For instance, populations of *Lacerta viridis viridis* from Hungary, like *L. schreiberi*, have a intermediate-sized W chromosome, but completely C-banded (Olmo et al. 1986). Yet some other species, such as *L. agilis*, *L. trilineata*, *L. strigata* and perhaps different populations of *L. viridis* have a micro-W chromosome (Gorman 1969; Ivanov and Fedorova 1970 1970; De Smet 1981; Olmo et al. 1987; Srikulnath et al. 2014). Intraspecific variability in sex chromosomes has also been reported for *T. lepidus*: specimens from a population in Northeastern Spain have been found to possess a homomorphic and heterochromatic W chromosome, whilst specimens from a different, but unknown, Spanish population have a W microchromosome, as the one found in the present study (Olmo et al. 1987). Altogether, these findings, and the general lack of phylogenetic correlation in the degree of heteromorphism, have lead to suggest that the transition from a primitive stage of sex chromosome differentiation, where both homologues are cytologically indistinguishable, to a more advanced stage, with a W chromosome heteromorphic and heterochromatic, might have happened independently in different species, and even in subspecies or populations of the same species (Olmo et al. 1987; Odierna et al. 1993).

However, no studies have evaluated so far the homology between the sex chromosomes of different species. Here, we found that the probe containing the W chromosome of *I. monticola* painted the euchromatic region of the W chromosome in *I. galani*, as well as the q arm of the W chromosome in *I. bonnali*, which indicates that the female-specific chromosomes are conserved among the three species. Lack of hybridization of the W-derived probe on the Z counterparts in either species suggests that the Z and W chromosomes share few or no sequences, and further supports the advances stage of degeneration of W chromosomes detected by C-banding.

As mentioned before, *I. bonnali* differs from the two other *Iberolacerta* species analyzed in bearing a biarmed W chromosome and a multiple Z_1Z_2W sex chromosome system. Multiple

sex chromosomes are thought to evolve via autosome-sex chromosome fusions (Wright 1973; King 1977; Olmo 1986; Odierna et al. 2001; Leaché and Sites 2009; Ezaz et al. 2009). Indeed, a screening with the remaining flow-sorted fractions of *I. monticola* revealed that the autosome which fused to the primitive W to form the neo-W chromosome in *I. bonnali* was chromosome pair 15 or 16. The homologous chromosomes now serve as Z_1 and Z_2 sex chromosomes. This multiple sex chromosome system is also present in at least another of the three Pyrenean species of *Iberolacerta*, *I. aurelioi* (Odierna et al. 1996). Since a simple ZW system seems to be the ancestral condition for this genus (see Chapter I), the occurrence of multiple sex chromosomes, like the abundance of biarmed elements in their karyotypes, would be a plesiomorphic character of this clade.

Even though multiple sex chromosomes are common in lizards with XY systems, they are rather unusual in ZW species (Ezaz et al. 2010). As far as we know, multiple sex chromosomes are only present in the family Lacertidae, with the exception of an unusual $Z_1Z_2W_1W_2$ system recently described in the gecko *Paroedura gracilis* (Koubová et al. 2014). Among lacertids, Z_1Z_2W sex chromosomes have evolved independently not only in the Pyrenean *Iberolacerta*, but also in *Podarcis taurica* and in *Zootoca vivipara* (Olmo and Signorino 2005). The latter case is especially interesting, since multiple and simple sex chromosomes have been found in different populations of the same species (e.g., Odierna et al. 2001; Kupriyanova et al. 2006).

Chromosome painting with the IMOW probe produced no hybridization signal either on the W chromosome of *L. schreiberi* or on the W microchromosome of *T. lepidus.* The putative differences between the W chromosomes of the three species were further investigated by using comparative genomic hybridization. These reciprocal cross-species hybridization experiments demonstrated that female-specific sequences are not conserved between the three species and, hence, they may have been amplified independently on the W chromosomes after the separation of these three genera. In fact, FISH experiments with a probe of TaqI satellite DNA, isolated from *Iberolacerta* (see Chapter III), showed that the this satellite family, which is widely distributed in the genomes of the three species, has been subsequently amplified only in the W chromosome of *L. schreiberi* (Fig. S4; Supplementary Material). Since *Lacerta* s. str. and *Timon* are sister taxa (e.g., Arnold et al. 2007; Pyron et al. 2013), the sex-specific accumulation of TaqI repeats may be a feature exclusive of the genus *Lacerta* s. str., or even only of *L. schreiberi*. Similarly, a recent study documented a rapid and independent amplification of different microsatellite motifs on the W chromosomes in two clades of Australian monitor lizards (Matsubara et al. 2014a). These observations suggest that differentiation of sex chromosomes, even between closely related species with the same type

of sex chromosome system, may follow independent pathways, perhaps associated with the accumulation of different repetitive sequences and lineage-specific rates of W chromosome degeneration.

In order to determine whether the lack of conservatism of the W chromosomes was extensive to their Z counterparts, chromosome painting using the Z chromosome probe from *I. monticola* (IMOZ) was also performed on *L. schreiberi* and *T. lepidus* metaphases. The probe labelled an odd number of small acrocentric chromosomes in *T. lepidus*, identifying an unpaired chromosome which is tentatively the Z. Therefore, the Z chromosome of *T. lepidus* seems to be homologous to the Z chromosome of *I. monticola*. On the contrary, the IMOZ probe hybridized to an even number of chromosomes in *L. schreiberi*, and the putative Z chromosome of this species was detected with the IMO probe containing chromosome pairs 15-16. Recently, the Z chromosome of a congeneric species, *L. agilis*, has been identified through chromosome banding and gene mapping as the fifth largest chromosome of the karyotype (Srikulnath et al. 2014). It appears to be somewhat larger in size than the putative Z chromosome of *L. schreiberi*, which in our karyotype reconstruction is as large as chromosome 10.

Even if these results are yet to be confirmed in males of *L. schreiberi* and *T. lepidus*, our preliminary data suggest that sex determination in *L. schreiberi* involves a ZW chromosome pair that is different from the ZW chromosomes of *I. monticola* and *T. lepidus*. This apparent lack of homology is stricking, considering the strong conservatism of lacertid karyotypes, the advanced stage of degeneration of *L. schreiberi* W chromosome and the short divergence time and the rapid diversification of Lacertini lineages (12-16 mya) (Arnold et al. 2007; Pavlicev and Mayer 2009). However, it would not be unique among lizards: rapid evolution of non-homologous ZW sex chromosomes has been reported in Australian dragon lizards which diverged at most around 25 mya (Ezaz et al. 2009b). Another recent study using chromosome painting showed that the ZW sex chromosomes of two gecko species are not homologous and represent independently derived ZW sex chromosomes within the Gekkonidae (Matsubara et al. 2014b).

Since the genus *Lacerta* s. str. is nested among lineages with homologous sex chromosomes (i.e., *Timon* and *Iberolacerta*), the sex chromosome pair of *L. schreiberi* seems to be represent a derived condition. Thus, a possible evolutionary scenario would be the presence of an original, poorly differentiated ZW system in the common ancestor of Lacertini, which is still present in *T. lepidus* and *Iberolacerta*. In *L. schreiberi*, or perhaps in the ancestral *Lacerta*

species, a shift from one ZW system to a different ZW system may have occurred after the translocation or transposition of the sex determining gene to a non-homologous chromosomal region, or when the appearance of a neo-sex determining gene on an autosomal pair defined a new W chromosome, as proposed for salmonid fishes (Woram et al. 2003; Phillips 2013).

Alternatively, it is also possible that the ancestral situation was the presence of a differentiated ZW chromosome pair, which was subsequently lost in the lineage leading to *L. schreiberi*. An analogous scenario has been recently proposed to explain transitions of sex chromosomes in Madagascar geckos of the genus *Paroedura* (Koubová et al. 2014) or in *Drosophila* (Vicoso and Bachtrog 2013). For instance, loss of differentiated sex chromosomes in *Drosophila* involved the reversal of an ancient sex chromosome back to an autosome, and takeover of the sex determining function by a formely autosomal pair (Vicoso and Bachtrog 2013).

Even if these hypotheses are merely speculative, the putative independent evolution of sex chromosomes among closely related taxa suggests that there may be cryptic complexity in the evolution of lacertid sex chromosomes. Hence, assessing sex chromosome homology among other closely related species and genera, as well as in outgroups to Lacertidae, should be a priority for future research. The Z and W chromosome paints developed in the present work will be valuable for this purpose.

PCR-assisted gene mapping and karyotype evolution in squamate reptiles

A recent cytogenetic map of *Lacerta agilis* (LAG) showed that the largest acrocentric chromosomes of this lacertid species are largely syntenic with macrochromosomes and/or macrochromosome segments of *Gallus gallus* (GGA) and four Toxicofera species (*Anolis carolinensis*, ACA; *Varanus salvator macromaculatus*, VSA; *Leiolepis reevesii rubritaeniata*, LRE; and *Elaphe quadrivirgata*, EQU). Most of the genes located on the microchromosomes of Toxicofera were localized to *L. agilis* chromosome 6 (LAG6), small acrocentric chromosomes (LAG11-18), and a microchromosome (LAG19) (Srikulnath et al. 2014).

Linkage homology of IMO chromosomes with the macrochromosomes of the other species based on our results of PCR-assisted gene mapping remains largely unresolved, because this approach had several caveats. Firstly, almost a third of the selected markers could be amplified on genomic DNA, but not on the chromosomal templates. It has been reported that the efficiency of PCR on chromosomes consisting of condensed DNA complexes with proteins may be lower in comparison to PCR using pure DNA (Kejnovský et al. 2001). In addition, non-specific methods of DNA amplification from flow-sorted chromosomes, such as DOP-

PCR, are characterized by high amplification bias and provide incomplete genome coverage (Dean et al. 2002; Pinard et al. 2006), so it is possible that some genomic regions containing target genes were absent in the chromosomal fractions.

Secondly, more than half of the genes with successful amplification were detected in several chromosomal templates and could not be unambiguously assigned to particular chromosomes. In some of these cases, the specific PCR product was amplified in chromosomal templates which were contiguous in the flow karyotype (e.g., *GMPPA* in chromosomes 1 and 2-3), suggesting that there may be contamination from DNA of the neighboring peaks. A different pattern was found for some other genes, such as *DMRT1*. The *DMRT1* gene is a conserved component of the sex-determining pathway of vertebrates and is a strong candidate for male sex determination in birds (Smith et al. 2009). It also lies on the Z and W chromosomes in the gecko lizard *Gekko hokouensis* (Kawai et al. 2009). However, *DMRT1* has been mapped to autosomes in the other Toxicofera species [ACA2p, VSA1p, *Pogona vitticeps* (PVI) 2p, LRE2p and EQU2p] (Matsubara et al. 2006 ; Srikulnath et al. 2009, 2013; Ezaz et al. 2009c; Alföldi et al. 2011; Young et al. 2013), and to one of the smallest acrocentric macrochromosomes in *L. agilis* (LAG11-18). In contrast, it was amplified on IMO chromosomes 1 and 5-7. This pattern of co-amplification on both chromosomes 1 and 5-7 was also found for genes *SH3PXD2A* [LAG5(Z)] and *IPO5* (LAG4), and it might indicate contamination of chromosome peak IMO1 with fragments of chromosomes 5-7, or vice versa. Alternatively, the pattern oberved for these genes may not be due to contamination of flow sorted fractions, but to structural rearrangements, like interchromosomal segmental duplications, in the genome of *I. monticola*. Even though our results of chromosome painting show a high degree of chromosome conservation between *I. monticola* and *L. schreiberi*, such structural changes might have not been detected at the resolution of whole chromosome painting (see, e.g., Bailey et al. 2002, for a demonstration of segmental duplications in human chromosome 22 previously undetected by chromosome painting). Physical mapping of cDNA fragments of these genes would be useful to corroborate their chromosomal location and examine the extent of chromosomal rearrangements in *I. monticola*.

The shortcomings of this experimental approach hampered the investigation of linkage homology between the chromosomes of *I. monticola* and the macrochromosome and/or macrochromosomal arms of the Toxicofera clade, except for chromosome 1. Four genes mapped to IMO1 were localized to ACA1, and to chicken chromosomes 3, 5, 7 and 13. Homology with GGA3, GGA5 and GGA7 was also found in VSA2, LRE1, PVI and EQU1

(Pokorná et al. 2012; Srikulnath et al. 2009, 2013; Young et al. 2013). In all these species, an arm of a large of a large bi-armed chromosome (1 or 2) is sytenic with GGA3, while the proximal and distal parts of the other arm are syntenic with GGA7 and GGA5, respectively. Indeed, this synteny is conserved across most major squamate lineages, which suggests that the association of the avian ancestral chromosomes 3, 5 and 7 can be dated back to the common ancestor of the lineage Unidentata (Serpentes, Iguania, Anguimorpha, Laterata and Scinciformata) (Pokorná et al. 2012). In the lacertid *Eremias velox*, GGA5 and GGA7 probes hybridized to part of chromosome 1, while the GGA3 probe painted a different pair of acrocentric chromosomes (Pokorná et al. 2012). The only gene located on GGA3 in our study (*WDR43*) was mapped to IMO1, whereas two different GGA3-linked genes were localized to chromosome 3 in *L. agilis*. Altogether, these data suggest that the fission of the ancestral bi-armed chromosome took place in the ancestral lacertid karyotype but it might have been preceded by intrachromosomal rearrangements, which anyway need to be confirmed by high-resolution gene mapping.

It is also interesting to note that three genes (*SKIL*, *SH3PXD2A* and *ADAM12*) linked to the Z chromosome of *L. agilis* (LAG5) were mapped to IMO1 and/or IMO5-7. They were amplified neither in the sample containing the Z chromosome of *I. monticola* (IMOZ,11,12), nor in the sample that painted the putative Z chrosomome in *L. schreiberi* (IMO15,16). Physical gene mapping of LAGZ orthologs, along with comparative chromosome painting with the IMOZ probe in more species representative of different lacertid lineages, will be necesssary to elucidate sex chromosome evolution in this group.

One of the characteristic features of lacertid karyotypes, in comparison with the karyotypes of Toxicofera species, is the reduction in the number or even the absence of microchromosomes (e.g., only one chromosome pair in *L. agilis* and no microchromosomes in *I. monticola*). In agreement with the cytogenetic map of *L. agilis*, our results show that genes localized on the microchromosomes of *A. carolinensis* were mostly located on IMO6 and IMO17. In fact, both chromosomes, and perhaps some other small chromosome pairs (11-18), appear to be syntenic only to Toxicofera microchromosomes. This suggests that the dissappearance of microchromosomes in the lacertid karyotype resulted from repeated fusions between these elements in the ancestral karyotype, rather than from translocation of microchromosomes to macrochromosomes. Interestingly, two genes (*ATP2A2* and *SBNO1*) anchored to the X chromosome of *A. carolinensis* (microchromosome LGb) were mapped to both IMO6 and IMO17. According to the results of *L. agilis*, which localized both genes to LAG11-18, it seems reasonable to supposse that their chromosomal location is IMO17.

Anyhow, the micro-X chromosome of *A. carolinensis* is not homologous to the Z chromosome of either *I. monticola* or *L. agilis*. These new data fill an important gap in the knowledge of sex chromosome homology between lacertids and other squamate groups, and further support the multiple and independent origin of lizard sex chromosomes from different autosomal pairs of the common ancestor.

References

Alföldi J, Di Palma F, Grabherr M, Williams C, Kong L, Russell EP, Lowe CB, Glor RE, Jaffe JD, Ray DA, Boissinot S, Shedlock AM, Botka C, Castoe TA, Colbourne JK, Fujita MK, Godinez- Moreno R, Ten-Hallers BF, Haussler D, Heger A, Heiman D, Janes DE, Johnson J, De Jong PJ, Koriabine MY, Lara M, Novick PA, Organ CL, Peach SE, Poe S, Pollock DD, De Queiroz K, Sanger T, Searle S, Smith JD, Smith Z, Swofford R, Turner-Maier J, Young JS, Zadissa A, Edwards SV, Glenn TC, Schneider CJ, Losos JB, Lander ES, Breen M, Ponting CP, Lindblad-Toh K (2011) The genome of the green anole lizard and a comparative analysis with birds and mammals. Nature 477: 587–591.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-410.

Arnold EN, Arribas O, Carranza S (2007) Systematics of the Palaearctic and Oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa 1430: 1-86.

Arribas O, Carranza S, Odierna G (2006) Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240: 1-55.

Bailey, Jeffrey A, Amy M Yavor, Luigi Viggiano, Misceo D, Horvath JE, Archidiacono N, Schwartz S, Rocchi M, Eichler EE (2002) Human-Specific Duplication and Mosaic Transcripts: The Recent Paralogous Structure of Chromosome 22. Am J Hum Genet 70: 83-100.

Bosch HAJ in den, Odierna G, Aprea G, Barucca M, Canapa A, Capriglione T, Olmo E (2003) Karyological and genetic variation in Middle Eastern lacertid lizards, *Lacerta laevis* and the *Lacerta kulzeri* complex: a case of chromosomal allopatric speciation. Chromosome Res 11: 165-78.

Brunner B, Hornung U, Shan Z, Nanda I, Kondo M, Zend-Ajusch E, Haaf T, Ropers HH, Shima A, Schmid M (2001) Genomic organization and expression of the doublesex-

related gene cluster in vertebrates and detection of putative regulatory regions for DMRT1. Genomics 77: 8-17.

Cobror O (1984). The chromosome complement of some insular lacertid lizards. Atti Accad Naz Lincei, Rend 76: 189-192.

Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS (2002) Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA 99: 5261-5266.

De Smet WHO (1981) Description of the orcein stained karyotypes of 36 lizard species (Lacertilia, Reptilia) belonging to the families Teiidae, Scincidae, Lacertidae, Cordylidae and Varanidae (Autarchoglossa). Acta Zool Pathol Antverpiensia 76: 73-118.

Edgar RC (2004) MUSCLE: nultiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792-1797.

Ezaz T, Quinn AE, Miura I, Sarre D, Georges A, Graves JAM (2005) The dragon lizard Pogona vitticeps has ZZ/ZW micro-sex chromosomes. Chromosome Res 13: 763-776.

Ezaz T, O'Meally D, Quinn AE, Sarre SD, Georges A, Graves JAM (2008) A simple non-invasive protocol to establish primary cell lines from tail and toe explants for cytogenetic studies in australian dragon lizards (Squamata: Agamidae). Cytotechnology 58: 135–39.

Ezaz T, Sarre SD, O'Meally D, Graves JAM, Georges A (2009a.) Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet Genome Res 127: 249-60.

Ezaz T, Quinn AE, Sarre SD, O'Meally D, Georges A, Graves JAM (2009b) Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards. Chromosome Res 17: 91-98.

Ezaz T, Moritz B, Waters PD, Graves JAM, Georges A, Sarre SD (2009c) The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. Chromosome Res 17: 965-973.

Fu J (2000) Toward the phylogeny of the family Lacertidae - Why 4708 base pairs of mtDNA sequences cannot draw the picture. Biol J Linnean Soc 71: 203-17.

Gadberry MD, Malcomber ST, Doust AN, Kellogg EA (2005) Primaclade—a flexible tool to find conserved PCR primers across multiple species. Bioinformatics 21: 1263-64.

Gamble T (2010) A review of sex determining mechanisms in geckos (Gekkota: Squamata).

Sex Dev 4: 88-103.

Gamble T, Coryell J, Ezaz T, Lynch J, Scantlebury DP, Zarkower D (2015) Restriction site-associated DNA sequencing *(*RAD-seq*)* reveals *an* extraordinary number *of* transitions among gecko sex-determining systems. *Mol Biol Evol 32: 1296-1309.*

Gamble T, Geneva AJ, Glor RE, Zarkower D (2014) *Anolis* sex chromosomes are derived from a single ancestral pair. Evolution 68: 1027-1041.

Gorman GC (1969) New chromosome data for 12 species of lacertid lizards. J Herpetol 3: 49-54.

Gorman GC (1973). The chromosomes of the Reptilia, a cytotaxonomic interpretation. In: Chiarelli AB, Capanna E, ed. Cytotaxonomy and vertebrate evolution. London: Academic Press, pp 349-424.

Harris DJ, Arnold EN, Thomas RH (1998) Relationships of lacertid lizards (Reptilia: Lacertidae) estimated from mitochondrial DNA sequences and morphology. Proc R Soc Lond *B* Biol Sci 265: 1939-48.

Hipsley CA, Lin Himmelmann, Dirk Metzler, and Johannes Müller. 2009 Integration of Bayesian Molecular Clock Methods and Fossil-Based Soft Bounds Reveals Early Cenozoic Origin of African Lacertid Lizards. BMC Evolutionary Biology 9: 151.

Hipsley CA, Himmelmann L, Metzler D, Mueller J (2009) Integration of Bayesian molecular clock methods and fossil-based soft bounds reveals Early Cenozoic origin of African lacertid lizards. BMC Evol Biol 9: 1-13.

Ivanov VG, Fedorova TA (1970) Sex heteromorphism of chromosomes in *Lacerta strigata*. *Tsitologiia* 12: 1582–85.

Ivanov VG, Fedorova TA (1973) Heterochromosomes in the karyotype of *Eremias arguta* Pall. Tsitologiya 15: 762-765.

Kawai A, Nishida-Umehara C, Ishijima J, Tsuda Y, Ota H, Matsuda Y (2007) Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. Cytogenet Genome Res 117: 92-102.

Kejnovský E, Vrána J, Matsunaga S, Soucek P, Siroký J, Dolezel J, Vyskot B (2001) Localization of male-specifically expressed *MROS* genes of *Silene latifolia* by PCR on flow-sorted sex chromosomes and autosomes. Genetics 158: 1269-77.

King M (1977) The evolution of sex chromosomes in lizards. In: Calaly J, Tyndale-Biscoe H, eds. Evolution and Reproduction. Canberra: Australian Academy of Science, pp 55-60.

Koubová M, Johnson Pokorná M, Rovatsos M, Farkačová K, Altmanová M, Kratochvíl L

(2014) Sex determination in Madagascar geckos of the genus *Paroedura* (Squamata: Gekkonidae): are differentiated sex chromosomes indeed so evolutionary stable? Chromosome Res 22: 441-452.

Leaché AD, Sites JW Jr (2009) Chromosome evolution and diversification in North American spiny lizards (genus *Sceloporus*). Cytogenet Genome Res 127: 166-81.

Mateo JA, Cano J (1991) Sobre el cariotipo de tres especies de lacértidos: *Acanthodactylus erythrurus* (Schinz), *Lacerta schreiberi* Bedriaga y *Lacerta perspicillata* (Dum. y Bib.). Rev Esp Herpetol 5: 141-147.

Matsubara K, Kuraku S, Tarui H, Nishimura O, Nishida C, Agata K, Kumazawa Y, Matsuda Y (2012) Intra-genomic GC heterogeneity in sauropsids: evolutionary insights from cDNA mapping and GC3 profiling in snake. BMC Genomics 13: 604.

Matsubara K, Tarui H, Toriba M, Yamada K, Nishida-Umehara Ch, Agata K, Matsuda Y (2006) Evidence for different origin of sex chromosomes in snakes, birds, and mammals and stepwise differentiation of snake sex chromosomes. Proc Natl Acad Sci USA 103: 18190-18195.

Matsubara K, Sarre SD, Georges A, Matsuda Y, Graves JAM, Ezaz T (2014a) Highly di fferentiated ZW sex microchromosomes in the Australian *Varanus* species evolved through rapid amplification of repetitive sequences. PLoS One 9: e95226

Matsubara, K, Gamble T, Matsuda Y, Zarkower D, Sarre SD, Georges A, Graves JAM, Ezaz T. (2014b) Non-homologous sex chromosomes in two geckos (Gekkonidae: Gekkota) with female heterogamety. Cytogenet Genome Res 143: 251-58.

Matsuda Y, Nishida-Umehara Ch, Tarui H, Kuroiwa A, Yamada K, Isobe T, Ando J, Fujiwara A, Hirao Y, Nishimura O, Ishijima J, Hayashi A, Saito T, Murakami T, Murakami Y, Kuratani S, Agata K (2005) Highly conserved linkage homology between birds and turtles: Bird and turtle chromosomes are precise counterparts of each other. Chromosome Res 13: 601-615.

Odierna G, Kupriyanova LA, Caprigilone T, Olmo E (1993) Further data on sex chromosomes of Lacertidae and a hypothesis on their evolutionary trend. Amphibia-Reptilia 14: 1–11.

Odierna G, Aprea G, Arribas O, Capriglione T, Caputo V, Olmo E (1996) The karyology of the Iberian rock lizards. Herpetologica 52: 542-550.

Odierna G, Heulin B, Guillaume CP, Vogrin N, Aprea G, Capriglione T, Surget˜Groba Y, Kupriyanova L (2001) Evolutionary and biogeographical implications of the karyological variations in the oviparous and viviparous forms of the lizard *Lacerta* (*Zootoca*) *vivipara*. Ecography 24: 332–340.

Olmo E, Odierna G, Cobror O (1986) C-band variability and phylogeny of Lacertidae. Genetica 71: 63–74.

Olmo E, Odierna G, Capriglione T (1987) Evolution of sex-chromosomes in lacertid lizards. Chromosoma 96: 33-38.

Olmo E, Signorino GG (2005) Chromorep: A reptile chromosomes database. Available at http: //chromorep.univpm.it. Accessed 20 May 2015.

Pinard R, de Winter A, Sarkis GJ, Gerstein MB, Tartaro KR, Plant RN, Egholm M, Rothberg JM, Leamon JH (2006) Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. BMC Genomics 7: 216.

Pokorná M, Kratochvíl L (2009) Phylogeny of sex-dermining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? Zool J Linn Soc 156: 168–183.

Pokorná M, Giovannotti M, Kratochvíl L, Kasai F, Trifonov VA, O'Brien PC, Caputo V, Olmo E, Ferguson-Smith MA, Rens W (2011a) Strong conservation of the bird Z chromosome in reptilian genomes is revealed by comparative painting despite 275 million years divergence. Chromosoma 120: 455–468.

Pokorná M, Kratochvíl L, Kejnovský E (2011b) Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). BMC Genet 12: 90.

Pokorná M, Giovannotti M, Kratochvíl L, Caputo V, Olmo E, Ferguson-Smith MA, Rens W (2012) Conservation of chromosomes syntenic with avian autosomes in squamate reptiles revealed by comparative chromosome painting. Chromosoma 121: 409–418.

Pokorná M, Rens W, Rovatsos M, Kratochvíl L (2014) A ZZ/ZW sex chromosome system in the thick-tailed gecko (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a member of the ancient gecko lineage. Cytogenet Genome Res 142: 190–196.

Pyron RA, Burbrink FT, Wiens JJ (2013) A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol Biol 13: 93.

RensW, Fu B, O'Brien PCM, Ferguson-Smith MA (2006) Cross-species chromosome painting. Nat Protoc 1: 783–790.

Rovatsos M, Altmanová M, Pokorná M, Kratochvíl L (2014a) Conserved sex chromosomes across adaptively radiated *Anolis* lizards. Evolution 68: 2079–2085.

Rovatsos M, Pokorná M, Altmanová M, Kratochvíl L (2014b) Cretaceous park of sex determination: sex chromosomes are conserved across iguanas. Biol Lett 10: 20131093.

Sarre SD, Ezaz T, Georges A (2011) Transitions between sex-determining systems in reptiles and amphibians. Annu Rev Genomics Hum Genet 12: 391-406.

Shedlock AM, Edwards SV (2009) Amniota. In: Hedges SB, Kumar S, eds. The timetree of life. New York: Oxford University Press, pp 375-379.

Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH (2009) The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. Nature 461: 267-271.

Srikulnath K, Nishida C, Matsubara K, Uno Y, Thongpan A, Suputtitada S, Apisitwanich S, Matsuda Y (2009) Karyotypic evolution in squamate reptiles: comparative gene mapping revealed highly conserved linkage homology between the butterfly lizard (*Leiolepis reevesii rubritaeniata*, Agamidae, Lacertilia) and the Japanese four-striped rat snake (*Elaphe quadrivirgata*, Colubridae, Serpentes). Chromosome Res 17: 975–986.

Srikulnath K, Uno Y, Nishida C, Matsuda Y (2013) Karyotype evolution in monitor lizards: cross-species chromosome mapping of cDNA reveals highly conserved synteny and gene order in the Toxicofera clade. Chromosome Res 21: 805-819.

Srikulnath K, Matsubara K, Uno Y, Nishida C, Olsson M, Matsuda Y (2014) Identification of the linkage group of the Z sex chromosomes of the sand lizard (Lacerta agilis, Lacertidae) and elucidation of karyotype evolution in lacertid lizards. Chromosoma 123: 563–575.

Telenius H, Pelmear AH, Tunnacliffe A, Carter NP, Behmel A, Ferguson-Smith MA, Nordenskjöld M, Pfragner R, Ponder BA (1992) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. Genes Chromosomes Cancer 4: 257-263.

Uetz P, Hošek J (eds) (2015) The reptile database. Available at http: //www.reptile-database.org. Accessed 1 June 2015.

Uno Y, Nishida C, Tarui H, Ishishita S, Takagi C, Nishimura O, Ishijima J, Ota H, Kosaka A, Matsubara K, Murakami Y, Kuratani S,Ueno N, Agata K,MatsudaY (2012) Inference of the protokaryotypes of amniotes and tetrapods and the evolutionary processes of microchromosomes from comparative gene mapping. PLoS One 7: e53027.

Vicoso B, Bachtrog D (2013) Reversal of an ancient sex chromosome to an autosome in *Drosophila*. Nature 499: 332-335.

Woram RA, Gharbi K, Sakamoto T, Hoyheim B, Holm LE, Naish K, McGowan C, Ferguson MM, Phillips RB Stein J (2003) Comparative genome analysis of the primary sex-determining locus in salmonid fishes. Genome Res 13: 272-280.

Wright JW (1973) Evolution of the X_1X_2Y sex chromosome mechanism in the scincid lizard *Scincella laterale* (Say). Chromosoma 43: 101-108.

Yang F, Carter NP, Shi L, Ferguson-Smith MA (1995) A comparative study of karyotypes of muntjacs by chromosome painting. Chromosoma 103: 642-652.

Young MJ, O'Meally D, Sarre SD, Georges A Ezaz T (2013) Molecular cytogenetic map of the central bearded dragon, *Pogona vitticeps* (Squamata: Agamidae). Chromosome Res 21: 361-374.

Table. S1. Accession numbers of reference sequences used for primer design, nucleotide sequences, expected PCR product sizes (in base pairs) and annealing temperatures (Ta; expressed in degrees Celsius) of primers used for PCR-assisted gene mapping in *I. monticola*. IUPAC nucleotide code for degenerate bases: Y=C+T; R=A+G; S=G+C; W=A+T; ; V=G+A+C; H=A+C+T; K=G+T; D=G+A+T; M=A+C.

Gene name	Reference gene IDs for primer design	Primer sequence (5'-3')		PCR product size (base pairs)	Ta (ºC)
		Forward	Reverse		
WT1	ENSACAG00000002480, ENSGALG00000012115, ENSPSIG00000013679	YTGARGCATTTTGSTCATTCC	ATGGGTGTGTATTCTRTATTGGGC	415	57
GMPPA	ENSACAG00000014108, ENSGALG00000002417, XM_006035385	WCCCAGTGACCCCAAYCC	GTGAGGVAGGACAATGGART	264	59
FBXW11	ENSACAG00000015716, ENSGALG00000002229, ENSPSIG00000011975	AGGTGCTGTGTAGATGCAAA	CTTTGAAAGCAAAGCYCTGT	349	54
WDR43	ENSACAG00000009264, ENSGALG00000008989, ENSPSIG00000014217, XM_007431858 XM_006271802.1	TTCACAGGHCATGCWACAKC	CTCYTCCTTGCKTTCTGAYA	810	58
ATP5A1	-[a]	TACCGYCAGATGTCYCTGCT	CCTGYCCATCDGTGATRGAG	217	57
DMRT1	-[b]	TTAATTTCATCCACCAGTGG	GGCAATAAAACTCAATCTTC	212	56
TKT	ENSACAG00000010885, ENSGALG00000005309, ENSPSIG00000003277	GTGGSATTGGAGAAGCWGT	TGMTCCARRCTAYTCTGCATT	198	58
SKIL	AB794070.1, ENSACAG00000016018, XM_007432652	CCACTRCTTGTTCCTTCCGA	ACCCTGRCACTTKCCCAAGC	426	58
SH3PXD2A	ENSACAG00000004932, ENSGALG00000008293, ENSPSIG00000005648	CARCTCAGAGGTGGACTTAAAGG	TTGTTGTGGATGAACTGGGA	849	59

Table. S1 (cont.) Accession numbers of reference sequences used for primer design, nucleotide sequences, expected PCR product sizes (in base pairs) and annealing temperatures (Ta; expressed in degrees Celsius) of primers used for PCR-assisted gene mapping in *I. monticola*. IUPAC nucleotide code for degenerate bases: Y=C +T; R=A+G; S=G+C; W=A+T; ; V=G+A+C; H=A+C+T; K=G+T; D=G+A+T; M=A+C.

ADAM12	AB794067.1 ENSACAG00000000456, ENSPSIG00000005992	AACCCAGAGATGCTAAGTGTGG	TGGGTTCCACGGCATAGA	136	55
IPO5	AB793729, ENSACAG00000016245	YAGACATGCTGGCTTGATGG	AAGCCTGGTGCAAAGTCTGT	180	58
OCA2	ENSACAG00000011098 , ENSGALG00000016740, ENSPSIG00000010807	ATGGCTSTTCATAATMCAGAT	ACCTCATCTTKGATAAATTGA A	180	50
SS18	ENSACAG00000001853, ENSGALG00000015119, ENSPSIG00000002550	CCCACACAGAATATGCCKA	TGTCAGCTACATCAAAGCCC	355	58
ZNF326	ENSACAG00000001151, ENSGALG00000006113, ENSPSIG00000002449	TTCGGAGGTAGTTATGGTGGTC	CTCTGCCTGTTGATGGTCCT	285	59
SOX5	ENSACAG00000017320, ENSGALG00000013204, ENSPSIG00000002789	CTGTTCTGTTGCTACCTGCTTG	TACCATTGTGTTKTGCTGAGARG	422	59
ACSL1	ENSACAG00000002400, ENSGALG00000010628, ENSPSIG00000015828	ACTATGATGATGTGAGAACAGT	TCCAYTCATATGGYTGRTTTG	592	55
WAC	ENSACAG00000007583, ENSGALG00000007379, ENSPSIG00000004660	TGAGTAGACTTTGGACCGTTGA	GTGCCATAGCAACAGTGCCT	603	57
TOP2A	AB793736, ENSACAG00000001904 ENSGALG00000003922	AGTCACAGGTGGCCGAAATG	AGCTCTCCGAGACAGCAGAG	1396	59
MYST2	ENSACAG00000005025 ENSPSIG00000003897	AGARGAGCCTGYHTATTCTAC	GGACAYTTCATGTTGAARTTGT	706	54

Table. S1 (cont.) Accession numbers of reference sequences used for primer design, nucleotide sequences, expected PCR product sizes (in base pairs) and annealing temperatures (Ta; expressed in degrees Celsius) of primers used for PCR-assisted gene mapping in *I. monticola*. IUPAC nucleotide code for degenerate bases: Y=C+T; R=A +G; S=G+C; W=A+T; ; V=G+A+C; H=A+C+T; K=G+T; D=G+A+T; M=A+C.

OAF	ENSACAG00000003470 ENSGALG00000006715 ENSPSIG00000014368	TGTTTGAGGTCTTGCCCCAG	ATGCCGCACTTGTAGGAGGA	181	59
SBNO1	ENSACAG00000015441 ENSGALG00000003392 ENSPSIG00000009572	TCTACCTCWGGACATGTMGAA	AGTYGCTTCTTCCCAAGACA	195	56
ATP2A2	ENSACAG00000016106 ENSGALG00000003835 ENSPSIG00000012143	TGGATTCGRGGYGCTATCTA	CTGACATCTGGTTGGTKGTG	226	57
OSGIN1	ENSFM00250000002711 ENSFM00250000002711 ENSFM00250000002711	AGGGGCYTCAGAAAYAACAG	TCTGGTGGACTTTGTGRTAC	620	56
CDH8	ENSACAG00000014921 ENSGALG00000005319 ENSPSIG00000002557	AGTYATTGTGGTGCTSTTTGT	CCACTGTCAAGTTTCTTTGTCA	502	55
C19orf47	ENSACAG00000003004 ENSPSIG00000006644	AGCCCACAGGRGTSTTCAGC	ACCCCMGSTTTGGMRCTCTC	160	61
COL5A1	ENSACAG00000007221 ENSGALG00000002546 XM_007061007	AATGGGYCATCCTGGMCTAC	ACTGGCTGGCATCGATGTTT	202	58
PIK3CD	ENSACAG00000013317 ENSGALG00000002583 ENSPSIG00000004597	GAGAAGTTTGAAMGGTAAG	GCAAACARRTGYAGGAAGA	1143	52
		TGRACGACTTCCGTGYCAAA	GSACKGGGAAGCTGTAYTCCA	102	57

Table. S1 (cont.) Accession numbers of reference sequences used for primer design, nucleotide sequences, expected PCR product sizes (in base pairs) and annealing temperatures (Ta; expressed in degrees Celsius) of primers used for PCR-assisted gene mapping in *I. monticola*. IUPAC nucleotide code for degenerate bases: Y=C+T; R=A+G; S=G+C; W=A+T; ; V=G+A+C; H=A+C+T; K=G+T; D=G+A+T; M=A+C.

PIK3CD	ENSACAG00000013317 ENSGALG00000002583 ENSPSIG00000004597	GAGAAGTTTGAAMGGTAAG	GCAAACARRTGYAGGAAGA	1143	52
		TGRACGACTTCCGTGYCAAA	GSACKGGGAAGCTGTAYTCCA	102	57
RNF19B	AB793732 XM_007441109 ENSACAG00000003508 ENSPSIG00000006015	TAGAGACCAAGCCSAGCCAT	TGGTTGCAYATCTGCTCCTG	130	58
TRIM37	AB490382 AB792699 XM_007441825	KACKCCTGTCCCTCCAGACT	AACCTTYARCCKCCAGCATA	154	60
EEF2	AB490384, AB792689, ENSACAG00000012275, ENSGALG00000001830	AGGAAGATCTCTACCTGAAGCC	GCTCMACATARCGRCCCATCA	369	58

-a Primers designed by Brunner et al. (2001)
-b Primers designed by Pokorná et al. (2011)

Table. S2b. Nucleotide sequences, expected PCR product sizes, and annealing temperatures of the internal primers designed from sequences of *I. monticola*. IUPAC nucleotide code for degenerate bases: Y=C+T; R=A+G; S=G+C; W=A+T; ; V=G+A+C; H=A+C+T; K=G+T; D=G+A+T; M=A+C.

Gene name	Reference gene IDs for primer design	Internal primer sequence (5'-3')		PCR product size (base pairs)	Ta (°C)
		Forward	Reverse		
ATP5A1	-[c]	CGTCAGATGTCTCTGCTGCT	TTGGTTGGAATGTAGGCTGA	188	54
TKT	-[c]	GTGGCATTGGAGAAGCAGTC	GGATGATGCCATCCTTATCG	144	58
SH3PXD2A	-[c]	TTTGAGATGCGGAGACACAG	TGCTCTCTTGCTCTGGTTGA	199	59
OCA2	-[c]	CAGATTTCACCACAGATGATGAC	TTGAATTTGACATGGAAGTTGTC	151	55
SS18	-[c]	CACACAGAATATGCCGATGG	TACTCAGGGGGCATTCAAAG	268	57
ZNF326	-[c]	GCTGGGAAGCACCTTACTCA	TGATGGTCCTCCAAAAGCAT	200	55
SOX5	-[c]	ATGGAGAGGTAGCCATGGTG	GCTGAGAGGTGGGAGTTCTG	180	59
WAC	-[c]	ACTTTGGACCGTTGAGTTGG	TTTAGCCACCTCAGCATTGA	227	55
CDH8	-[c]	CAATTTATGCCGAGGCAGGG	AGCTACTGAGCCTCTCCCTT	156	59

-[c] Primers designed from sequences of *I. monticola* obtained in this work.

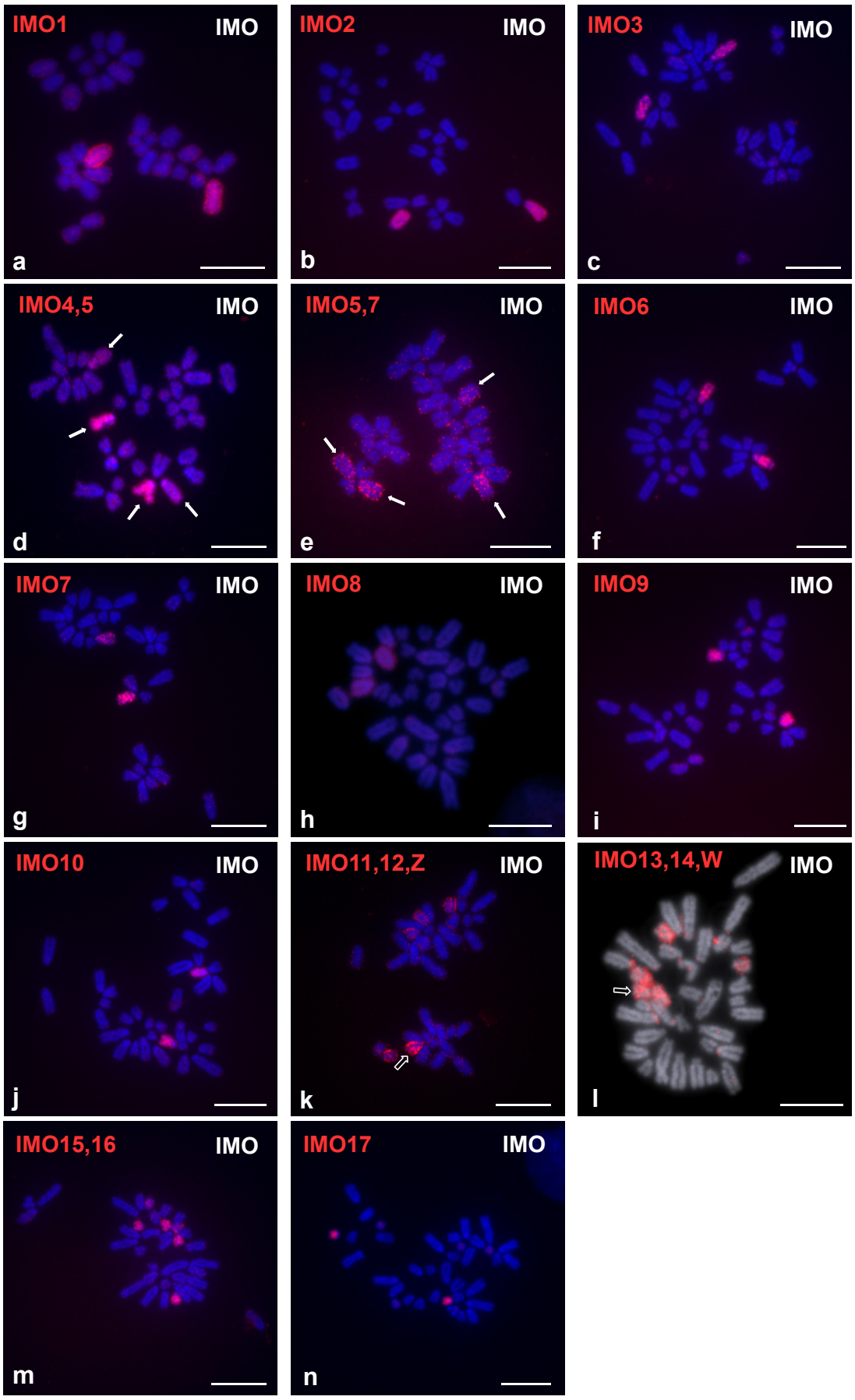


Fig. S1. Chromosomal assignment of flow karyotype peaks on female *I. monticola*. Arrows in **k** and **l** point to Z and W sex chromosomes, respectively. Scale bars=10 µm.

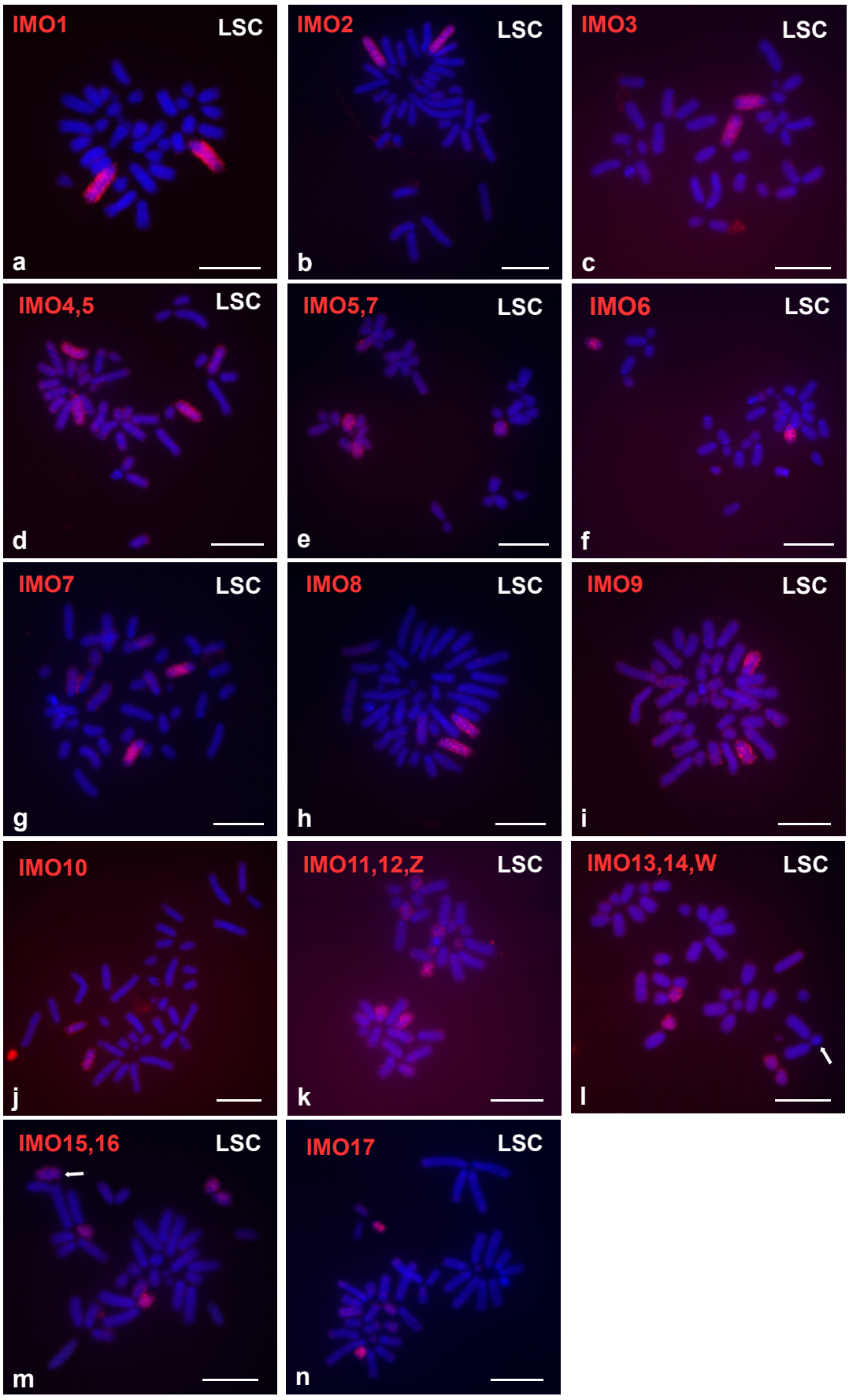


Fig. S2. Chromosome painting with the whole set of *I. monticola* probes on female *L. schreiberi*. Arrows in **l** and **m** point to W and Z sex chromosomes, respectively. Scale bars=10 μm.

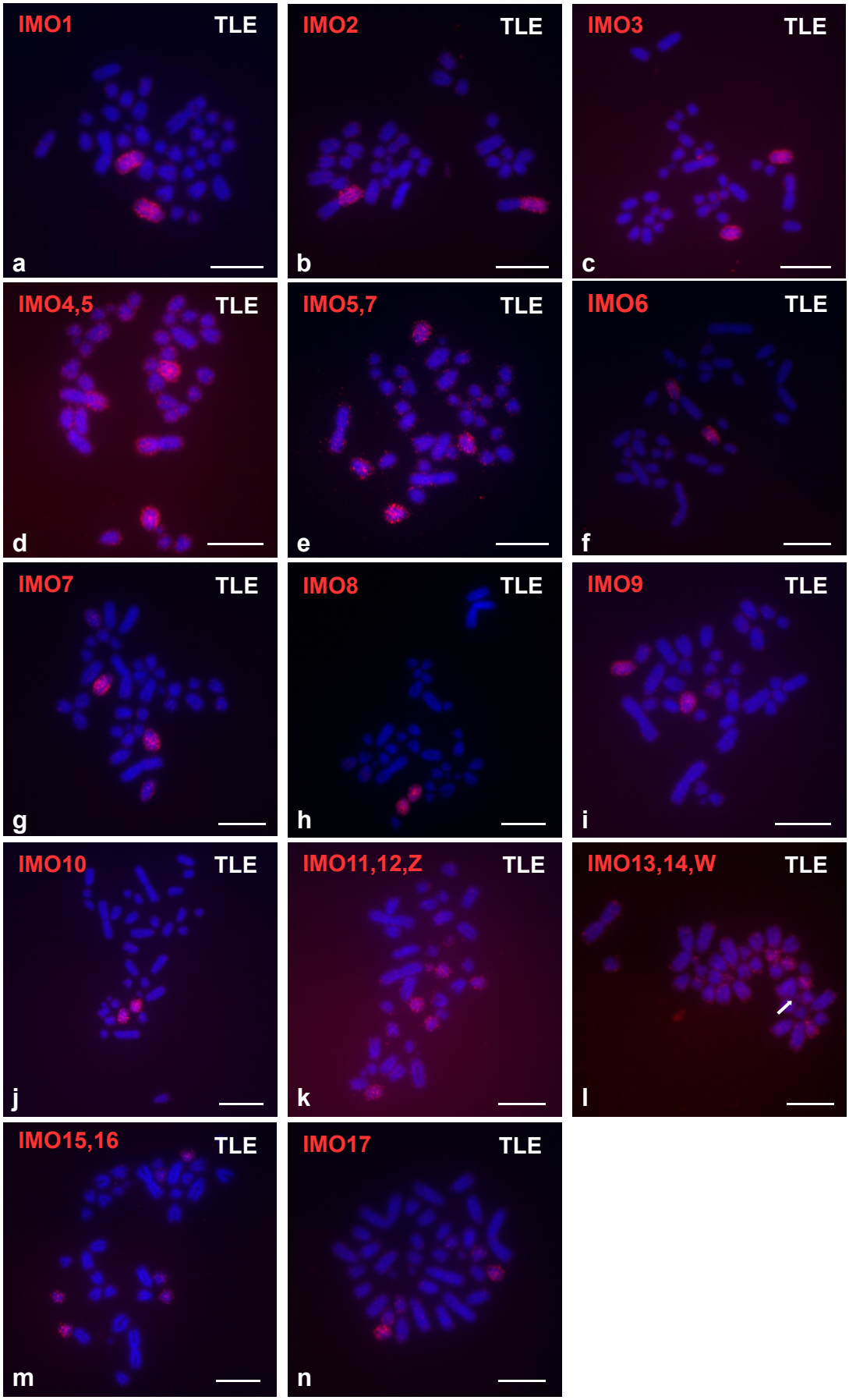


Fig. S3. Chromosome painting with the whole set of *I. monticola* probes on female *T. lepidus*. Arrow in l points to W and Z sex chromosomes, respectively. Scale bars=10 μm.

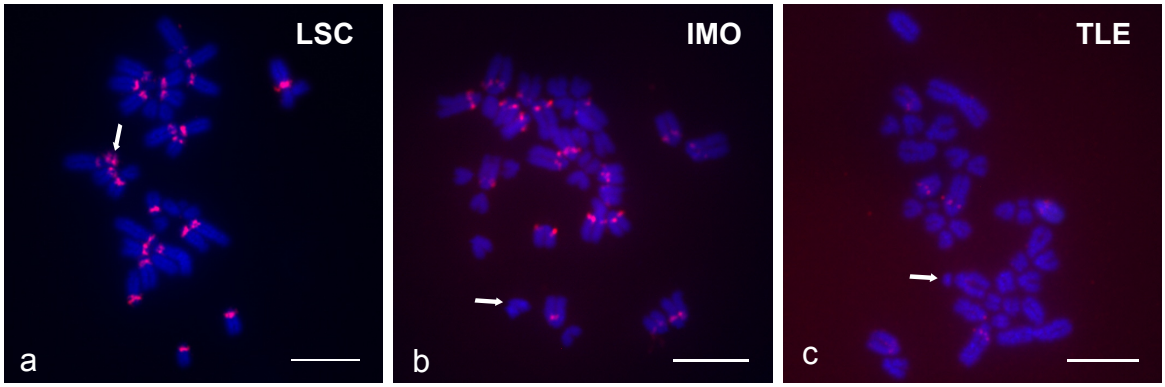


Fig. S4. Hybridization pattern of the TaqI satellite DNA probe on female metaphase spreads of **a** *L. schreiberi*, **b** *I. monticola*, and **c** *T. lepidus*. Arrows point to W chromosomes. Scale bars=10 μm.

CHAPTER III

Evolutionary dynamics of two satellite DNA families in Rock lizards of the genus *Iberolacerta* (Squamata, Lacertidae): different histories but common traits

Abstract

Satellite DNAs compose a large portion of all high eukaryotic genomes. The turnover of these highly repetitive sequences is an important element in genome organization and evolution. However, information about the structure and dynamics of reptilian satellite DNA is still scarce. Two satellite DNA families, HindIII and TaqI, have been previously characterized in four species of the genus *Iberolacerta*. These families showed different chromosomal locations, abundances and evolutionary rates. Here, we extend the study of both satDNAs to the remaining *Iberolacerta* species, with the aim to investigate the patterns of variability and factors influencing the evolution of these repetitive sequences. Our results revealed disparate patterns, but also common traits in the evolutionary histories of these satellite families: (i) each satellite DNA is made up of a library of monomer variants or subfamilies shared by related species; (ii) species-specific profiles of satellite repeats are shaped by expansions and/or contractions of different variants from the library; (iii) different turnover rates, even among closely related species, result in great differences in overall sequence homogeneity, and in concerted or non-concerted evolution patterns, which may not reflect the phylogenetic relationships among taxa. Contrasting turnover rates are possibly related to genomic constraints such as karyotype architecture and the interspersed organization of diverging repeat variants in satellite arrays. Moreover, rapid changes in copy number, especially in the centromeric HindIII satDNA, may have been associated with chromosomal rearrangements, and even contributed to speciation within *Iberolacerta*.

Keywords: Concerted evolution · FISH · *Iberolacerta* · Library model · Satellite DNA · Squamate reptiles.

Introduction

Satellite DNAs (satDNAs) represent one of the major classes of repetitive sequences in almost all eukaryotic genomes. They consist of tandemly repeated non-coding DNA sequences, typically arranged in large clusters of hundreds or thousands of copies usually located in the heterochromatic regions of chromosomes, close to the centromeres and telomeres (Charlesworth et al. 1994). Several satDNA families of independent origin are commonly found in the genome of a species or group of species, and they usually differ in nucleotide sequence, monomer length, and complexity, as well as in evolutionary history (Ugarković and Plohl 2002; Kuhn et al. 2008, 2010). The biological function of these sequences is not yet fully understood, although numerous reports point out the role of certain satellites in centromeric condensation, chromosome organization, or chromosome pairing (see Plohl et al. 2008). A growing field of research is also addressing the role of satDNA transcripts in the formation and maintenance of heterochromatin, and even in regulation of gene expression (Ugarković 2009; Pezer et al. 2012). In addition, several examples support the hypothesis that the rapid evolution of satDNAs can act as a driver of population and species divergence (Ugarković and Plohl 2002; Feliciello et al. 2015).

Despite their biological significance, satDNAs are still the least understood genomic component, underrepresented in outputs of most genome projects (Plohl et al. 2012). A common feature of many of them is that, even though monomers can be present in many thousand copies per genome, sequence divergence between repeats of the same family is often very low, usually less than 15% (Plohl et al. 2008). The non-independent or concerted evolution of repeat units is postulated to be a consequence of a two-step process called molecular drive, consisting of the gradual spread of a sequence variant (1) through a genome (homogenization) and (2) through a species (fixation) (Dover 1982). Sequence homogenization is due to diverse molecular mechanisms of nonreciprocal transfer, such as unequal crossing-over, gene conversion, rolling circle replication and reinsertion, and transposon-mediated exchange (Stephan 1986; Dover 2002), while fixation results from random chromosomal assortment in sexual reproduction, depending thus on population factors. This process results in rapid divergence of satellite sequences in reproductively isolated groups of organisms, and in this case satDNAs can be used as phylogenetically informative markers (Plohl et al. 2012).

Accumulation of mutations in satellite families is not the only way to alter specific profiles of satellite repeats in short evolutionary periods. In addition to sequence changes, satDNAs are permanently altered in copy number by expanding and contracting arrays of satellite

monomers (Ugarković and Plohl 2002; Plohl et al. 2012). Because usually more than one satellite family exists in a genome, fluctuations in their copy numbers can change very efficiently and rapidly any profile of genomic satDNA. The library model of satDNA evolution explains the occurrence of species-specific satellite profiles as a result of differential amplifications and/or contractions within a collection, or library, of satellite sequences shared by related species (Fry and Salser 1977; Meštrovic et al. 1998; Ugarković and Plohl 2002). Not only distinct satDNAs, but also monomer variants or subfamilies from a single family can be distributed in genomes in the form of a library (Cesari et al. 2003).

SatDNAs have been extensively studied in insects (Palomeque and Lorite 2008) and mammals (Enukashvily and Ponomartsev 2013), and less so in other taxa, although there are several exceptions. Squamata, by far the largest reptile order, is one of them (see, for example, Giovannotti et al. 2009, 2013; Chaiprasertsri et al. 2013). It includes the Lacertidae, a widespread species-rich group restricted to the Palearctic region, formed by two subfamilies, Gallotiinae and Lacertinae (Arnold et al. 2007; Sindaco and Jeremčenko 2008). So far, five satDNA families have been described in Lacertinae, with different taxonomic distributions. Three satellite families are genus-specific, namely pLHS in *Podarcis* (Capriglione et al. 1994; Capriglione 2000), CLsat in *Darevskia* (Ciobanu et al. 2003; Grechko et al. 2006), and Agi160 in *Lacerta* (Ciobanu et al. 2004; Grechko et al. 2005). The other two families, on the contrary, are broadly distributed in Lacertinae: pLCS, shared by *Algyroides*, *Teira*, *Lacerta*, and *Podarcis* (Capriglione et al. 1989, 1991; Capriglione 2000), and pGPS, present in *Podarcis*, *Archaeolacerta*, *Algyroides*, *Lacerta*, and *Zootoca* (Capriglione et al. 1998).

In a previous work (see AnnexII; Giovannotti et al. 2014), we isolated two new satDNA families in the lacertid genus *Iberolacerta*, a monophyletic group of rock lizards mainly distributed in highland areas of Western Europe. This genus comprises eight species, which can be subdivided into three main units: (1) *I. horvathi*, occurring in the Eastern Alps and the north of the Dinaric Chains; (2) the subgenus *Pyrenesaura*, which includes the three species found in the Pyrenees, (*I. aranica*, *I. aurelioi* and *I. bonnali*); and (3) the four species included in the 'Iberian group' (*I. cyreni*, *I. martinezricai*, *I. galani*, and *I. monticola*), with disjunct distributions in central and northern mountain ranges of the Iberian Peninsula. Previous cytogenetic surveys of the *Iberolacerta* species (Capula et al. 1989; Odierna et al. 1996; Arribas and Odierna 2005; Arribas et al. 2006; Rojo et al. 2014) showed them to possess a diploid number of 2n = 36, and a similar karyotypic macrostructure, with all chromosomes acrocentric. Only the karyotypes of the three Pyrenean species differ from this formula, with reduced diploid numbers that range from 2n = 24 to 26 in males, and from 2n = 23 to 26 in

females, and many biarmed chromosomes that probably evolved from the ancestral acrocentric complement through a series of Robertsonian fusions (Odierna et al. 1996).

According to the most recently published phylogeny (Arribas et al. 2014), speciation within *Iberolacerta* started *ca.* 13.5 million years ago (mya; 95% credibility interval 11.6–15.6), with the split between the clades formed by *I. horvathi* and the Iberian group, on one side, and by the Pyrenean species, on the other. This event was most likely to be quickly followed by the separation of *I. horvathi*, which took place approximately 11.5 mya (9.6–13.7). Within the Iberian group, *I. cyreni* split earlier (7.3–8.5 mya), while the speciation events within the clade formed by *I. martinezricai*, *I. galani*, and *I. monticola* occurred considerably later, at the beginning of the Pleistocene, 2.1–2.9 mya. The three Pyrenean species probably originated in rapid succession *ca.* 3.8 mya (2.7–4.9), although this phylogenetic analysis suggests that *I. bonnali* split first, shortly before the separation between *I. aranica* and *I. aurelioi*, 3.3 mya (2.3–4.3). Notwithstanding minor uncertainties still remaining, the mapping of satDNA differences on that species tree is likely to provide valuable information about the time and mode of evolution of these repetitive sequences. In our previous work (Giovannotti et al. 2014), we analyzed two unrelated satDNA arrays in the Iberian clade of *Iberolacerta*: (1) the centromeric HindIII family, which comprises two subfamilies (I and II) and represents 5%–10% of the genome, and (2) the TaqI family, which shows only interstitial loci and represents 2.5%–5% of the genome. The nucleotide sequences of the two families were presumably evolving at different rates, almost tenfold higher for centromeric than for instertitial repeats, after comparing *I. cyreni vs*. the other, relatively closer, species of the Iberian clade. In agreement with this conclusion, the HindIII family seems to be specific to the genus *Iberolacerta* (Capriglione et al. 1989, 1991, 1998; Capriglione 2000), whereas the TaqI satDNA has also been detected in representatives of three other genera of the subfamily Lacertinae (*Lacerta*, *Podarcis* and *Timon*).

Here, we extend the study of both satDNAs to the remaining *Iberolacerta* species, and increase our dataset for HindIII satDNA, to further investigate the occurrence of two divergent subfamilies in the genomes of all these taxa. The results obtained offer a more complete portrait of the intra- and interspecific variability of these highly repetitive sequences, their genomic organization and chromosomal distribution, with the ultimate objective of contributing to assess the relative strength of the processes that determine their structure and mode of evolution.

Material and Methods

Animals

Genomic DNA was isolated from a total of 20 specimens, representing all eight *Iberolacerta* species. The number of specimens per species and their geographical origin are given in Supplementary Table S1. In addition, one male and one female of *I. horvathi*, and one female of *I. bonnali* were used to make metaphase chromosomes. Permissions for field work and experimental procedures were issued by the competent authorities: Xunta de Galicia (for *I. monticola* and *I. galani*), Junta de Castilla y León (for *I. cyreni* and *I. martinezricai*), Gobierno de Aragón (for *I. bonnali*) and Italian Environment Ministry (for *I. horvathi*). All institutional and national guidelines for the care and use of laboratory animals were followed.

DNA extraction, PCR, cloning and sequencing

Genomic DNA was extracted from ethanol preserved tissues using standard protocols with proteinase K digestion followed by phenol/chloroform extraction (see Sambrook et al. 1989). Two primer pairs designed in our previous work (HindIII-F: 5'-TGAGTGTTTTACAGTTGAAAAGCT-3'; HindIII-R: 5'-CATTGTGTTATTTGAGCGCAA-3'; TaqI-F: 5'-ATTCTGACCCTGGGGGTTAG-3'; TaqI-R: 5'-CATATTTAAAGAAATCAG GCCTCG-3') were used for isolation of both satellite families from the genomes of *I. horvathi*, *I. bonnali*, *I. aranica* and *I. aurelioi*. An additional primer pair was designed to specifically amplify HindIII-subfamily II in all eight *Iberolacerta* species. (Hind_sfII-F: 5'-CTCTTGCTTATTTCGCTCCAAATGA-3'; Hind_sfII-R: 5'-ATTTCTGTGTGCAGCATGCA TTGG-3'). PCR reactions were performed in a final volume of 25 µl containing ~25 ng of genomic DNA, 0.625 U of *Taq* DNA polymerase and 1x PCR buffer (Roche Diagnostics), 5 nmol of each dNTP (Roche Diagnostics), and 20 pmol of each primer. The general reaction conditions were as follows: initial denaturation at 94ºC for 5 min; 35 cycles of denaturation at 94ºC for 30 s, annealing at the following temperatures (HindIII-F/HindIII-R, 55 ºC; TaqI-F/TaqI-R, 47 ºC; Hind_sfII-F/Hind_sfII-R, 58 ºC) for 30 s, extension at 72ºC for 30-60 s; and a final extension at 72ºC for 7 min. The obtained PCR products were run on 1.5% agarose gels; DNA in bands of interest was eluted using Pure Link Quick Gel Extraction Kit (Invitrogen) and cloned in the T&A cloning vector with T&A cloning kit (Yeastern Biotech) following manufacturer's recommendations. Positive clones were selected through PCR amplification using the M13 forward and M13 reverse primers. Bidirectional sequencing with the M13 primers was performed on an ABI PRISM 3730XL (Applied Biosystems) automatic sequencer.

Sequence analysis

The newly sequenced repeats were analyzed together with the previously reported sequences of the HindIII and TaqI satDNA families from *I. cyreni*, *I. monticola*, *I. galani* and *I. martinezricai* (DDBJ/EMBL/GenBank accession numbers for HindIII: from KF453637 to KF453681; accession numbers for TaqI: from KF453682 to KF453723) (Giovannotti et al. 2014). Multiple sequence alignment was performed with MUSCLE (Edgar 2004), using default parameters, as implemented in Geneious version 8.0.5 (Kearse et al. 2012). After visual inspection of alignments, sequences were classified into different sets according to shared nucleotide changes and indels.

Intraspecific nucleotide diversity (π) was estimated using DnaSP v. 5 (Librado and Rozas 2009). Net average genetic distances between groups were calculated using the Maximum Composite Likelihood model (Tamura et al. 2004) in MEGA v. 6.0 (Tamura et al. 2013). Sequence variability among satellite repeats was further investigated by performing a factorial correspondence analysis (FCA), carried out with Genetix v. 4.05.2 (Belkhir et al. 2004). For this analysis, we constructed a matrix with all the sequences, where the nucleotide present at each diagnostic position was coded with a unique integer (100, 120, 140 or 160).

For the subsequent phylogenetic analysis, a consensus sequence was obtained for each sequence set by choosing the most frequent nucleotide at each position, except when a combination of dinucleotides of the three pairs CpG, CpA, and TpG was present at the same doublet position. In that case, the CpG dinucleotide was chosen as the consensus unless the T or A nucleotides were present in >70% of the sequences. A phylogenetic network of the consensus sequences was constructed with TCS v. 1.21 (Clement et al. 2000) using the statistical parsimony algorithm under the 95% parsimony criterion (Templeton et al. 1992).

Chromosome analysis

Metaphase chromosome spreads were prepared as described previously (Giovannotti et al. 2014). As for *I. horvathi*, individuals of this species were induced to autotomize their tail tips, the tissues were collected in the field following the protocol by Waters et al. (2008), and transferred to the laboratory for the establishment of primary cell cultures. For fluorescence in situ hybridization (FISH) experiments we developed species-specific probes obtained by PCR amplification of HindIII and TaqI satDNA clones. The probes were labeled either with Cy3, using a PCR labeling kit (Jena Bioscience), or with FITC, using the Platinum Bright 495 labeling kit (KREATECH Biotechnology). Slide pretreatment, denaturation, hybridization, post-hybridization washes and detection were performed according to Schwarzacher and Heslop-Harrison (2000). Images were captured using the epifluorescence microscopes (Nikon

Microphot-FXA; Leica Leitz DMRBE) equipped with monochrome cameras (Nikon DS-Qi1Mc; JAI CV-M4+CL). The NIS-Elements D 3.10 (Nikon Instruments) and Leica CytoVision version 7.2 (Leica Microsystems) softwares were used to process the images and reconstruct the karyotypes.

Results

Isolation and characterization of satellite DNAs

PCR amplification using primers specific for HindIII and TaqI satDNA was successful in all tested species, and produced a ladder-like banding pattern, which is typical for satellite DNA. PCR products included complete monomers and multimers (from dimers up to hexamers), flanked by partial monomer sequences. Only clones with complete repeat units were sequenced and, for further analyses, multimers were separated into individual monomers. A total of 187 new sequences were obtained for HindIII, whereas 109 clones were sequenced for TaqI. Comparison of these new sequences with the HindIII and TaqI monomers isolated from *I. cyreni*, *I. monticola*, *I. galani* and *I. martinezricai* in our previous study (Giovannotti et al. 2014) indicated that all of them belong to the same satDNA families. Altogether, our dataset comprises 232 HindIII and 151 TaqI monomers from all eight *Iberolacerta* species, which are likely to reflect the overall variability of the two satellite families in the genus.

Both HindIII and TaqI satDNAs are characterized by an AT bias (average AT content of 58.9% and 59.1%, respectively) and by the occurrence of short repeat motifs such as A and T stretches, dinucleotide TG and CA, and trinucleotide CAA and TTC (Supplementary Figs. 1a and 1b). The size of HindIII repeats ranged between 169 and 172 bp, with the exception of two monomers with lengths of 151 bp (IAR_99b) and 161 bp (ICY_209c) (Table 1). TaqI repeats showed a broader range of length variation, from 155 to 191 bp (Table 1). Several indels varying in size from 1 bp to 31 bp are the cause of the repeat length variation in this satDNA family.

After alignment, monomers within each satDNA family were classified into subfamilies, according to the state of diagnostic positions, characterized by nucleotide substitutions or indels shared by at least 90% of all the members grouped in the same subfamily. The subfamilies were designated with Roman numerals following the nomenclature previously used in Giovannotti et al. (2014) for HindIII subfamilies I and II. Additional diagnostic positions further divided each subfamily into several sequence groups and subgroups, denoted by a latin letter and a numeral, respectively, after the subfamily name (Table 2).

Table 1 (next page). Summary of repeat features of HindIII and TaqI satDNAs. Number of monomeric repeats sequenced (n), length of repeats (expressed in base pairs), and nucleotide diversities (π) ± S.E. for both satDNAs for each *Iberolacerta* species investigated.

Table 2 (pages 137 and 138). Nucleotide differences among the consensus sequences of the different groups of **a** HindIII subfamilies HI, HII and HIII and **b** TaqI subfamilies TI and TII. The second row refers to base positions relative to the alignment shown in Supplementary Fig. 1a (HindIII) and 1b (TaqI). The general consensus sequence of each satDNA was used as reference. Dots indicate identity with this reference sequence.

			*Hind*III				*Taq*I	
Species	**Subfamily**	**n**	**Repeat length**	**Nucleotide diversity (π)**	**Subfamily**	**n**	**Repeat length**	**Nucleotide diversity (π)**
I. monticola		**34**		**0.0151 ± 0.0018**		**10**		**0.0600 ± 0.0089**
	HI	30	171	0.0142 ± 0.0023	TI	10	171 - 188	0.0600 ± 0.0089
	HII	4	170	0.0177 ± 0.0060				
I. galani		**31**		**0.0331 ± 0.0040**		**16**		**0.0489 ± 0.0001**
	HI	23	171	0.0148 ± 0.0019	TI	16	186 - 188	0.0489 ± 0.0001
	HII	8	169 - 170	0.0211 ± 0.0082				
I. martinezricai		**33**		**0.0151 ± 0.0018**		**7**		**0.0541 ± 0.0103**
	HI	33	171 - 172	0.0151 ± 0.0018	TI	7	187 - 188	0.0541 ± 0.0103
I. cyreni		**40**		**0.0356 ± 0.0037**		**9**		**0.0406 ± 0.0001**
	HI	7		0.0180 ± 0.0030	TI	9	186 - 187	0.0406 ± 0.0001
	HIII	33	161 - 171	0.0240 ± 0.0029				
I. horvathi		**12**		**0.0116 ± 0.0028**		**33**		**0.1218 ± 0.0079**
	HI	12	171	0.0116 ± 0.0028	TI	31	167 - 191	0.1184 ± 0.0083
					TII	2	189 - 191	0.0699 ± 0.0349
I. aurelioi		**25**		**0.0396 ± 0.0034**		**20**		**0.0976 ± 0.0086**
	HI	14	171	0.0290 ± 0.0048	TI	1	187	
	HII	11	170	0.0262 ± 0.0026	TII	19	177 - 188	0.0908 ± 0.0074
I. aranica		**22**		**0.0355 ± 0.0043**		**34**		**0.1209 ± 0.0070**
	HI	7	151 - 171	0.0265 ± 0.0055	TI	14	175 - 190	0.1082 ± 0.0126
	HII	15	170	0.0164 ± 0.0028	TII	20	177 - 190	0.0960 ± 0.0059
I. bonnali		**35**		**0.0491 ± 0.0050**		**22**		**0.1204 ± 0.0096**
	HI	17	171	0.0257 ± 0.0027	TI	17	155 - 188	0.1060 ± 0.0102
	HII	15	169 - 170	0.0230 ± 0.0076	TII	5	177 - 190	0.0983 ± 0.0156
	HIII	3	171	0.0195 ± 0.0033				
	HI	154		0.0241 ± 0.0015	TI	105		0.1342 ± 0.0060
	HII	53		0.0230 ± 0.0018	TII	46		0.0961 ± 0.0044
	HIII	25		0.0254 ± 0.0029				
	TOTAL	232		0.0539 ± 0.0020	TOTAL	151		0.1567 ± 0.0038

	1	2	3	4	6	7	8	10	11	12	13	14	15	17	18	19	20	21	22	23	24	26	27	28	29	30	31	32	33	35
Positions	14	15	21	27	38	39	56	73	83	84	85	86	87	95	98	99	101	113	114	117	119	129	140	144	145	147	149	150	151	165
Consensus	T	C	A	T	T	T	C	A	A	A	T	T	T	C	T	G	A	G	A	T	A	T	G	C	A	A	G	A	G	-
HI_a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_b	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_c	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_d1	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_d2	.	.	G	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_e	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_f	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	-
HI_g	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_h	.	.	.	C	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_i	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_j	.	G	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_k	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.	.	-
HI_L	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	-
HI_m	.	.	.	C	.	G	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
HII_a	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	A	-	-	T	T	G	.	A
HII_b	.	.	.	C	.	G	T	.	C	.	.	.	.	.	.	.	G	.	.	G	.	.	A	-	-	T	T	G	.	A
HII_c	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	A	-	-	T	T	G	C	A
HII_d	.	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	A	-	-	T	T	G	C	A
HII_e	A	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	A	-	-	T	T	G	.	A
HII_f	.	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	A	-	-	T	T	G	.	A
HII_g	.	.	G	.	C	.	T	.	C	.	.	.	.	.	.	.	G	.	.	G	.	.	A	-	-	T	T	G	.	A
HII_h	.	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	G	.	.	G	.	.	A	-	-	T	T	G	.	A
HIII_a	.	.	.	C	.	G	T	G	C	.	.	.	G	A	C	A	.	A	T	.	.	.	.	.	.	.	.	.	.	-
HIII_b	.	.	.	C	.	G	T	G	C	.	.	.	G	A	C	A	.	A	T	.	.	C	A	.	.	.	.	.	.	-
HIII_c	.	.	.	C	.	G	T	G	C	.	.	.	G	A	C	A	.	A	G	.	.	C	A	.	.	.	.	.	.	-
HIII_d	.	.	.	C	.	G	T	G	C	.	.	G	G	A	C	A	.	A	G	.	.	C	A	.	.	.	.	.	.	-
HIII_e	.	.	.	C	.	G	T	G	C	.	.	G	G	A	C	A	.	A	G	.	C	C	A	.	.	.	.	.	.	-

Table 2a

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Positions	1	5	6	9	14	16	17	18	19	20	21	22	31	33	43	54	56	58	59	60	61	66	71	72	83	93	101	110	114	117	120	121	123	125	126	127	139	142	150	157	163	167	168	169	170	175	181	184	187	188
Consensus	C	G	C	G	C	T	A	A	C	C	T	A	A	C	C	T	T	C	C	A	G	G	C	G	G	T	T	G	G	T	T	G	T	A	T	C	G	T	C	A	A	G	T	T	G	T	G	G	T
TI_A1	.	.	.	.	.	.	C	.	G	G	.	.	.	.	T	.	C	.	.	G	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	T	.	.	.	T	G	.	.	.
TI_A2	.	.	.	.	.	.	C	.	G	G	.	.	.	.	T	.	C	.	.	G	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	T	.	.	.	T	G	.	C	.
TI_B1	.	.	.	.	.	.	C	.	G	G	.	.	.	.	T	.	C	.	.	.	C	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	G	.	.	.
TI_B2	.	.	.	.	.	.	C	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	G	.	.	.
TI_C1	G	.	T	T	.	.	C	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
TI_C2	.	.	.	.	.	.	C	.	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
TI_D	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
TI_E	.	.	.	.	.	.	G	.	.	G	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	A	A	T	.	.	.	.
TI_F1	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TI_F2	.	.	.	.	.	.	G	.	.	.	C	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TI_G1	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TI_G2	.	.	.	C	.	.	G	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TI_H1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	A	.	C	A	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
TI_H2	.	.	.	.	.	.	T	.	A	A	.	.	G	.	T	.	.	.	.	.	.	C	.	C	.	.	.	.	A	.	C	A	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
TI_I1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	T	.	T	C	.	A	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
TI_I2	.	.	.	.	.	.	T	.	A	A	.	.	G	.	.	.	.	.	T	.	T	C	.	A	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
TI_J1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	A	.	C	A	.	.	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.
TI_J2	.	.	.	.	.	.	T	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	A	.	C	A	.	.	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.
TI_K	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	A	.	C	A	.	.	.	.	.	C	.	.	.	.	.	.	.	T	G	.	.	.
TI_L1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
TI_L2	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
TI_L3	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
TII_A	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G
TII_B1	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	A	.	.	G
TII_B2	.	.	.	.	T	C	.	T	.	.	.	.	G	.	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	A	.	.	G
TII_C1	.	.	.	.	T	T	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G
TII_C2	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G
TII_D	.	.	.	.	T	C	.	T	.	G	.	.	.	G	.	C	A	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G
TII_E1a	.	.	.	.	T	C	.	T	.	.	.	G	.	.	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	C	.	.	.	G	G	.	.	.	.	.	.	T	C	.	.	.	.	.	.	G
TII_E1b	.	.	.	.	T	C	.	T	.	.	.	G	.	.	.	C	.	T	.	.	.	.	T	.	.	.	C	.	.	C	.	.	.	G	G	.	.	.	.	.	.	T	C	.	.	.	.	.	.	G
TII_E2	T	.	.	.	T	C	.	T	.	.	.	G	.	.	.	C	.	T	.	.	.	.	T	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G
TII_F	.	.	.	.	T	C	.	T	.	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G
TII_G1	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
TII_G2a	T	.	T	.	T	C	.	T	.	.	.	.	.	A	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	G
TII_G2b	T	.	T	.	T	C	T	T	.	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	G
TII_G3	.	T	.	.	T	C	.	T	G	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	G
TII_G4	T	A	.	.	T	C	.	T	.	.	.	.	.	.	.	C	.	T	.	.	.	T	T	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	A	.	.	G

Table 2b

Fig. 1. Distribution and abundance of HindIII (a) and TaqI (b) subfamilies in Iberolacerta coupled to a Bayesian tree obtained from two mitochondrial loci (Cyt b, cytochrome b; CR, control region) (adapted from Arribas et al. 2014). Node bars indicate 95% credibility intervals (regions of highest posterior density) for the corresponding divergence time (in million years). Numbers in the table indicate the number of repeats of each subfamily retrieved from each species. Colours identify different subfamilies.

	HI_A	HI_B	HI_C	HI_D1	HI_D2	HI_E	HI_F	HI_G	HI_H	HI_I	HI_J	HI_K	HI_L	HI_M	HII_A	HII_B	HII_C	HII_D	HII_E	HII_F	HII_G	HII_H	HIII_A	HIII_B	HIII_C	HIII_D	HIII_E
I. monticola			13	1		5	2	1	2	3	1	2			3						1						
I. galani	1		4	1	3	7		3	1		1		2		1				1								
I. martinezricai	2	4	13			3		7	1	2	1																
I. cyreni				2		1	5					2		7					1	3		1					7
I. horvathi			6			4		1		1																	
I. aurellioi	2		2	2		1	5					2			4	1	2		1	3		1		1			6
I. aranica				1			3	2				1			3				1	7	3						3
I. bonnali			1	4	3	1	3		1	3		3	1		3	2	1	2		5	2		4	10	6	7	5	8
	5	4	39	9	3	21	13	14	5	9	3	8	3	7	14	3	3	3	3	17	6	4					

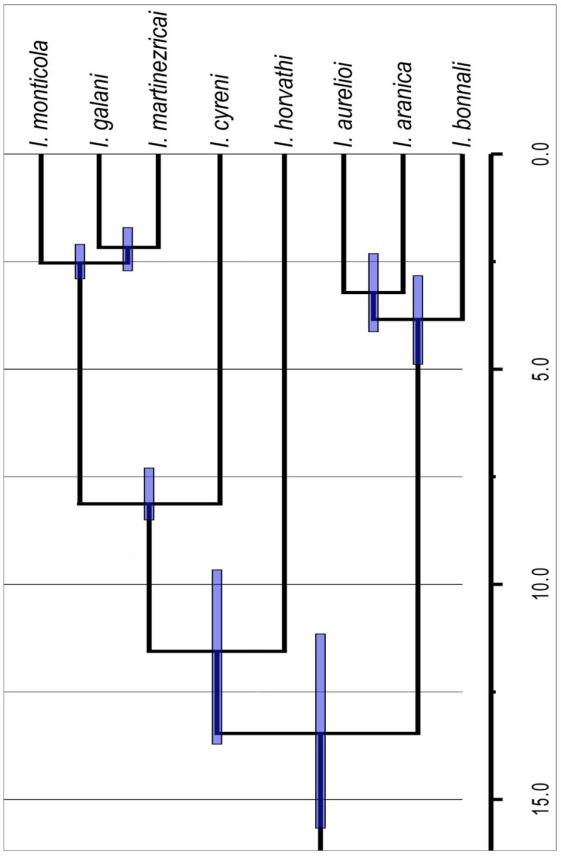
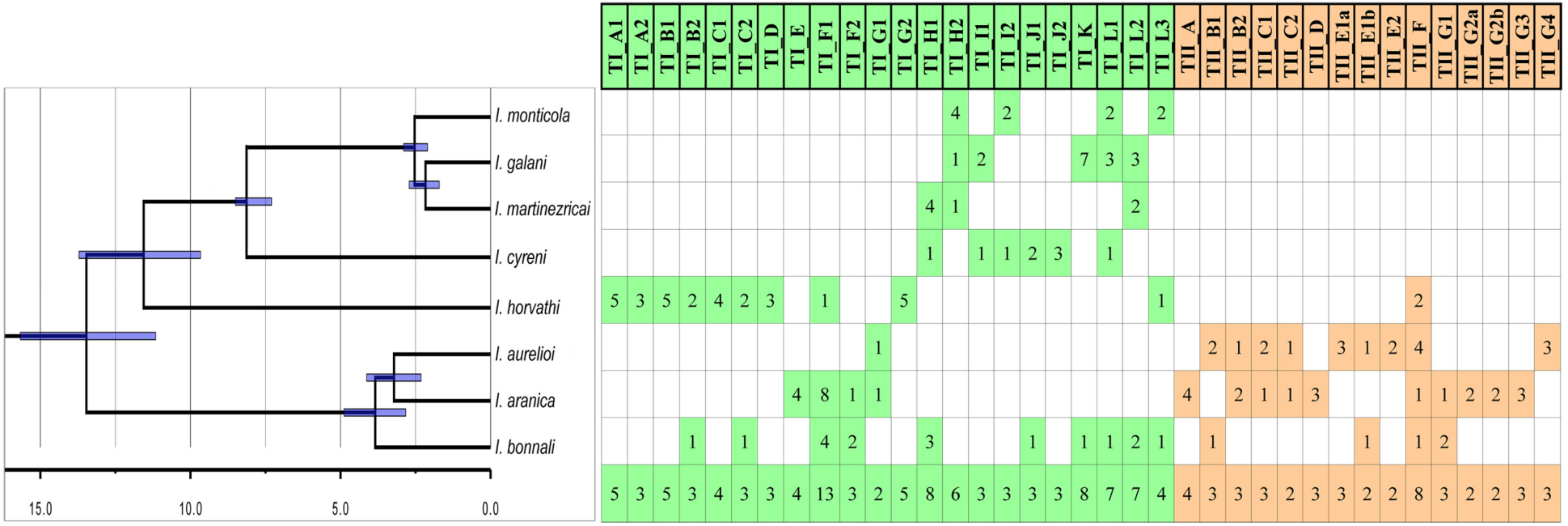


Fig. 1b


I. monticola
I. galani
I. martinezricai
I. cyreni
I. horvathi
I. aurelioi
I. aranica
I. bonnali
15.0
10.0
5.0
0.0
TI_A1 TI_A2 TI_B1 TI_B2 TI_C1 TI_C2 TI_D TI_E TI_F1 TI_F2 TI_G1 TI_G2 TI_H1 TI_H2 TI_I1 TI_I2 TI_J1 TI_J2 TI_K TI_L1 TI_L2 TI_L3 TII_A TII_B1 TII_B2 TII_C1 TII_C2 TII_D TII_E1a TII_E1b TII_E2 TII_F TII_G1 TII_G2a TII_G2b TII_G3 TII_G4
4 2 2
1 2 7 3 3
4 1 2
1 1 1 2 3 1
5 3 5 2 4 2 3 1 5 1 2
1 2 1 2 1 3 1 2 4 3
4 8 1 1 4 2 1 1 2 4 1 1 2 3
1 1 4 2 3 1 1 1 2 1 1 1 2
5 3 5 3 4 3 3 4 13 3 2 5 8 6 3 3 3 3 8 7 7 4 4 3 3 3 2 3 3 2 2 8 3 2 2 3 3

Sequence variability within HindIII satDNA

Within HindIII satDNA, we found a total of 30 diagnostic positions, which identified three subfamilies—namely HI, HII and HIII—and 27 sequence groups (Table 2a and Supplementary Fig. S1a). Their abundances ranged from 1.3% to 17% (3–39 representatives) of the examined sequences. Figure 1a overlies data on the abundance and distribution of HindIII sequence groups onto a phylogenetic tree for *Iberolacerta* derived from mitochondrial markers (Arribas et al. 2014). As evidenced in this figure, sequence groups were not equally represented in the different species. The Pyrenean species (*I. aurelioi*, *I. aranica* and *I. bonnali*) harbor a wide diversity of HindIII repeats, mainly belonging to subfamilies HI and HII. Only 12 monomers were retrieved from *I. horvathi*, and they are all members of subfamily HI. Similarly, subfamily HI is also the most abundant variant of the HindIII family in the Iberian species *I. martinezricai*, *I. monticola* and *I. galani*. A strikingly different profile of HindIII repeats was found in *I. cyreni*, also an Iberian species, which is characterized by the presence of several private sequence groups belonging to subfamily HIII, and one exclusive sequence group within subfamily HI.

The coexistence of more than one subfamily explains the higher nucleotide diversity values (π) in species such as *I. bonnali* (4.91%) or *I. aurelioi* (3.96%), in comparison with the values obtained for those species in which all their HindIII repeats belonged to a single subfamily, i.e., *I. horvathi* (1.16%) and *I. martinezricai* (1.51%) (Table 1). Interestingly, despite their different abundances, mean π values for each subfamily were roughly similar (from 2.30% in subfamily HII to 2.54% in subfamily HIII).

Sequence variability within TaqI satDNA

The factorial correspondence analysis (FCA) based on diagnostic positions highlighted the differentiation among the three HindIII subfamilies, lending further support to our classification. Altogether, the three main axes of variation explain 96.53% of the observed variation (Fig. 2a). The most informative is axis 1 (69.70%), which identifies two main clusters, corresponding to subfamily HIII repeats of *I. cyreni* and *I. bonnali* on one side, and to subfamilies HI and HII on the other. Axis 2, which accounts for 24.60% of the observed variation, separates subfamilies HI and HII. Finally, axis 3, with 2.23% of the observed variation, probably corresponds to sequence heterogeneity within each subfamily. The clustering of HindIII repeats revealed by the FCA matches the estimates of interspecies and inter-subfamilies net genetic distances, shown in Table 3a. Monomers of subfamily HIII are

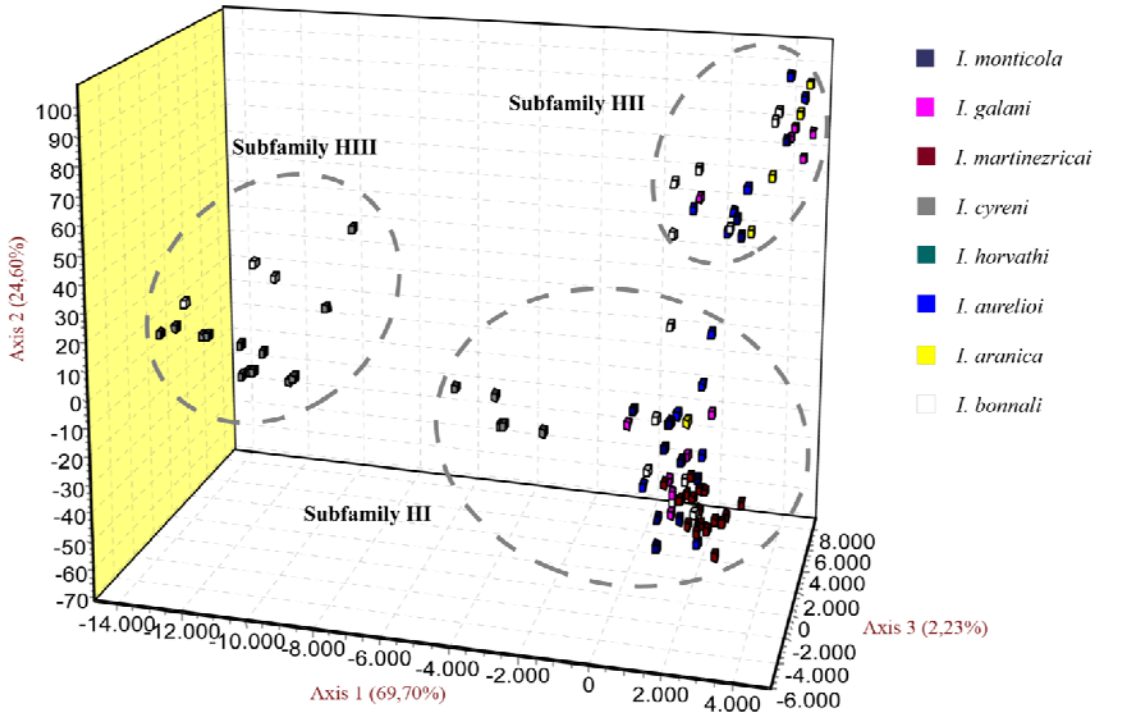
the most divergent, with average genetic distances of 7.50% and 9.90% from subfamily HI and HII, respectively. These values are substantially higher than the average distance between subfamilies HI and HII (around 4.0%). When *I. cyreni* is excluded from the analysis, pair-wise interspecies genetic distances within each subfamily are all very low and uncorrelated with relative divergence times between species, with average values of 1.0% within subfamily HIII, 0.34% within subfamily HII, and 0.33% within subfamily HI. Net genetic distances between HI repeats involving *I. cyreni* are always considerably higher (from 2.0% between *I. cyreni* and *I. aranica* to 3.40% between *I. cyreni* and *I. horvathi*).

From the alignment of TaqI sequences we identified a total of 49 diagnostic positions, which defined two main subfamilies—namely TI and TII—and 37 sequence groups, whose abundances ranged from 1.3% to 8.5% (2–13 representatives) of the examined sequences (Table 2b and Supplementary Fig. S1b).

In general, the species of the Iberian clade were characterized by the presence of TaqI repeats belonging only to subfamily TI (Fig. 1b), with a substantial proportion of private sequence groups (four groups, comprising 15 out of 42 sequences). Conversely, subfamily TII is essentially characteristic of the subgenus *Pyrenesaura*, although it has been residually observed also in *I. horvathi*. This subfamily appears to be the most abundant variant in the genomes of *I. aranica* and, above all, *I. aurelioi*, which show both species-specific and shared sequence groups. The sampled loci from *I. bonnali* and *I. horvathi* contain mostly T1 repeats. However, the clustering pattern of TI repeats differs markedly between the two species: while all the monomers retrieved from *I. bonnali* were grouped together with monomers from other species, *I. horvathi* shows the highest proportion of species-specific repeats (25 out of 33), allocated to six private sequence groups.

As expected from the distribution of subfamilies TI and TII in the genomes of the *Iberolacerta* species, intraspecific nucleotide diversity values are higher for *I. horvathi* and the Pyrenean species, which harbor both types of TaqI repeats in their genomes (Table 1). When each subfamily is analyzed separately, π values within subfamily TI are two to three-fold greater in these species than in the species of the Iberian clade (from 4.06% in *I. cyreni* to 11.84% in *I. horvathi*). High π values were also obtained for subfamily TII in those species with a large number of monomers examined (9.08% in *I. aurelioi*, and 9.60% in *I. aranica*).

a)



b)

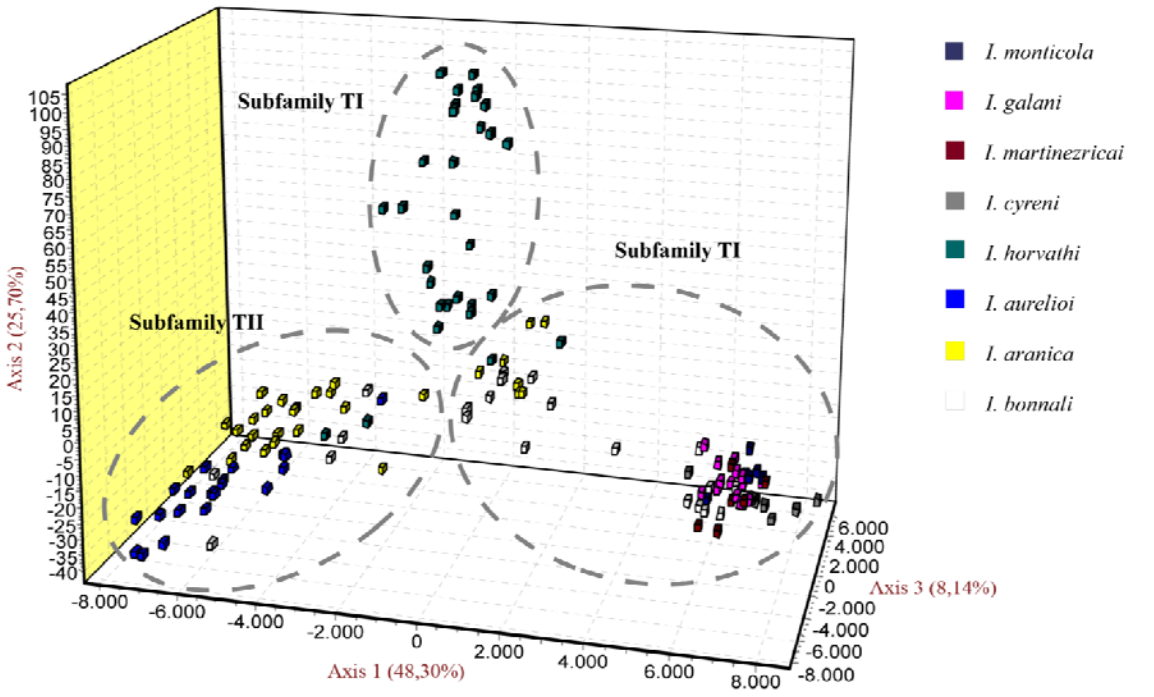


Fig. 2. Three-dimensional representation of a Factorial Correspondence Analysis based on monomeric sequences of HindIII (**a**) and TaqI (**b**) satDNAs.

The factorial analysis of TaqI monomers identified a main axis of variation (axis 1 at Fig. 2b, explaining 48.30% of the observed variation), corresponding to the separation between three groups of repeats: 1) subfamily TII (i.e., essentially Pyrenesaura); 2) a subset of subfamily TI, including all the monomers of Iberian species and a few monomers of *I. bonnali*; and 3) a subset of subfamily TI, made up of monomers from *I. horvathi*, *I. aranica* and *I. bonnali*. Axis 2 in the FCA, which accounts for 25.70% of the total variation, separates a fourth group of repeats, comprising the remaining TI monomers of *I. horvathi*. Net genetic distances between repeats from the different species (Table 3b) give additional support to the FCA results. Leaving aside the comparisons involving the single monomer of TI in *I. aurelioi*, larger distances between T1 repeats correspond to pairs of the Iberian species with both *I. aranica* (4.70%–5.10%) and, above all, *I. horvathi* (6.10%–7.0%). As for the TII repeats, all the pair-wise comparisons, involving the subgenus *Pyrenesaura* and *I. horvathi*, produce rather low values, (0.0%–1.30%).

Organization of consecutive monomeric units

The cloning and sequencing of multimeric products allowed us to characterize the organization of consecutive monomeric repeats. In both satDNA families, and in all the species analyzed, we observed that adjacent monomers in a satellite array usually belong to different sequence groups, and even to different subfamilies (for a list of all HindIII and TaqI composite arrays sampled in the *Iberolacerta* species, see Supplementary Tables S2 and S3, respectively).

Phylogenetic analysis

The statistical parsimony network obtained for HindIII satDNA showed a high degree of reticulation among the members of subfamily HI (Fig. 3a). This pattern suggests that rearrangements due to recombination events are an important force generating new monomers in subfamily HI. According to this phylogenetic reconstruction, HI—the most widespread subfamily among the *Iberolacerta* species—was found to occupy the central position of the parsimony network. Two sequence groups within this subfamily, HI_K and HI_M, branched into two separate lineages, corresponding to subfamilies HII and HIII, respectively. In contrast to subfamily HI, no evidence for recombination events has been found within subfamilies HII and HIII.

Fig. 3. Statistical parsimony network constructed from the consensus sequences of the different sequence groups of **a** HindIII satDNA and **b** TaqI satDNA.

a)

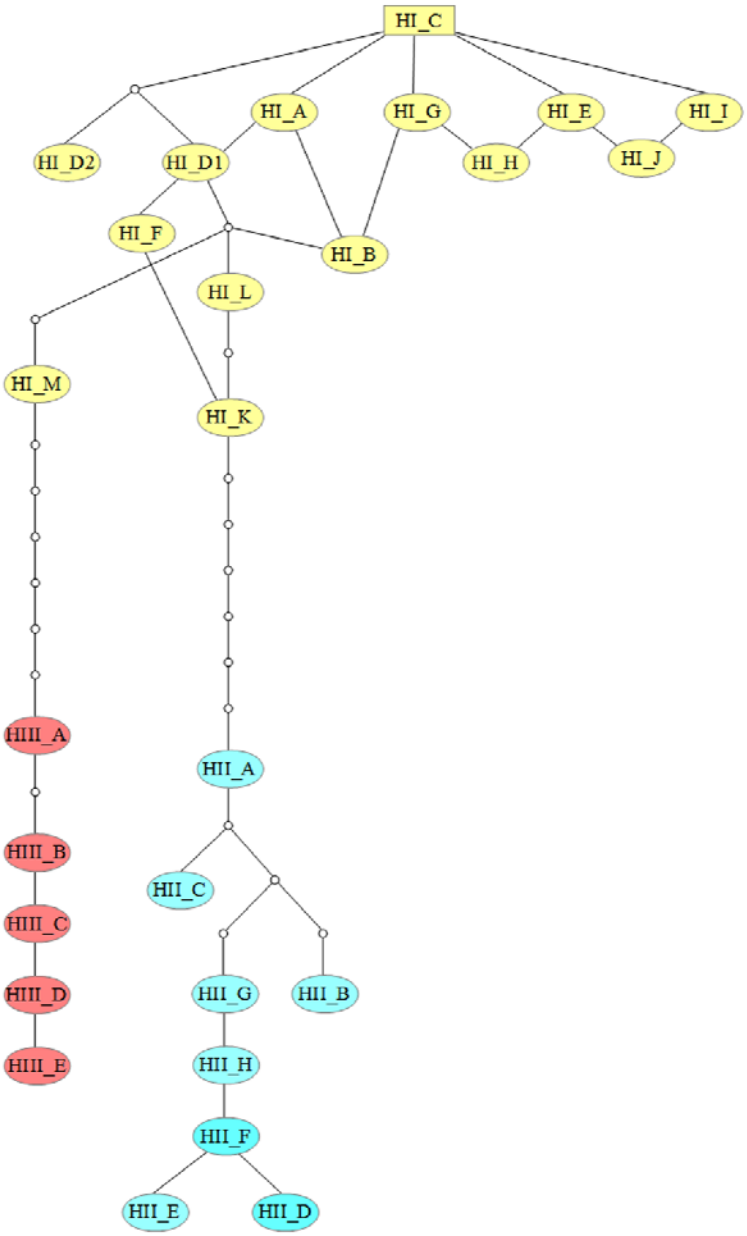
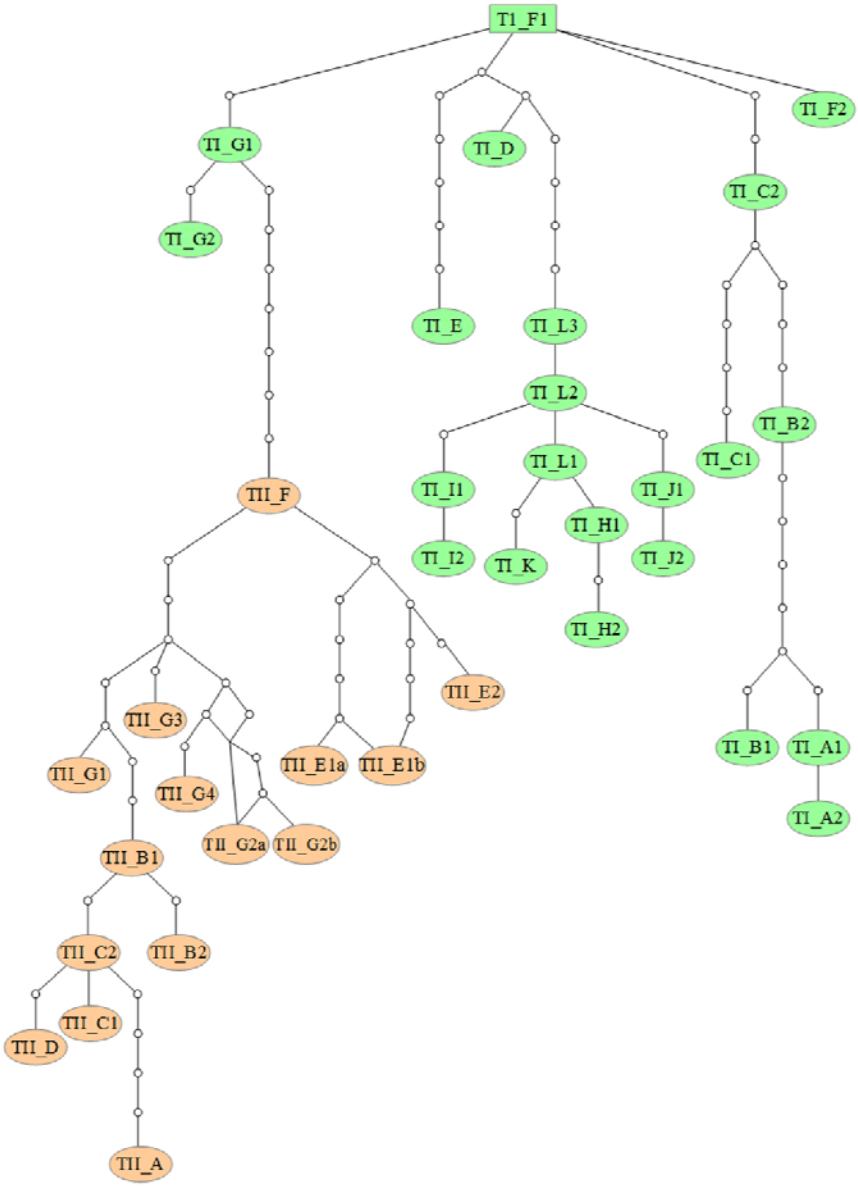


Fig. 3 (continued).

b)



In the network of TaqI satDNA, all sequence groups converge on a group belonging to subfamily T1 (T1_ FI, Fig. 3b). The network shows a major separation of four clusters, connected to group TI_F1 by a few mutational steps. Three of them (T1_F2, T1_C2 and T1_G1, together with their related variants) include sequences only found in *I. horvathi* and in the subgenus *Pyrenesaura*. All sequence groups belonging to subfamily TII occupy a peripheral position within cluster G1. The extensive diversification within subfamily TII has been promoted, in some cases, by recombination events that created new monomer variants (e.g., TII_E1b or TII_G2a). Within the fourth cluster, the prolific lineage TI_L3 includes all the sequence groups characteristic of the Iberian clade.

Chromosomal location of HindIII and TaqI satDNA families

FISH with HindIII satDNA probe on metaphase chromosomes of *I. monticola* and *I. galani* revealed that this repetitive element is present at centromeres of all the 36 chromosomes of the diploid complement (Fig. 4; Giovannotti et al. 2014). FISH on female metaphases of *I. bonnali,* carried out in this work, showed hybridization signals in the centromeric regions of all the 23 chromosomes of the karyotype, although with variable signal strength in different chromosome pairs (Fig. 4). Moreover, the overall intensity of HindIII signals in *I. bonnali* was noticeably lower than in *I. monticola* and *I. galani*. No hybridization signals were observed in the chromosomes of *I. horvathi*.

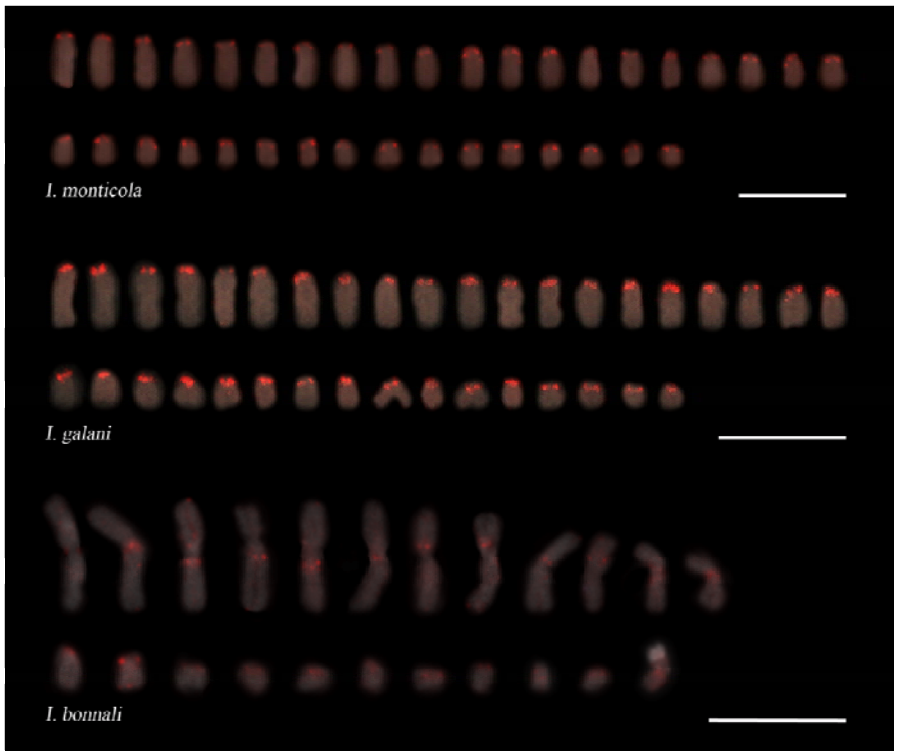


Fig. 4. Hybridization pattern of the HindIII probe in the karyotypes of *I. monticola*, *I. galani* and *I. bonnali*. Scale bar = 10 μm.

FISH with TaqI satDNA probe in *I. monticola* and *I. galani* produced bright signals in interstitial position in a subset of 20 and 18 chromosomes, respectively (Fig. 5). In *I. bonnali*, similarly intense signals were detected interstitially on both arms of 10 meta-/submetacentric chromosomes. In some metaphases, an additional faint signal could be observed in a medium-sized chromosome pair (Fig. 5). In *I. horvathi*, strong hybridization signals were also observed in interstitial position, but just in six chromosomes. However, after increased exposure times, 10 additional chromosomes appeared weakly labeled (Fig. 5).

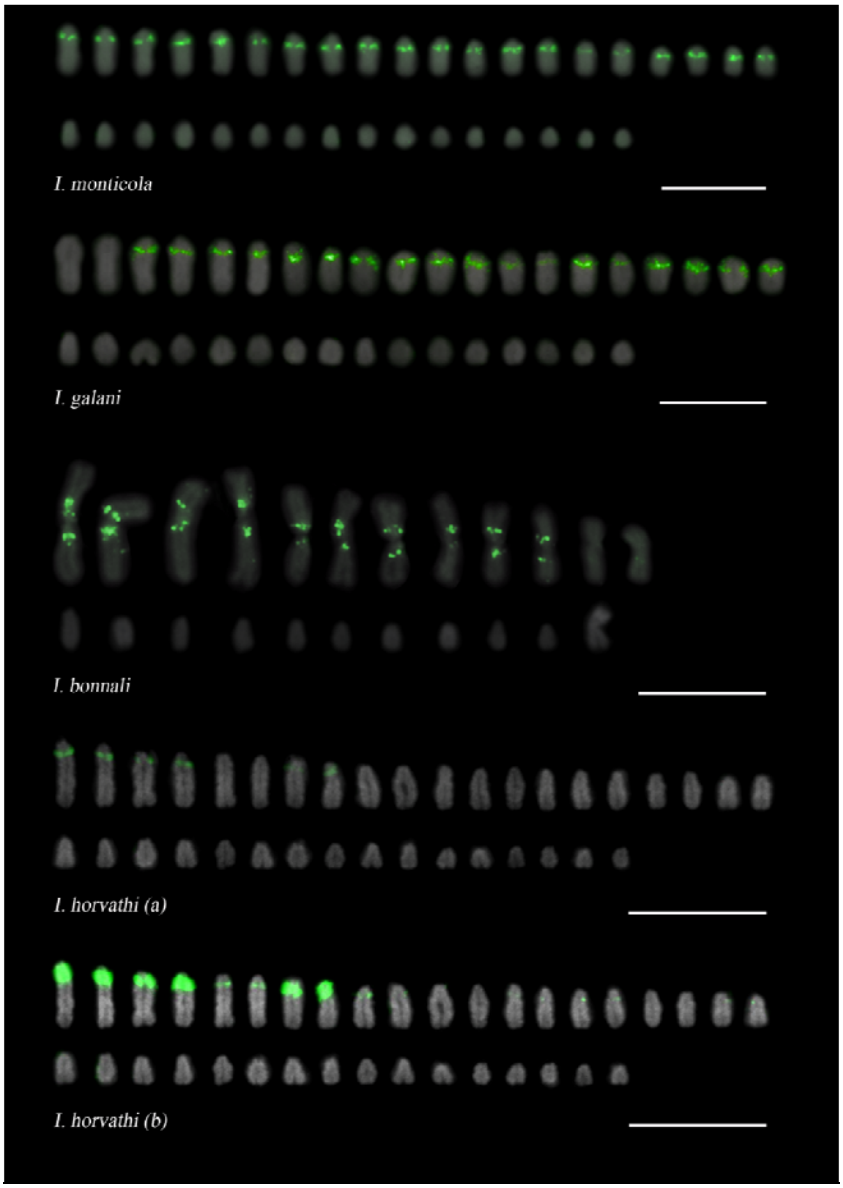


Fig. 5. Hybridization pattern of the TaqI probe in the karyotypes of *I. monticola*, *I. galani*, *I. bonnali* and *I. horvathi*. FISH signals on *I. horvathi* chromosomes are shown at standard (a) and increased (b) exposure times. Scale bar = 10 μm.

Discussion

The turnover rate of a satDNA family is a complex feature that depends on many parameters, such interchromosomal and intrachromosomal recombination rates, copy number and long-range organization of repeat units, genome location and distribution, putative functional interactions, reproductive mode and population factors (Strachan et al. 1985; Dover 2002; Luchetti et al. 2003; Robles et al. 2004; Meštrović et al. 2006; Kuhn et al. 2008; Navajas-Pérez et al. 2009; Giovannotti et al. 2013). In consequence, sequence dynamics of satDNA families may differ not only among families, but also, for a given family, among genomic

regions (Kuhn et al. 2011), or among populations (Wei et al. 2014), species, or higher taxonomic groups (e.g., Macas et al. 2006; Kuhn et al. 2008; Martinsen et al. 2009; Plohl et al. 2010).

In agreement with Giovannotti et al. (2014), the results of the present work show that overall variability of TaqI repeats in the whole genus *Iberolacerta* is on average three times higher than the variability of HindIII repeats, which suggests a faster homogenization/fixation rate for the latter satDNA family. However, the detailed characterization of both satDNA families in all eight *Iberolacerta* species reveals that their evolutionary patterns are more complex than previously anticipated. The presence of HindIII_HI in all the species, and its central position in the phylogenetic network, suggests that this is the most ancestral variant of HindIII satDNA, from which subfamilies HII and HIII were derived. Interestingly, with the exception of *I. cyreni*, no intraspecific homogenization for any particular subfamily was detected in our study, and most different sequence groups of subfamilies HI and HII are widespread and shared by even distantly related species. Indeed, interspecific genetic distances within each subfamily are substantially lower than intraspecific genetic distances between repeats belonging to different subfamilies. On the contrary, *I. cyreni* shows a high proportion of private sequence groups belonging to subfamily HIII, and a well-differentiated subset of HI repeats, which explains the evidence of concerted evolution found for this species in our previous study. However, the finding of HIII repeats also in *I. bonnali* indicates that this subfamily is not exclusive of *I. cyreni*, but was already present in the common ancestral library of HindIII variants. Combining these data with the results of FISH experiments, the most parsimonious interpretation of HindIII satDNA evolution is that the diversification of HindIII repeats—which generated most of the extant variants—took place in the common ancestor of *Iberolacerta*, before species radiation, i.e., from 11.6 to 15.6 mya (Arribas et al. 2014). In the ancestral species, HindIII satDNA might have been widely distributed in the centromeres of all chromosome pairs, with a subsequent decrease in copy number in *I. horvathi* and, at least, in the Pyrenean *I. bonnali*. In the latter species, and maybe also in the other two Pyrenean taxa, the reduced amounts of HindIII satDNA might obey to the possible involvement of this centromeric element in the Robertsonian fusions that originated the biarmed chromosomes characteristic of *Pyrenesaura* from the ancestral acrocentric karyotype, as has been suggested for other centromeric repeats in marsupials (Bulazel et al. 2007). Alternatively, HindIII could represent a minor satDNA family in the centromeres of the ancestral species, which was differentially amplified in the Iberian clade. In either case, the turnover of HindIII repeats in the different lineages mainly involved the same pool of “old” repeat variants. Long-term

conservation of ancestral repeats could be a consequence of selective constraints imposed on functional motifs or structural features of satellite monomers (see, for example, Meštrović et al. 2006; Plohl et al. 2012), involved in any of the roles ascribed to satDNAs (reviewed in Ugarković 2009). Thus, even if we did not find any evidence of function in HindIII satDNA, selection may have favored the maintenance of some repeat variants and/or limited the diversification of this repetitive element. Nevertheless, the loss of HindIII repeats in *I. horvathi* and *I. bonnali* (or, alternatively, the amplification in the Iberian species) suggests that, even if functional, a satellite family may be replaced by another in a relatively short evolutionary time.

Actually, and in contrast to the highly conserved function of the centromers, the rapid evolution and extensive changes in copy number of satDNAs is a general characteristic of centromeric regions (Henikoff et al. 2001). The detection of recombinant sequences within subfamily HI suggests that mechanisms such as unequal crossovers between sister chromatids and gene conversion may have been an important source of new sequence variants in HindIII satDNA (e.g. Smith 1976; Talbert and Henikoff 2010). Moreover, unequal crossover occurring between highly homogeneous arrays can induce copy number alterations of satDNA repeats, as those observed in the *Iberolacerta* species (Stephan 1986). This fast evolution of centromeric satDNAs can be linked with reproductive isolation and speciation (Bachmann et al. 1989, 1993). For example, divergence of centromeric satDNA in *Drosophila* species can inhibit chromosome segregation in hybrids and thus directly cause hybrid incompatibilies and postzygotic isolation (Ferree and Barbash 2009). Likewise, the high copy number polymorphisms and rapid shifts in centromere sequence composition could have contributed and even triggered species radiation within *Iberolacerta*.

The TaqI satDNA family has a very different evolutionary history from the HindIII family, and appears to evolve much faster in the lineage that leads to *I. horvathi*. According to the parsimony network, TaqI_TI, the most widespread subfamily among the analyzed species, would also be the most ancestral variant, from which subfamily TII was derived. The phylogenetic distribution of the different sequence sets suggests that both subfamilies were present in the common ancestor of *Iberolacerta.* Subsequently, subfamily TII spread in the Pyrenean species, whereas it was progressively lost in *I. horvathi*, and maybe even completely removed from the genomes of the Iberian species. Altogether, TI repeats retrieved from *I. horvathi* show a general pattern of concerted evolution, with high interspecific distance values in all pairwise comparisons and a large subset of species-specific sequence groups. The allocation of these private groups (e.g., TI_A2 or TI_C1) in terminal clades of the statistical

parsimony network indicates that they probably arose after the early separation of *I. horvathi* from the remaining species, about 11.5 mya (9.6–13.7) (Arribas et al. 2014). The evolution of TaqI satDNA in *I. horvathi* was probably accompanied by a reduction in the abundance and chromosomal distribution, as inferred from the results of FISH experiments. TaqI satDNA also seems to evolve in concert in the Iberian clade, but with a distinct pattern from that found in *I. horvathi*. In this case, the profile of TI repeats and the low levels of nucleotide diversity indicate that concerted evolution in the Iberian clade involved the preferential homogenization of a reduced subset of TaqI variants, all of which evolved from a single sequence lineage, TI_L3. After cladogenesis, however, the rate at which TI repeats evolve within the Iberian clade is presumably low, since TaqI sequences are poorly differentiated between the four taxa and we found almost no species-specific sequence sets.

In contrast with *I. horvathi* and in the Iberian species, the turnover process of TaqI satDNA seems to be remarkably slow in the Pyrenean *I. bonnali*. TaqI repeats from this species belong mainly to "old" sequence sets of subfamily TI and lack species-specific diagnostic positions, which indicates that most of the variability found in *I. bonnali* obeys to synapomorphisms, and that TaqI repeats have been evolving with a low rate of sequence change after speciation. Conversely, the evolution of TaqI satDNA in the other two Pyrenean species, *I. aranica* and *I. aurelioi*, is characterized by the amplification of subfamily TII. Phylogenetic studies suggest that the three species of the Pyrenean clade originated in rapid succession, though *I. bonnali* probably split first, roughly 3.8 mya (2.7–4.9) (Arribas et al. 2006, 2014). According to this phylogenetic reconstruction, the amplification of subfamily TII in the genomes of *I. aranica* and *I. aurelioi* may have occurred in a short time, after the separation of *I bonnali* and before the divergence of both species, *ca*. 3.3 mya (2.3–4.3). A rapid expansion of subfamily TII agrees well with the high levels of intraspecific nucleotide diversity and interspecific sequence conservation observed for this subfamily in both species.

The different turnover rates of TaqI repeats among the Pyrenean species, *I. horvathi* and the Iberian species, could be related to differences in their karyotypes. It is possible that interchromosomal exchange and homogenization between the asymmetric meta-/submetacentric chromosomes of the Pyrenean species is more limited than in the species with all acrocentric chromosomes, more homogeneous in shape and size. Similar considerations have been proposed to explain the lower evolutionary rate of satDNAs in sturgeons as compared to sparids (de la Herrán et al. 2001). Limited interchromosomal exchange would lead to a progressive compartmentalization of satellite repeats, followed by a reduction in their interactions and, eventually, by a lack of homogenization of different sequence variants.

However, this hypothesis is at least partially contradicted by our analysis of consecutive monomeric units, which revealed that, in both HindIII and TaqI satDNA families, adjacent repeats are not necessarily more similar than are repeats selected at random, and that members of different sequence groups or even subfamilies can be interspersed in the same array.

In fact, this pattern of composite repeats may be a key factor explaining the disparate turnover rates of each satDNA family in different species. In eukaryotes, homologous recombination within or between chromosomes can be inhibited by only one mutation per 200 bp (Nijman and Lenstra 2001, and references therein). Likewise, mutations in new monomer variants would inhibit the interactions of repeat units, leading to sequence diversification, divergent evolution and the formation of satDNA subfamilies. Accordingly, our estimates of intraspecific genetic distances between repeats belonging to different subfamilies suggest that each subfamily within HindIII and TaqI satDNAs is evolving independently. In this context, the intermixing between subfamilies HI and HII within HindIII arrays in most of the species analyzed, and between TaqI subfamilies TI and TII in the Pyrenean taxa, would strongly reduce recombination and homogenization within each subfamily, resulting in the pattern of non-concerted evolution observed in our study. Conversely, the amplification of subfamily HIII in *I. cyreni*, and the preponderance of subfamily TI in *I. horvathi* and the Iberian species, allows a more efficient homogenization of HindIII and TaqI repeats, respectively, which translates into the overall patterns of concerted evolution observed for these satDNA families in the species mentioned above.

Taken together, our results on the dynamics of HindIII and TaqI satDNAs in *Iberolacerta* are congruent with proposed models of satDNA evolution and life history, intended to explain the considerable fluctuations in copy number and variability of satDNAs shared by related species (Nijman and Lenstra 2001; Plohl et al. 2010). They also support the idea that the "library model" may be extended to monomer variants of the same satDNA family, which were already present in a common ancestor and are currently distributed in related species in variant copy numbers (Cesari et al., 2003). As observed in *Iberolacerta*, this particular evolutionary pattern may result in species-specific profiles of satDNAs which do not reflect the phylogenetic relationships among taxa.

In conclusion, an in-depth analysis of intragenomic variability of HindIII and TaqI satDNAs in *Iberolacerta* revealed two disparate evolutionary histories which, nevertheless, showed some common traits: (i) each satDNA family is made up of a library of monomer variants or subfamilies shared by related species; (ii) species-specific profiles of satellite repeats are shaped by expansions and/or contractions of different variants from the library; (iii) different

turnover rates, even among closely related species, result in great differences in overall sequence homogeneity, and in concerted or non-concerted evolution patterns. Contrasting turnover rates are possibly related to genomic constraints such as karyotype architecture and the interspersed organization of diverging repeat variants in satellite arrays, and maybe also to functional interactions. On the whole, these satDNA families constitute highly dynamic systems, which may have a critical role on the evolution of genome and species. Further studies aimed at investigating the genome-wide variability and organization of reptilian satDNAs may not only be useful to test current hypothesis and identify mechanisms influencing the evolution of this genomic component, but also to improve its application as a molecular marker in phylogenetic studies.

References

Arnold EN, Arribas O, Carranza S (2007) Systematics of the Palaearctic and Oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa 1430: 1–86.

Arribas O, Odierna G (2004) Karyological and osteological data supporting the specific status of *Iberolacerta* (*cyreni*) *martinezricai* (Arribas, 1996). Amphibia-Reptilia 25: 359–367.

Arribas O, Carranza S, Odierna G (2006) Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240: 1–55.

Arribas O, Galán P, Remón, Naveira H (2014) A new mountain lizard from Montes de León (NW Iberian Peninsula): *Iberolacerta monticola* astur ssp. nov. (Squamata: Lacertidae). Zootaxa 3796: 201–236.

Bachmann L, Raab M, Sperlich D (1989) Satellite DNA and speciation: a species specific satellite DNA of Drosophila guanchel. J Zool Sys Evol Res 27: 84–93.

Bachmann L, Sperlich D (1993) Gradual evolution of a specific satellite DNA family in *Drosophila ambigua*, *D. tristis*, and *D. obscura*. Mol Biol Evol 10: 647–659.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Retrieved from: www.genetix.univ-montp2.fr/genetix/genetix.htm 18/3/2015.

Bulazel KV, Ferreri GC, Eldridge MDB, O'Neill RJ (2007) Species-specific shifts in centromere sequence composition are coincident with breakpoint reuse in karyotypically divergent lineages. Genome Biol 8: R170.

Capriglione T (2000) Repetitive DNA as a tool to study the phylogeny of cold-blooded

vertebrates. In: Olmo E, Redi CA, eds. Chromosomes Today. Basel: Birkhäuser Verlag, pp 183–194.

Capriglione T, Olmo E, Odierna G, Smith DI, Miller OJ (1989) Genome composition and tandemly repetitive sequence at some centromeres in the lizard *Podarcis s. sicula* Raf. Genetica 79: 85–91.

Capriglione T, Cardone A, Odierna G, Olmo E (1991) Evolution of a centromeric satellite DNA and phylogeny of lacertid lizards. Comp Biochem Physiol B 100: 641–645.

Capriglione T, Cardone A, Odierna G, Olmo E (1994) Further data on the occurrence and evolution of satellite DNA families in the lacertid genome. Chromosome Res 2: 327–330.

Capriglione, T, de Santo MG, Odierna G, Olmo E (1998) An alphoid-like satellite DNA sequence is present in the genome of a lacertid lizard. J Mol Evol 46: 240–244.

Capula M, Lapini L, Capanna E (1989) The karyotype of *Lacerta horvathi* (Reptilia, Sauria, Lacertidae). Genetica 79: 11–16.

Cesari M, Luchetti A, Passamonti M, Scali V, Mantovani B (2003) Polymerase chain reaction amplification of the Bag320 satellite family reveals the ancestral library and past gene conversion events in *Bacillus rossius* (Insecta Phasmatodea). Gene 312: 289–295.

Chaiprasertsri N, Uno Y, Peyachokinagui S, Peyachoknagul S, Prakhongcheep O, Baicharoen S, Charernsuk S, Nishida C, Matsuda Y, Koga A, Srikulnath K (2013) Highly species-specific centromeric repetitive DNA sequences in lizards: Molecular cytogenetic chracterization of a novel family of satellite DNA sequences isolated from the Water Monitor Lizard (*Varanus salvator macromaculatus*, Platynota). J Hered 104: 798–806.

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371: 215–220.

Ciobanu D, Grechko VV, Darevsky IS (2003) Molecular Evolution of Satellite DNA CLsat in Lizards from the Genus *Darevskia* (Sauria: Lacertidae): Correlation with Species Diversity. Russ J Genetics 39: 1527–1541.

Ciobanu D, Grechko VV, Darevsky IS, Kramerov DA (2004) New satellite DNA in *Lacerta* s. str. lizards (Sauria: Lacertidae): Evolutionary pathways and phylogenetic impact. J Exp Zool B Mol Dev Evol 302: 505–516.

Clement M, Posada D, Crandall KA (2000) TCS: A computer program to estimate gene genealogies. Mol Ecol 9: 1657–1659.

de la Herrán R, Fontana F, Lanfredi M, Congiu L, Leis M, Rossi R, Ruiz Rejón C, Ruiz Rejón M, Garrido-Ramos MA (2001) Slow rates of evolution and sequence homogenization

in an ancient satellite DNA family of sturgeons. Mol Biol Evol 18: 432–436.

Dover GA (1982) Molecular drive: a cohesive mode of species evolution. Nature 299: 111-117.

Dover GA (2002) Molecular drive. Trends Genet 18: 587–589.

Edgar RC (2004) MUSCLE: nultiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792–1797.

Enukashvily NI, Ponomartsev NV (2013) Mammalian satellite DNA: a speaking dumb. Adv Protein Chem Struct Biol 90: 31–65.

Feliciello I, Akrap I, Brajković J, Zlatar I, Ugarković Đ (2015) Satellite DNA as a driver of population divergence in the red flour beetle *Tribolium castaneum*. Genome Biol Evol 7: 228–239.

Ferree PM, Barbash DA (2009) Species-specific heterochromatin prevents mitotic chromosome segregation to cause hybrid lethality in *Drosophila*. PLoS Biol 7: e1000234.

Fry K, Salser W (1977) Nucleotide sequences of HS-A satellite DNA from kangaroo rat *Dipodomys ordii* and characterization of similar sequences in other rodents. Cell 12: 1069–1084.

Giovannotti M, Nisi Cerioni P, Caputo V, Olmo E (2009) Characterisation of a GC-rich telomeric satellite DNA in *Eumeces schneideri* Daudin (Reptilia, Scincidae). Cytogenet Genome Res 125: 272–278.

Giovannotti M, Nisi Cerioni P, Splendiani A, Ruggeri P, Olmo E, and Caputo Barucchi V (2013) Slow evolving satellite DNAs: the case of a centromeric satellite in *Chalcides ocellatus* (Forskål, 1775) (Reptilia, Scincidae). Amphibia-Reptilia 34: 401–411.

Giovannotti M, Rojo V, Nisi Cerioni P, González-Tizón A, Martínez-Lage A, Splendiani A, Naveira H, Ruggeri P, Arribas Ó, Olmo E, Caputo Barucchi V (2014) Isolation and characterization of two satellite DNAs in some Iberian rock lizards (Squamata, Lacertidae). J Exp Zool B Mol Dev Evol 322: 13–26.

Grechko VV, Ciobanu DG, Darevsky IS, Kramerov DA (2005) Satellite DNA of lizards of the genus *Lacerta* s. str. (the group *L. agilis*), the family Lacertidae. Dokl Biochem Biophys 400: 44–47.

Grechko VV, Ciobanu DG, Darevsky IS, Kosushkin SA, Kramerov DA (2006) Molecular evolution of satellite DNA repeats and speciation of lizards of the genus *Darevskia* (Sauria: Lacertidae). Genome 49: 1297–1307.

Henikoff S, Ahmad K, Malik HS (2001) The Centromere Paradox: Stable Inheritance with

Rapidly Evolving DNA. Science 293: 1098–1102.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649.

Kuhn GCS, Sene FM, Moreira-Filho O, Schwarzacher T, Heslop-Harrison JS (2008) Sequence analysis, chromosomal distribution and long-range organization show that rapid turnover of new and old pBuM satellite DNA repeats leads to different patterns of variation in seven species of the *Drosophila buzzatii* cluster. Chromosome Res 16: 307–324.

Kuhn GCS, Schwarzacher T, Heslop-Harrison JS (2010) The non-regular orbit: three satellite DNAs in *Drosophila martensis* (*buzzatii* complex, *repleta* group) followed three different evolutionary pathways. Mol Genet Genomics 284: 251–262.

Kuhn GCS, Küttler H, Moreira-Filho O, Heslop-Harrison JS (2011) The 1.688 repetitive DNA of *Drosophila*: Concerted evolution at different genomic scales and association with genes. Mol Biol Evol msr173.

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.

Luchetti A, Cesari M, Carrara G, Cavicchi S, Passamonti M, Scali V, Mantovani B (2003) Unisexuality and molecular drive: Bag320 sequence diversity in *Bacillus* taxa (Insecta Phasmatodea). J Mol Evol 56: 587–589.

Macas J, Navrátilová A, Koblížková A (2006) Sequence homogenization and chromosomal localization of VicTR-B satellites differ between closely related *Vicia* species. Chromosoma 115: 437–447.

Martinsen L, Venanzetti F, Johnsen A, Sbordoni V, Bachman L (2009) Molecular evolution of the *pDo500* satellite DNA family in *Dolichopoda* cave crickets (Rhaphidophoridae). BMC Evolutionary Biology 9: 1–14.

Meštrović N, Plohl M, Mravinac B, Ugarković D (1998) Evolution of Satellite DNAs from the Genus Palorus--Experimental Evidence for the 'Library' Hypothesis. Mol Biol Evol 15: 1062–1068.

Meštrović N, Castagnone-Sereno P, Plohl M (2006) Interplay of Selective Pressure and Stochastic Events Directs Evolution of the MEL172 Satellite DNA Library in Root-Knot Nematodes. Mol Biol Evol 23: 2316–2325.

Navajas-Pérez R, Quesada del Bosque ME, Garrido-Ramos MA (2009) Effect of location,

organization, and repeat-copy number in satellite-DNA evolution. Mol Genet Genomics 282: 395–406.

Nijman IJ, Lenstra JA (2001) Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite repeats. J Mol Evol 52: 361–271.

Odierna G, Aprea G, Arribas O, Capriglione T, Caputo V, Olmo E (1996) The karyology of the Iberian rock lizards. Herpetologica 52: 542–550.

Palomeque T, Lorite P (2008) Satellite DNA in insects: a review. Heredity 100: 564–573.

Pezer Ž, Brajković J, Feliciello I, Ugarković D. Satellite DNA-mediated effects on genome regulation. In: Garrido-Ramos MA, ed. Repetitive DNA. Genome dynamics vol 7. Basel: Karger, pp 153–169.

Plohl M, Luchetti A, Meštrović N, Mantovani B (2008) Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. Gene 409: 72–82.

Plohl M, Petrovic V, Luchetti A, Ricci A, Satović E, Passamonti M, Mantovani B (2010) Long-term conservation vs high sequence divergence: the case of an extraordinarily old satellite DNA in bivalve mollusks. Heredity 104: 543-551.

Plohl M, Meštrović N, Mravinac B (2012) Satellite DNA evolution. In: Garrido-Ramos MA, ed. Repetitive DNA. Genome dynamics vol 7. Basel: Karger, pp 126–152.

Robles F, de la Herrán R, Ludwig A, Rejón CR, Rejón MR, Garrido-Ramos, MA (2004) Evolution of ancient satellite DNAs in sturgeon genomes. Gene 338: 133–142.

Rojo V, Giovannotti M, Naveira H, Nisi Cerioni P, González-Tizón AM, Caputo Barucchi V, Galán P, Olmo E, Martínez-Lage A (2014) Karyological characterization of the endemic Iberian Rock lizard, *Iberolacerta monticola* (Squamata, Lacertidae): Insights into sex chromosome evolution. Cytogenet Genome Res 142: 28–39.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor.

Schwarzacher T, Heslop-Harrison P (2000) Practical *in Situ* Hybridization. Oxford: BIOS Scientific Publishers Ltd.

Sindaco R, Jeremčenko VK (2008) The Reptiles of the Western Paleartic, Volume 1: Annotated Checklist and Distributional Atlas of the Turtles, Crocodiles, Amphisbaenians and Lizards of Europe, North Africa, Middle East and Central Asia. Latina: Edizioni Belvedere.

Smith GP (1976) Evolution of repeated DNA sequences by unequal crossover. Science 191: 528–535.

Stephan W (1986) Recombination and the evolution of satellite DNA. Genet Res 47: 167-174.

Strachan T, Webb D, Dover G (1985) Transition stages of molecular drive in multiple-copy DNA families in *Drosophila*. EMBO J 4: 1701–1708.

Talbert PB, Henikoff S (2010) Centromeres convert but don't cross. PLoS Biol 8: e1000326.

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101*:* 11030–11035.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30: 2725–2729.

Templeton AR, Crandall KA, Sing CF (1992) A Cladistic Analysis of Phenotypic Associations with Haplotypes Inferred from Restriction Endonuclease Mapping and DNA Sequence Data. III. Cladogram Estimation. Genetics 132: 619–633.

Ugarković D (2009) Centromere-competent DNA: structure and evolution. Prog Mol Subcell Biol 48: 53–76.

Ugarković D, Plohl M (2002) Variation in satellite DNA profiles—causes and effects. EMBO J 21: 5955–5959.

Waters PD, Marshall Graves JA, Thompson K, Sankovic N, Ezaz T (2008) Identification of cryptic sex chromosomes and isolation of X- and Y-borne genes. *Methods Mol Biol 422: 239–251.*

Wei KH-C, Grenier JK, Barbash DA, Clark AG (2014) Correlated variation and population differentiation in satellite DNA abundance among lines of *Drosophila melanogaster*. PNAS 111: 18793–18798.

Supplementary Material

Table S1. *Iberolacerta* species and populations included in this study.

Species	Populations	Locality	Number of specimens
I. horvathi	2	Sella Nevea, Carnic Alps, Udine (Italy) Passo di Pramollo, Carnic Alps, Udine (Italy)	2
I. bonnali	2	Pico de Urdiceto, Pirineos, Huesca (Spain) Estany de Cavallers, Aigüestortes, Pirineos, Cataluña (Spain)	2
I. aranica	2	Estany de Liat, Vall d'Aran, Pirineos, Cataluña (Spain) Combe de la Montanyole, Pirineos (France)	2
I aurelioi	2	Pica d'Estats, Pirineos, Cataluña (Spain) Circ de Comapedrosa, Pirineos (Andorra)	2
I. cyreni	3	Navacerrada, Sierra de Guadarrama, Segovia-Madrid (Spain), Pico Zapatero, Sierra de la Paramera, Ávila (Spain) Puerto de Peña Negra, Sierra de Villafranca, Ávila (Spain)	3
I. monticola	1	Fragas do Eume, A Capela, Galicia (Spain)	4
I. galani	1	A Ponte, Pena Trevinca, A Veiga, Galicia, Spain	4
I. martinezricai	1	Puerto El Portillo, Salamanca (Spain)	1

Fig. S1a. Sequence alignment of consensus sequences of the different HindIII sequence groups. The first line shows the general consensus for all the sequences of each satDNA. Diagnostic positions for each group are indicated by coloured shading.

```
                  *        20         *        40         *        60
          AGCTTATTTGCGCtcAAATA-CACAA-TGTGCCCAAAt-GCAAAACGACGCACAA-AAGC
HI_A    : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_B    : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_C    : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_D1   : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAATAAGC :  60
HI_D2   : AGCTTATTTGCGCTCAAATAGCACAATTGTGCCCAAATTGCAAAACGACGCACAATAAGC :  60
HI_E    : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_F    : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAATAAGC :  60
HI_G    : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_H    : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_I    : AGCTTATTTGCGCTGAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_J    : AGCTTATTTGCGCTGAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAACAAGC :  60
HI_K    : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAATAAGC :  60
HI_L    : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATTGCAAAACGACGCACAATAAGC :  60
HI_M    : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATGGCAAAACGACGCACAATAAGC :  60
HII_A   : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAATAAGC :  60
HII_B   : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATGGCAAAACGACGCACAATAAGC :  60
HII_C   : AGCTTATTTGCGCTCAAATAACACAATTGTGCCCAAATTGCAAAACGACGCACAATAAGC :  60
HII_D   : AGCTTATTTGCGCTCAAATAGCACAATTGTGCCCAAACTGCAAAACGACGCACAACAAGC :  60
HII_E   : AGCTTATTTGCGCACAAATAGCACAATTGTGCCCAAACTGCAAAACGACGCACAACAAGC :  60
HII_F   : AGCTTATTTGCGCTCAAATAGCACAATTGTGCCCAAACTGCAAAACGACGCACAACAAGC :  60
HII_G   : AGCTTATTTGCGCTCAAATAGCACAATTGTGCCCAAACTGCAAAACGACGCACAATAAGC :  60
HII_H   : AGCTTATTTGCGCTCAAATAGCACAATTGTGCCCAAACTGCAAAACGACGCACAACAAGC :  60
HIII_A  : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATGGCAAAACGACGCACAATAAGC :  60
HIII_B  : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATGGCAAAACGACGCACAATAAGC :  60
HIII_C  : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATGGCAAAACGACGCACAATAAGC :  60
HIII_D  : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATGGCAAAACGACGCACAATAAGC :  60
HIII_E  : AGCTTATTTGCGCTCAAATAACACAACTGTGCCCAAATGGCAAAACGACGCACAATAAGC :  60
```

```
                  *        80         *       100         *       120
          CTCAGAATGATGaGAATAAGCCaa-t-AGCGTCCcAAtgCaTGCTGCACACAgaAA-CaG
HI_A    : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_B    : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_C    : CTCAGAATGATGAGAATAAGCCAACTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_D1   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_D2   : CTCAGAATGATGAGAATAAGCCAACTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_E    : CTCAGAATGATGAGAATAAGCCACCTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_F    : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAACCAG : 120
HI_G    : CTCAGAATGATGAGAATAAGCCAACTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_H    : CTCAGAATGATGAGAATAAGCCACCTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_I    : CTCAGAATGATGAGAATAAGCCAACTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_J    : CTCAGAATGATGAGAATAAGCCACCTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_K    : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAACCAG : 120
HI_L    : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HI_M    : CTCAGAATGATGAGAATAAGCCAATTGAGCGTCCCAATGCATGCTGCACACAGAAATCAG : 120
HII_A   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAACCAG : 120
HII_B   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCGTGCTGCACACAGAAAGCAG : 120
HII_C   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAAGCAG : 120
HII_D   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAAGCAG : 120
HII_E   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAAGCAG : 120
HII_F   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCATGCTGCACACAGAAAGCAG : 120
HII_G   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCGTGCTGCACACAGAAAGCAG : 120
HII_H   : CTCAGAATGATGAGAATAAGCCAATTTAGCGTCCCAATGCGTGCTGCACACAGAAAGCAG : 120
HIII_A  : CTCAGAATGATGGGAATAAGCCCATTGAGCGTCCAAACACATGCTGCACACAATAATCAG : 120
HIII_B  : CTCAGAATGATGGGAATAAGCCCATTGAGCGTCCAAACACATGCTGCACACAATAATCAG : 120
HIII_C  : CTCAGAATGATGGGAATAAGCCCATTGAGCGTCCAAACACATGCTGCACACAAGAATCAG : 120
HIII_D  : CTCAGAATGATGGGAATAAGCCCATGGAGCGTCCAAACACATGCTGCACACAAGAATCAG : 120
HIII_E  : CTCAGAATGATGGGAATAAGCCCATGGAGCGTCCAAACACATGCTGCACACAAGAATCCG : 120

                  *       140         *       160         *
          TGTTTCTCtTGCTTATTTC-CTC—A--T--gTGTTTTACAGTTG-AAAAGCT
HI_A    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_B    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_C    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_D1   : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_D2   : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_E    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_F    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_G    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_H    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_I    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_J    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_K    : TGTTTCTCTTGCTTATTTCACTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_L    : TGTTTCTCTTGCTTATTTCACTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HI_M    : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HII_A   : TGTTTCTCTTGCTTATTTCACTC--ATTTGGTGTTTTACAGTTGAAAAAGCT : 170
HII_B   : TGTTTCTCTTGCTTATTTCACTC--ATTTGGTGTTTTACAGTTGAAAAAGCT : 170
HII_C   : TGTTTCTCTTGCTTATTTCACTC--ATTTGCTGTTTTACAGTTGAAAAAGCT : 170
HII_D   : TGTTTCTCTTGCTTATTTCACTC--ATTTGCTGTTTTACAGTTGAAAAAGCT : 170
HII_E   : TGTTTCTCTTGCTTATTTCACTC--ATTTGGTGTTTTACAGTTGAAAAAGCT : 170
HII_F   : TGTTTCTCTTGCTTATTTCACTC--ATTTGGTGTTTTACAGTTGAAAAAGCT : 170
HII_G   : TGTTTCTCTTGCTTATTTCACTC--ATTTGGTGTTTTACAGTTGAAAAAGCT : 170
HII_H   : TGTTTCTCTTGCTTATTTCACTC--ATTTGGTGTTTTACAGTTGAAAAAGCT : 170
HIII_A  : TGTTTCTCTTGCTTATTTCGCTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HIII_B  : TGTTTCTCCTGCTTATTTCACTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HIII_C  : TGTTTCTCCTGCTTATTTCACTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HIII_D  : TGTTTCTCCTGCTTATTTCACTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
HIII_E  : TGTTTCTCCTGCTTATTTCACTCCAAATGAGTGTTTTACAGTTG-AAAAGCT : 171
```

Fig. S1b. Sequence alignment of consensus sequences of the different TaqI sequence groups. The first line shows the general consensus for all the sequences of each satDNA. Diagnostic positions for each group are indicated by coloured shading.

```
                  *        20         *        40         *        60
          cGAggcCTgATTT-T-----taTGATAaAA-C-cTtCTGTTTccACcGCCAAA-CtT-ca
TI_A1   : CGAGGCCTGATTTCTTCAGGTATGATAAAAACCCTTCTGTTTTCACCGCCAAATCCTCCG :  60
TI_A2   : CGAGGCCTGATTTCTTCAGGTATGATAAAAACCCTTCTGTTTTCACCGCCAAATCCTCCG :  60
TI_B1   : CGAGGCCTGATTTCTTCAGGTATGATAAAAACCCTTCTGTTTTCACTGCCAAATCCTCCA :  60
TI_B2   : CGAGGCCTGATTTCTTCAGGTATGATAAAAACCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_C1   : GGAGGTCTTATTTCTTCATCTGTGATAAAAACCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_C2   : CGAGGCCTGATTTCTTCATCTATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_D    : CGAGGCCTGATTTCTTAACTTATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_E    : CGAGGCCTGATTTCTTGACGTTTGATAAAAGCCCTTCTGTTTCCACAGCCAAATCTTCCA :  60
TI_F1   : CGAGGCCTGATTTCTTGACCTATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_F2   : CGAAGCCTGATTTCTTGACCCATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_G1   : CGATGCCTGATTTCTTGTCCTATGATAAAAACCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_G2   : CGAGGCCTCATTTCTTGTCCTATGATAAAAACCCTTCTGTTTCCACCGCCAAATCCTCCA :  60
TI_H1   : CGAGGCCTGATTTCTTAAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_H2   : CGAGGCCTGATTTCTTTAAATATGATAAAAGCCCTTCTGTTTTCACCGCCAAATCTTCCA :  60
TI_I1   : CGAGGCCTGATTTCTTAAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCTA :  60
T1_I2   : CGAGGCCTGATTTCTTTAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCTA :  60
TI_J1   : CGAGGCCTGATTTCTTAAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_J2   : CGAGGCCTGATTTCTTTAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_K    : CGAGGCCTGATTTCTTAAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_L1   : CGAGGCCTGATTTCTTAAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_L2   : CGAGGCCTGATTTCTTAAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TI_L3   : CGAGGCCTGATTTCTTAAAATATGATAAAAGCCCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TII_A   : CGAGGCCTGATTTTTCATCCTATGATAAAAACGCTTCTGTTTCCACCGCCAAATCTTCCA :  60
TII_B1  : CGAGGCCTGATTTTTCATCCTATGATAAAAACGCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_B2  : CGAGGCCTGATTTTTCATCCTATGATAAAAGCCCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_C1  : CGAGGCCTGATTTTTTATCCTATGATAAAAACGCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_C2  : CGAGGCCTGATTTTTCATCCTATGATAAAAACGCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_D   : CGAGGCCTGATTTTTCATCGTATGATAAAAACGCTTCTGTTTCCACCGCCAAACCATTCA :  60
TII_E1a : CGAGGCCTGATTTTTCATCCTGTGATAAAAACCCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_E1b : CGAGGCCTGATTTTTCATCCTGTGATAAAAACCCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_E2  : TGAGGCCTGATTTTTCATCCTGTGATAGAAACCTTCCTGTTTCCACCGCCAAACCTTTCA :  60
TII_F   : CGAGGCCTGATTTTTCATCCTATGATAAAAACCCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_G1  : CGAGGCCTGATTTTTCATCCTATGATAAAAACGCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_G2a : TGAGGTCTGATTTTTCATCCTATGATAAAAACACTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_G2b : TGAGGTCTGATTTTTCTTCCTATGATAAAAACCCTTCTGTTTCCACCGCCAAACCTTTCA :  60
TII_G3  : CGAGTCCTGATTTTTCATGCTATGATAAAAACGCTTCTGTTTCAACCGCCAAACCTTTCA :  60
TII_G4  : TGAGACCTGATTTTTCATCCTATGATAAAAACCCTTCTGTTTCCACCGCCAAACCTTTCA :  60
```

```
                  *        80         *       100         *       120
          gGGGA-AATt—CAACaGTTTGgCACCaTTTT-GAGTGAAtTGGAaAACgTCA-ATtTT--
TI_A1    : CGGGAGAATTTGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TI_A2    : CGGGAGAATTTGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAGAACGTCAGATTTTT : 120
TI_B1    : CGGGAGAATTTGCAACAGTTTGACACCATTTTTGAGTGAATTGGAGAACGTCAGATTTTT : 120
TI_B2    : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TI_C1    : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAGAACGTCAGATTTTT : 120
TI_C2    : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAGAACGTCAGATTTTT : 120
TI_D     : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TI_E     : GGGGAGAATGCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TI_F1    : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TI_F2    : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TI_G1    : GGGGAGAATTCGCAACAGTTTGGCACCTTTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TI_G2    : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAGAACGTCAGATTTTT : 120
TI_H1    : GGGGACAATTCCCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_H2    : GGGGACAATTCCCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_I1    : TGGGACAATTCACAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
T1_I2    : TGGGACAATTCACAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_J1    : GGGGACAATTCACAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_J2    : GGGGACAATTCACAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_K     : GGGGACAATTCCCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_L1    : GGGGACAATTCCCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_L2    : GGGGACAATTCACAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TI_L3    : GGGGAGAATTCACAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAAATTTTC : 120
TII_A    : GGGGAGAATTCGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_B1   : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_B2   : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_C1   : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_C2   : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_D    : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_E1a  : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATCTTT : 120
TII_E1b  : GGGGAGAATTTGCAACAGTTTGGCACCATTTTTGAGTGAACTGGAAAACGTCAGATCTTT : 120
TII_E2   : GGGGAGAATTTGCAACAGTTTGGCACCATTTTTGAGTGAACTGGAAAACCTCAGATTTTT : 120
TII_F    : GGGGAGAATTTGCAACAGTTTGGCACCATTTTTGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_G1   : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_G2a  : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_G2b  : GGGGAGAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
TII_G3   : GGGGAGAATGTGCAACTGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCATATTTTT : 120
TII_G4   : GGGGATAATTTGCAACAGTTTGGCACCATTTTGGAGTGAATTGGAAAACGTCAGATTTTT : 120
```

```
                    *       140         *       160         *       180
          -GtgaaaTTCTGACCCCG-GGgTTAgGgAtTTTTt-caaaaa------ttTt-C-CAgGT
TI_A1   : GGTGAACTTCTGACCCCGGGGGTTAGGGATTTTTT-CAAAAAAA--TGTTTTTCTCAGGT : 177
TI_A2   : GGTGAACTTCTGACCCCGGGGGTTAGGGATTTTTT-CAAAAAAA--TGTTTTTCTCAGGT : 177
TI_B1   : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-CAAAAAAA--AGTTTTTCTCAGGT : 177
TI_B2   : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-CAAAAAAA--TGTTTTTCGCAGGT : 177
TI_C1   : GGTGAAATTCTGACCCCGCGGGTTA-GGATTTTTT-CAAAAAA---TGTTTTTCGCAGGT : 175
TI_C2   : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---TGTTTTTCGCAGGT : 176
TI_D    : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAAA--AGTTTT-CACAGGT : 176
TI_E    : CGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTTTTAAAAAAA--AGAATT-CTCAGGT : 177
TI_F1   : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCGCAGGT : 176
TI_F2   : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAAA--AGTTTTTCGCAGGT : 177
TI_G1   : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAAA--AGTTTTTCGCAGGT : 177
TI_G2   : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCGCAGGT : 176
TI_H1   : AGGGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
TI_H2   : AGGGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
TI_I1   : AGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
T1_I2   : AGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
TI_J1   : AGTGAAATTCTGACCCCGCGGGTTAGGGAATTTTT-CAAAAAC---AGTTTTTCTCAAGT : 176
TI_J2   : AGTGAAATTCTGACCCCGCGGGTTAGGGAATTTTT-CAAAAAC---AGTTTTTCTCAGGT : 176
TI_K    : AGTGAAATTCTGACCCCGCGGCTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
TI_L1   : AGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
TI_L2   : AGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
TI_L3   : AGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCTCAGGT : 176
TII_A   : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-C-----------TTT--CGCAGGT : 166
TII_B1  : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-C-----------TTT--CGCAGGT : 166
TII_B2  : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-C-----------TTT--CGCAGGT : 166
TII_C1  : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-C-----------TTT--CGCAGGT : 166
TII_C2  : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-C-----------TTT--CGCAGGT : 166
TII_D   : GGTGAAATTCTGACCCCGGGGGTTAGGAATTTTTT-C-----------TTT--CGCAGGT : 166
TII_E1a : GGTGGGATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---TCTTTTTCGCAGGT : 176
TII_E1b : GGTGGGATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---TCTTTTTCGCAGGT : 176
TII_E2  : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---TGTTTTTCGCAGGT : 176
TII_F   : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAA---TGTTTTTCGCAGGT : 176
TII_G1  : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-CAAAAAA---AGTTTTTCGCAGGT : 176
TII_G2a : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAAAA-TGTTTTTCGCAGGT : 178
TII_G2b : GGTGAAATTCTGACCCCGCGGGTTAGGGATTTTTT-CAAAAAAAAATGTTTTTCGCAGGT : 179
TII_G3  : GGTCAAATTCTGACCCCGCGGGTTATGGATTTTT--CAAAAAAA--TGTTTTTCGCAGGT : 176
TII_G4  : GGTGAAATTCTGACCCCGGGGGTTAGGGATTTTTT-CAAAAAAA--TGTTTTTCGCAGGT : 177
```

```
                  *
          -aagTTg-cG
TI_A1    : GAAGTTGTCG : 187
TI_A2    : GAAGTTCTCG : 187
TI_B1    : GAAGTTGTCG : 187
TI_B2    : GAAGTTGTCG : 187
TI_C1    : TAAGTTGTCG : 185
TI_C2    : TAAGTTGTCG : 186
TI_D     : TAAGTTGTCG : 186
TI_E     : TACGTTGTCG : 187
TI_F1    : TAAGTTGTCG : 186
TI_F2    : TAAGTTGTCG : 187
TI_G1    : TAAGTTGTCG : 187
TI_G2    : TAAGTTGTCG : 186
TI_H1    : TAAGTTGTCG : 186
TI_H2    : TAAGTTGTCG : 186
TI_I1    : TAAGTTGTCG : 186
T1_I2    : TAAGTTGTCG : 186
TI_J1    : TAAGTTGTCG : 186
TI_J2    : TAAGTTGTCG : 186
TI_K     : GAAGTTGTCG : 186
TI_L1    : TAAGTTGTCG : 186
TI_L2    : TAAGTTGTCG : 186
TI_L3    : TAAGTTGTCG : 186
TII_A    : TAACTTGGCG : 176
TII_B1   : AAAGTTGGCG : 176
TII_B2   : AAAGTTGGCG : 176
TII_C1   : TAACTTGGCG : 176
TII_C2   : TAACTTGGCG : 176
TII_D    : TAACTTGGCG : 176
TII_E1a  : TAAGTTGGCG : 186
TII_E1b  : TAAGTTGGCG : 186
TII_E2   : TAAGTTGGCG : 186
TII_F    : TAAGTTGGCG : 186
TII_G1   : AAAGTTGTCG : 186
TII_G2a  : AAAGTTGGTG : 188
TII_G2b  : AGAGTTGGTG : 189
TII_G3   : AAAGTTGGCG : 186
TII_G4   : AAAGTTGGCG : 187
```

Species	Clone number	HindIII Constituent monomeric units	Subfamily
I. monticola	20	20a	HI_C
		20b	HI_C
	26	26a	HI_H
		26b	HI_H
		26c	HI_F
		26d	HI_I
	143	143a	HI_E
		143b	HI_K
	146	146a	HI_C
		146b	HI_E
		146c	HI_C
	155	155a	HI_D1
		155b	HI_F
		155c	HII_A
	181	181a	HII_A
		181b	HI_K
I. galani	160	160a	HI_E
		160b	HI_J
		160c	HI_G
	161	161a	HI_D1
		161b	HI_C
	168	168a	HI_H
		168b	HI_E
		168c	HI_C
	189	189a	HI_G
		189b	HI_C
	192	192a	HI_A
		192b	HI_E
I. martinezricai	170	170a	HI_G
		170b	HI_C
	174	174a	HI_C
		174b	HI_B
	196	196a	HI_A
		196b	HI_C
	249	249a	HI_B
		249b	HI_H
	278	278a	HI_A
		278b	HI_C
	279	279a	HI_I
		279b	HI_G
		279c	HI_C
	281	281a	HI_B
		281b	HI_G

Table S2. Description of cloned HindIII satDNA multimers. Lowercase letters after the clone name were assigned in alphabetical order according to the order of consecutive monomers in the satellite array.

Species	Clone number	HindIII Constituent monomeric units	Subfamily
I. cyreni	177	177a	HIII_C
		177b	HIII_B
	205	205a	HI_M
		205b	HIII_D
		205c	HIII_B
		205d	HIII_B
	207	207a	HIII_C
		207b	HI_M
		207c	HI_M
		207d	HIII_C
I. cyreni	209	209a	HIII_C
		209b	HIII_B
		209c	HIII_B
	283	283a	HIII_D
		283b	HIII_D
		283c	HIII_E
		283d	HI_M
	286	286a	HIII_D
		286b	HIII_B
		286c	HIII_B
		286d	HI_M
		286e	HIII_C
	290	290a	HI_M
		290b	HIII_B
I. horvathi	59	59a	HI_C
		59b	HI_C
	61	61a	HI_I
		61b	HI_E
I. aurelioi	32	32a	HII_E
		32b	HII_F
	55	55a	HI_E
		55b	HI_C
	59	59a	HI_F
		59b	HI_F
	266	266a	HII_A
		266b	HII_C
		266c	HII_F
		266d	HI_D1
I. aranica	61	61a	HI_F
		61b	HII_A
	98	98a	HI_F
		98b	HI_K
	99	99a	HII_A
		99b	HI_F
	108	108a	HII_F
		108b	HII_F
	173	173a	HI_G
		173b	HII_G

Table S2 (continued)

Species	Clone number	HindIII Constituent monomeric units	Subfamily
I. bonnali	50	50a	HII_D
		50b	HIII_E
	54	54a	HII_B
		54b	HIII_E
	55	55a	HIII_C
		55b	HIII_E
	140	140a	HII_A
		140b	HII_F
	101	101a	HI_D1
		101b	HI_H
		101c	HI_D1
		101d	HI_L
	104	104a	HI_D1
		104b	HI_F
		104c	HI_K
I. bonnali	110	110a	HI_D1
		110b	HI_C
	114	114a	HI_F
		114b	HI_I
		114c	HI_I
	134	134a	HI_F
		134b	HII_A

Table S2 (continued)

Species	Clone number	TaqI Constituent monomeric units	Subfamily
I. horvathi	3	3a	TI_L3
		3b	TI_A2
	5	5a	TI_B2
		5b	TI_A2
		5c	TI_B2
	9	9a	TI_C2
		9b	TI_C1
		9c	TI_G2
	10	10a	TI_A2
		10b	TI_A1
	14	14a	TI_G2
		14b	TI_G2
		14c	TI_G2
	15	15a	TI_D
		15c	TI_D
	23	23a	TI_F1
		23b	T1_C1
		23c	TI_C2
	24	24a	TII_F
		24b	TII_F
		24c	TI_A1
I. aurelioi	5	5a	TII_B1
		5b	TII_C1
	34	34a	TII_E2
		34b	TII_B1
	86	86a	TII_C1
		86b	TII_F
	166	166a	TII_B2
		166b	TII_E1a
		166c	TII_E1a
I. aranica	13	13a	TII_G3
		13b	TII_C2
	41	41a	TI_E
		41b	TI_F1
		41c	TII_A
		41d	TI_F1
		41e	TI_F1
	44	44a	TII_G2a
		44b	TII_B2
	108	108a	TII_G2a
		108b	TII_G2b
	114	114a	TI_F1
		114b	TI_F2
	117	117a	TI_F1
		117b	TI_F1

Table S3. Description of cloned TaqI satDNA multimers. Lowercase letters after the clone name were assigned in alphabetical order according to the order of consecutive monomers in the satellite array.

Species	Clone number	TaqI Constituent monomeric units	Subfamily
I. aranica	147	147a	TII_F
		147b	TII_A
		147c	TI_G1
		147d	TI_E
		147e	TI_F1
		147f	TII_A
	150	150a	TII_G3
		150b	TII_A
I. bonnali	25	25a	TI_H1
		25b	TI_L3
		25c	TI_H1
	61	61a	TI_F1
		61b	TI_F1
	103	103a	TII_G2
		103b	TI_L2
	138	138a	TII_E1b
		138b	TI_K
	139	139a	TI_C2
		139b	TI_F1

Table S3 (continued)

CONCLUSIONS

Conclusions

Main conclusions of Chapter I: Karyological characterization of *Iberolacerta monticola*.

I. The cytogenetic analysis of male and female specimens from four different populations of *I. monticola* showed a common karyotype, consisting of 2n=36 acrocentric chromosomes gradually decreasing in size.

II. C-banding and differential fluorochome staining evidenced conspicuous heterochromatic blocks in the centromeric and interstitial/pericentromeric regions, as well as a compartmentalization of GC-rich elements in the telomeric heterochromatin.

III. Comparisons of the C-banding patterns among *Iberolacerta* species revealed extensive heterogeneity in the amount, distribution and composition of the heterochromatic areas, even between species so closely related as *I. monticola*, *I. galani* and *I. martinezricai*, which emphasizes the dynamic nature of these genomic compartments. C-banding patterns may be useful to identify species-diagnostic characters, but do not necessarily reflect phylogenetic relationships among taxa.

IV. In contrast with previous works, C-banding and comparative genomic hybridization (CGH) uncovered a heteromorphic ZW sex chromosome pair in specimens of *I. monticola* from all four populations investigated. The heterogametic W chromosome is highly differentiated from the Z chromosome, both in size, heterochromatin content, and in the massive accumulation of female-specific sequences. The sex chromosome pair is superficially similar to that of other *Iberolacerta* species (*I. horvathi*, *I. cyreni*, and *I. galani*), which suggests that the presence of a differentiated ZW pair is the ancestral condition for this genus. The putative absence of heteromorphic sex chromosomes in *I. martinezricai* and *I. aranica* deserves further investigation. High-resolution molecular cytogenetic techniques, such as CGH, would be especially effective for identifying molecularly differentiated sex chromosomes, which may have been overlooked after conventional staining and C-banding.

V. Neither the major ribosomal genes nor telomeric $(TTAGGG)_n$ repeats are differentially amplified in the heterochromatin of the W chromosome. Instead, the major ribosomal genes were located in the subtelomeric region of chromosome pair 6. Hybridization

signals of the telomeric probe showed a telomere-typical pattern, as well as interstitial telomeric sites in five chromosome pairs, which could be remnants of chromosomal rearrangements that occurred during karyotype evolution.

Main conclusions of Chapter II: Comparative chromosome painting in lacertid lizards.

VI. Cross-species chromosome painting using *I. monticola* as the source genome revealed homology of sex chromosomes in the genus *Iberolacerta*. A fusion event involving the primitive W chromosome and a small acrocentric autosome (chromosome 15 or 16) originated a biarmed neo-W and a multiple Z_1Z_2W sex chromosome system in *I. bonnali*.

VII. The W chromosomes of *I. monticola* and representatives of two other lacertid genera (*Timon lepidus* and *Lacerta schreiberi*) are highly differentiated from each other, and probably evolved independently through rapid accumulation of female-specific sequences characteristic of each lineage.

VIII. A preliminary analysis of female metaphses with a Z chromosome paint suggest that the ZW pair of *L. schreiberi* is not homologous to that of *I. monticola* and *T. lepidus,* and represent an independent origin of sex chromosomes in Lacertidae, masked under morphologically similar karyotypes.

IX. Appart from the sex chromosomes, *I. monticola*, *T. lepidus* and *L. schreiberi* show a high degree of chromosome conservation. The main rearrangements in the studied species include a centric fusion of two acrocentric chromosomes in *T. lepidus*, and a translocation of microchromosomes to macrochromosomes in *I. monticola*.

X. Comparative gene mapping detected partial synteny of *I. monticola* chromosome 1 with chicken chromosomes 3, 5 and 7, a feature conserved across most Squamate lineages. The results of gene mapping in *I. monticola* also support lack of homology between the sex chromosomes of lacertids and *A. carolinensis*, and suggest that the loss of microchromosomes in Lacertidae was due to repeated fusions between microchromosomes that existed in the ancestral karyotype of squamate reptiles.

Main conclusions of Chapter III: Evolutionary dynamics of two satellite DNA families in the genus *Iberolacerta*.

XI. A detailed characterization of intragenomic variability of two satDNA families, namely HindIII and TaqI, in *Iberolacerta* unraveled complex evolutionary dynamics that depart from the expected patterns of concerted evolution.

XII. HindIII and TaqI satDNAs differ in their chromosomal locations, abundances and turnover rates; nevertheless, they share some common traits:

- Each satellite family is made up of a library of monomer variants or subfamilies, which were already present in the common ancestor of *Iberolacerta*.

- Species-specific profiles are mainly defined by the differential amplification of particular variants from the library, rather than by gradual accumulation and homogenization of single nucleotide changes.

- Long-term sequence conservation of satellite monomers might be related to putative functional constraints, but also to the interspersed organization of divergent monomer variants in satellite arrays, which could reduce the efficacy of homogenization mechanisms.

- Extensive fluctuations in copy number may also lead to a drastic reduction in the abundance of a satellite family, as would be the case of HindIII satDNA in *I. horvathi* and *I. bonnali*. In the latter species (and maybe also in the other Pyrenean taxa), rapid changes in this centromeric satDNA might be correlated with the exceptionally high rate of chromosomal rearrangements characteristic of this lineage.

XIII. As a result of this complex mode of evolution, both HindIII and TaqI satDNAs are poorly informative as phylogenetic markers for the genus *Iberolacerta*.

ANNEXES

ANNEX I

Original Article

Cytogenetic and Genome Research

Cytogenet Genome Res 2014;142:28–39
DOI: 10.1159/000356049

Accepted: June 24, 2013
by M. Schmid
Published online: November 28, 2013

Karyological Characterization of the Endemic Iberian Rock Lizard, *Iberolacerta monticola* (Squamata, Lacertidae): Insights into Sex Chromosome Evolution

V. Rojo[a] M. Giovannotti[c] H. Naveira[a] P. Nisi Cerioni[c] A.M. González-Tizón[a]
V. Caputo Barucchi[c] P. Galán[b] E. Olmo[c] A. Martínez-Lage[a]

[a]Departamento de Bioloxía Celular e Molecular, and [b]Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía, Grupo de Investigación en Bioloxía Evolutiva (GIBE), Universidade da Coruña, A Coruña, Spain; [c]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy

Key Words
Chromosome banding · Comparative cytogenetics · FISH · Rock lizards · Sex chromosomes

Abstract
Rock lizards of the genus *Iberolacerta* constitute a promising model to examine the process of sex chromosome evolution, as these closely related taxa exhibit remarkable diversity in the degree of sex chromosome differentiation with no clear phylogenetic segregation, ranging from cryptic to highly heteromorphic ZW chromosomes and even multiple chromosome systems ($Z_1Z_1Z_2Z_2/Z_1Z_2W$). To gain a deeper insight into the patterns of karyotype and sex chromosome evolution, we performed a cytogenetic analysis based on conventional staining, banding techniques and fluorescence in situ hybridization in the species *I. monticola*, for which previous cytogenetic investigations did not detect differentiated sex chromosomes. The karyotype is composed of 2n = 36 acrocentric chromosomes. NORs and the major ribosomal genes were located in the subtelomeric region of chromosome pair 6. Hybridization signals of the telomeric sequences $(TTAGGG)_n$ were visualized at the telomeres of all chromosomes and interstitially in 5 chromosome pairs. C-banding showed constitutive heterochromatin at the centromeres of all chromosomes, as well as clear pericentromeric and light telomeric C-bands in several chromosome pairs. These results highlight some chromosomal markers which can be useful to identify species-specific diagnostic characters, although they may not accurately reflect the phylogenetic relationships among the taxa. In addition, C-banding revealed the presence of a heteromorphic ZW sex chromosome pair, where W is smaller than Z and almost completely heterochromatic. This finding sheds light on sex chromosome evolution in the genus *Iberolacerta* and suggests that further comparative cytogenetic analyses are needed to understand the processes underlying the origin, differentiation and plasticity of sex chromosome systems in lacertid lizards.

The genus *Iberolacerta* is a group of rock lizards (family Lacertidae) mainly distributed in the highland areas of Western Europe. According to recent taxonomic revisions [Mayer and Arribas, 2003; Arribas and Carranza, 2004; Arribas and Odierna, 2004; Carranza et al., 2004; Crochet et al., 2004; Arribas et al., 2006], the genus *Iberolacerta* comprises 8 species, which can be subdivided into 3 main units: (1) *I. horvathi* (Méhely, 1904), occurring in the

KARGER

E-Mail karger@karger.com
www.karger.com/cgr

1424–8581/13/1421–0028$38.00/0

Andrés Martínez-Lage
Departamento de Bioloxía Celular e Molecular
Grupo de Investigación en Bioloxía Evolutiva (GIBE), Universidade da Coruña
A Fraga 10, ES–15008 A Coruña (Spain)
E-Mail andres@udc.es

Eastern Alps and the north of the Dinaric Chains; (2) the subgenus *Pyrenesaura* (Arribas, 1999), which includes the 3 species found in the Pyrenees Mountains, namely *I. aranica* (Arribas, 1993), *I. aurelioi* (Arribas, 1994) and *I. bonnali* (Lantz, 1927); and (3) the 4 species included in the 'Iberian group', i.e. *I. cyreni* (Müller and Hellmich, 1937), *I. martinezricai* (Arribas, 1996), *I. galani* (Arribas, Carranza and Odierna, 2006), and *I. monticola* (Boulenger, 1905), with disjunct distributions in central and northern mountain ranges of the Iberian Peninsula.

The phylogeny of this genus has been under continual revision, but the evolutionary relationships among some taxa still remain unresolved [Mayer and Arribas, 2003; Carranza et al., 2004; Arribas et al., 2006]. Within the Iberian group, data from mitochondrial and nuclear genes suggest that *I. cyreni* split earlier, between 6 and 7.5 mya, while the speciation events within the clade formed by *I. martinezricai*, *I. galani* and *I. monticola* occurred considerably later, at the beginning of the Pleistocene (roughly 2.5 mya). Recent molecular analyses support the hypothesis that *I. monticola* was the first lineage to diverge from the common branch, shortly before the separation of *I. martinezricai* and *I. galani,* approximately 1.8 mya (see www.karger.com/doi/10.1159/000356049 for online suppl. fig. 1) [Remón et al., 2013].

Karyological studies based on conventional staining and banding techniques have proven useful for establishing phylogenetic relationships and delimiting species and subspecies boundaries in the genus *Iberolacerta*, as well as in several other lacertid groups [e.g. Olmo et al., 1993; Odierna et al., 1996; in den Bosch et al., 2003; Kupriyanova and Melashchenko, 2011]. Previous cytogenetic surveys of the *Iberolacerta* species [Capula et al., 1989; Odierna et al., 1996; Arribas and Odierna, 2004; Arribas et al., 2006] showed a common diploid number of 2n = 36 and a similar karyotypic macrostructure, with all chromosomes acrocentric. Only the karyotypes of the 3 *Iberolacerta* species from the Pyrenees differ from this formula, with reduced diploid numbers that range from 2n = 24 to 26 in males and from 23 to 26 in females and numerous biarmed chromosomes, which probably evolved from the ancestral acrocentric complement through a series of Robertsonian fusions (online suppl. fig. 1) [Odierna et al., 1996].

Interestingly, C-banding analyses uncovered high levels of diversity regarding the sex chromosome system. A ZW sex chromosome pair, in which the W chromosome is smaller than the Z and highly heterochromatic, has been described in *I. horvathi*, *I. cyreni* and *I. galani* [Capula et al., 1989; Odierna et al., 1996; Arribas et al., 2006]. In contrast, the sex chromosomes of *I. aranica*, *I. martinezricai* and *I. monticola* are reported to be homomorphic and indistinguishable by differences in size, morphology or heterochromatinization [Odierna et al., 1996; Arribas and Odierna, 2004]. More significant differences are present in the Pyrenean species *I. bonnali* and *I. aurelioi*, with multiple $Z_1Z_1Z_2Z_2/Z_1Z_2W$ sex chromosome systems where the W chromosome is biarmed and the Z_1 and Z_2 counterparts are uniarmed (online suppl. fig. 1) [Odierna et al., 1996]. The presence of ZW-derived multiple sex chromosome systems is a particularly uncommon feature within lizards, so far reported for only 2 other species of lacertids, namely *Zootoca vivipara* and *Podarcis taurica* (Chromorep: A reptile chromosomes database, http://193.206.118.100/professori/chromorep.pdf).

The heterogeneous situation concerning sex chromosomes in the genus *Iberolacerta* is illustrative for the wide diversity of sex chromosomes found in the family Lacertidae. Female heterogamety is considered to be universal within this family. Even so, sex chromosomes at different stages of differentiation are frequently found between closely related species and even between populations of the same species, suggesting that sex chromosomes can have multiple and independent origins in related lacertid taxa [e.g. Olmo et al., 1987; Odierna et al., 1993, 2001; in den Bosch et al., 2003].

Typically, sex chromosomes are thought to evolve after suppression of recombination through increasing stages of differentiation, from a primitive form, in which nascent sex chromosomes differ only in a limited region and are otherwise indistinguishable, to an advanced state, in which sex chromosomes are highly heteromorphic [Charlesworth et al., 2005; recently reviewed in Charlesworth and Mank, 2010]. Reports on lacertid karyotypes, mainly accomplished through conventional banding techniques, suggest that lacertid sex chromosomes have evolved primarily via heterochromatinization followed by degeneration of the female-specific W chromosome, although this is probably not the only mechanism operating in this family [Olmo et al., 1986, 1987; Ezaz et al., 2009]. Chromosomal rearrangements, such as inversions or translocations, can be also involved in the primary differentiation of lizard sex chromosomes [for a review, see Olmo et al., 1987; Ezaz et al., 2009], implying that even newly evolved sex chromosomes can be heteromorphic [Charlesworth and Mank, 2010]. In this regard, comparative cytogenetic analyses within the genus *Iberolacerta* can provide valuable insights into the processes underlying the origin, differentiation and evolutionary transitions of sex chromosomes.

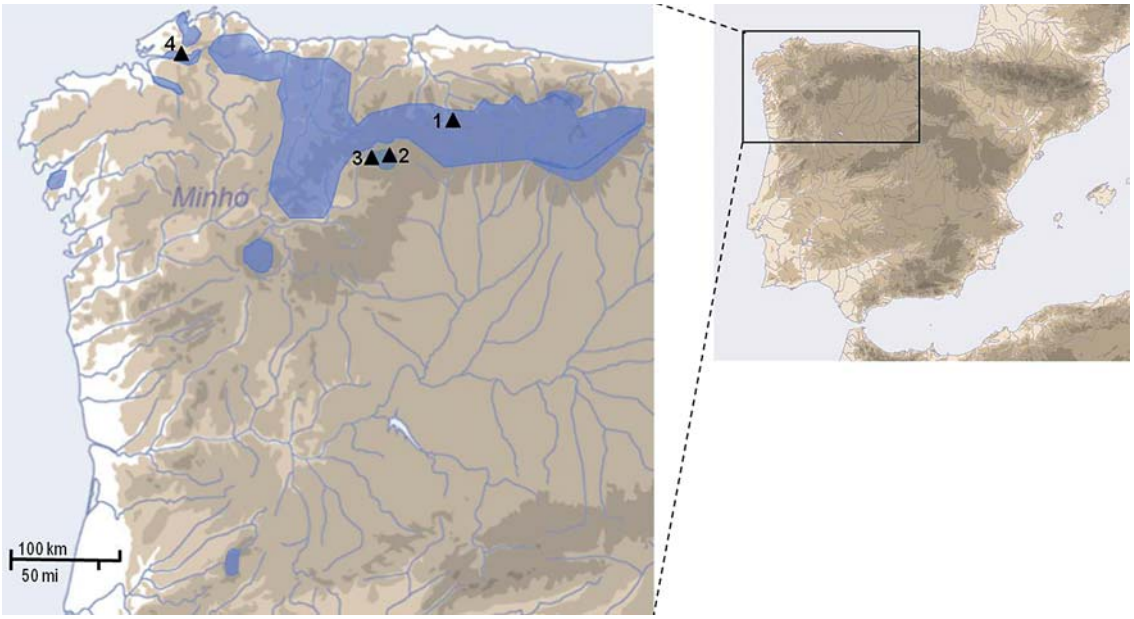


Fig. 1. Map of the Iberian Peninsula showing the current distribution area of *I. monticola* (blue areas). Numbers represent localities sampled in the present study: (1) Puerto de Vegarada, (2) Villabandín, (3) Salientes, and (4) Eume. See text for further details.

In this study, we focus on one of the *Iberolacerta* species, *I. monticola*, for which previous cytogenetic investigations did not detect differentiated sex chromosomes. This species is distributed across a wide area in the north of the Iberian Peninsula, along the Cantabrian Mountain range, where it inhabits mainly rocky habitats at middle-high altitudes [Mayer and Arribas, 2003; Carranza et al., 2004; Crochet et al., 2004]. Apart from this continuous area, there are several other isolated populations in the Serra da Estrela Mountains, in Portugal, and in Galicia, at the north-west corner of Spain (fig. 1). Some populations in this last region are found at areas of exceptionally low altitudes, most of them associated to Atlantic forests in shady fluvial gorges [Galán, 1999; Galán et al., 2007].

The karyotype of *I. monticola* has been previously described based on conventional staining and banding techniques (C-banding and silver-staining) for the populations of Puerto de Vegarada, in the Cantabrian Mountains, and Serra da Estrela [Odierna et al., 1996]. Here, we reinvestigate the specimens from the Cantabrian population (locality 1 in fig. 1) and extend the cytogenetic analysis to 2 additional isolated populations from the Cantabrian area, Villabandín and Salientes (localities 2 and 3 in fig. 1, respectively), as well as to the lowland population of Eume, in the northwesternmost edge of the species' range (locality 4 in fig. 1). The aim of this study was to (1) better characterize the karyotype of *I. monticola* and perform a comparative cytogenetic analysis within a phylogenetic framework, in order to clarify chromosome evolution within the genus *Iberolacerta*; and (2) search for sex-specific differences that enable the identification of cryptic sex chromosomes. This was accomplished by using conventional staining and banding techniques, differential fluorochrome staining and fluorescence in situ hybridization (FISH) with 18S-5.8S-28S rDNA and telomeric $(TTAGGG)_n$ probes.

Material and Methods

Specimens

One adult male and one adult female of *I. monticola* were collected from each of the following localities: (1) Puerto de Vegarada (43.04N, –5.46E), (2) Villabandín (42.90N, –6.14E), (3) Salientes (42.85N, –6.31E), and (4) the fluvial valley of the river Eume (43.41N, –8.07E) (fig. 1). Permissions for fieldwork and ethics approval of experimental procedures were issued by the competent authorities Xunta de Galicia and Junta de Castilla-León, in Spain, in accordance with the Spanish legislation (Royal Decree 1201/2005 and Law 32/2007, on the protection of animals used for experimentation and other scientific purposes).

Phenotypic sex was determined on the basis of external morphology and then confirmed via visual inspection of gonads upon dissection.

Cell Culture and Chromosome Preparations

Metaphase chromosome spreads were prepared according to previously described protocols [Giovannotti et al., 2009a]. Fibroblast cell lines were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Gibco). Cultures were incubated at 30°C in a humidified atmosphere of 5% CO_2 in air. When exponential cell growth was observed around the primary explants (usually after 2–3 weeks of culture), the cells were trypsinized and subcultivated at a 1:2 split ratio. Following this first passage, the cell lines were grown until 70–80% of overall confluence was reached. Six hours prior to harvesting, 0.1 μg/ml colcemid (Roche) was added to the cultures followed by 30 min of hypotonic treatment in 0.075 M KCl at 30°C and fixation in 3:1 methanol:glacial acetic acid. Fifteen microlitres of the cell suspension were dropped onto glass slides and air-dried.

Chromosome Analysis

Conventional chromosome staining was performed using a 5% Giemsa solution at pH 7. C-banding was carried out according to Sumner [1972]. C-banded chromosomes were independently stained with 10% Giemsa solution at pH 7 for 10 min and sequentially with both fluorochromes chromomycin A_3 (CMA_3), and 4′,6-diamidino-2-phenylindole (DAPI) [Schweizer, 1976; Schmid et al., 1983]. Silver-staining of nucleolar organizer regions (Ag-NORs) was performed as described by Howell and Black [1980].

Chromosomal locations of the 18S-5.8S-28S rRNA genes were determined by FISH as described in González-Tizón et al. [2000], with slight modifications, using the DNA probe p*Dm* 238 from *Drosophila melanogaster* [Roiha et al., 1981], labeled by nick translation with digoxigenin-11-dUTP (Roche).

Briefly, the slides were dehydrated by serial ethanol washes [twice for 2 min in 70% (v/v) ethanol, twice for 2 min in 90% ethanol and once for 5 min in 100% ethanol], air dried and aged at 65°C for 30 min. Subsequently, they were incubated in DNase-free RNase (100 μg/ml in 2× SSC) at 37°C for 30 min and washed in 2× SSC for 10 min. One hundred nanograms of labeled probe (2.5 μl) were made up to 30 μl with hybridization buffer (50% formamide, 2× SSC and 10% dextran sulphate), denatured at 75°C for 15 min, chilled on ice, placed onto each slide, covered with a coverslip, and finally sealed with rubber cement. Chromosome denaturation was performed in a slide PCR (MJ Research, MJ 100) as follows: 75°C for 7 min, 55°C for 2 min, 50°C for 30 s, 45°C for 1 min, 42°C for 2 min, 40°C for 5 min, 38°C for 5 min, and 37°C for 5 min. Hybridization took place at 37°C overnight in a humid chamber. Posthybridization washes consisted of two 5-min incubations in 2× SSC at 37°C and at room temperature, respectively, followed by a 5-min incubation in washing solution composed of 0.1 M Tris, 0.15 M NaCl and 0.05% Tween-20 at room temperature. Signal detection included 3 consecutive incubation steps, at 37°C for 30 min each, with: (i) mouse anti-digoxigenin antibody (Roche), (ii) fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) and (iii) FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich). After each incubation step, slides were washed 3 times for 5 min with washing solution at room temperature. Chromosomes were counterstained with 1.5 μg/ml propidium iodide in the anti-fade medium Vectashield (Vector Laboratories).

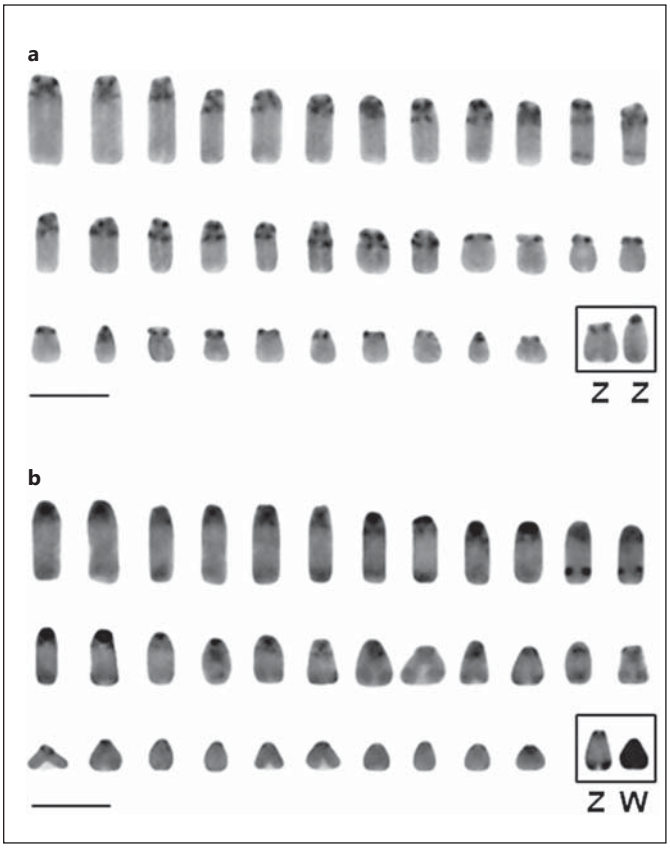


Fig. 2. C-banded karyotypes of male (**a**) and female (**b**) *I. monticola* from the population of Eume. Sex chromosome pairs ZZ and ZW (**inset**). Scale bars = 5 μm.

Chromosome mapping of the $(TTAGGG)_n$ sites was carried out with a Cy3-labeled pan-telomeric DNA probe (Cambio) following the manufacturer's instructions. The slides were mounted using the anti-fade medium Vectashield (Vector Laboratories), containing 1.5 μg/ml DAPI.

Images were captured using an epifluorescence microscope Nikon Microphot-FXA equipped with a Nikon DS-Qi1Mc digital camera and processed with the NIS-Elements D 3.10 software.

Results

Karyotypes, Heterochromatin Distribution and Fluorochrome Staining

All analyzed specimens of *I. monticola* showed a karyotype composed of 2n = 36 acrocentric chromosomes of gradually decreasing size (fig. 2).

C-banding evidenced constitutive heterochromatin at the centromeres of all chromosomes and interstitially at

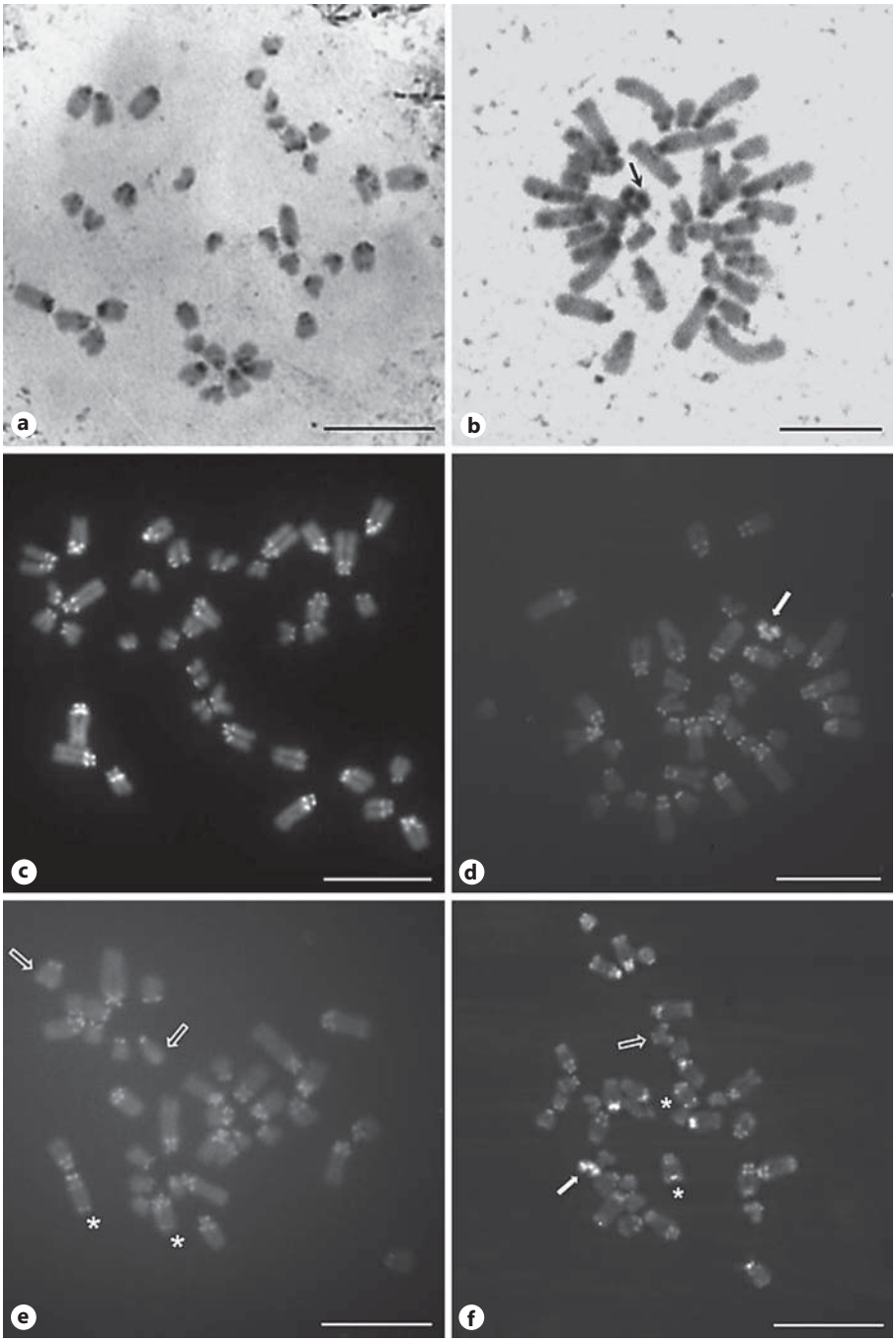


Fig. 3. Metaphase plates of male (**a**, **c**, **e**) and female (**b**, **d**, **f**) *I. monticola* from Eume, C-banded and stained with Giemsa (**a**, **b**), DAPI (**c**, **d**) and CMA_3 (**e**, **f**). Asterisks in **e** and **f** indicate CMA_3-positive signals associated with NORs. Empty and filled arrows point to Z and W sex chromosomes, respectively. Scale bars = 10 μm.

the pericentromeric regions of the 10 larger chromosome pairs (figs. 3, 4). These conspicuous heterochromatic blocks were uniformly stained with both DAPI and CMA_3, and hence, they do not seem to contain particularly AT- or GC-rich repetitive DNA families (figs. 3c–f, 4). Faint C-positive bands were also found at the ends of several chromosome pairs (tentatively, in the 12 larger chromosome pairs) and resulted only positively stained by CMA_3, indicating that this telomeric heterochromatin was composed of GC-rich sequences. In addition, CMA_3 staining produced an intense fluorescent signal in the subterminal region of a large chromosome pair, probably correlated with NOR-associated heterochromatin (figs. 3e, f, 4c, d).

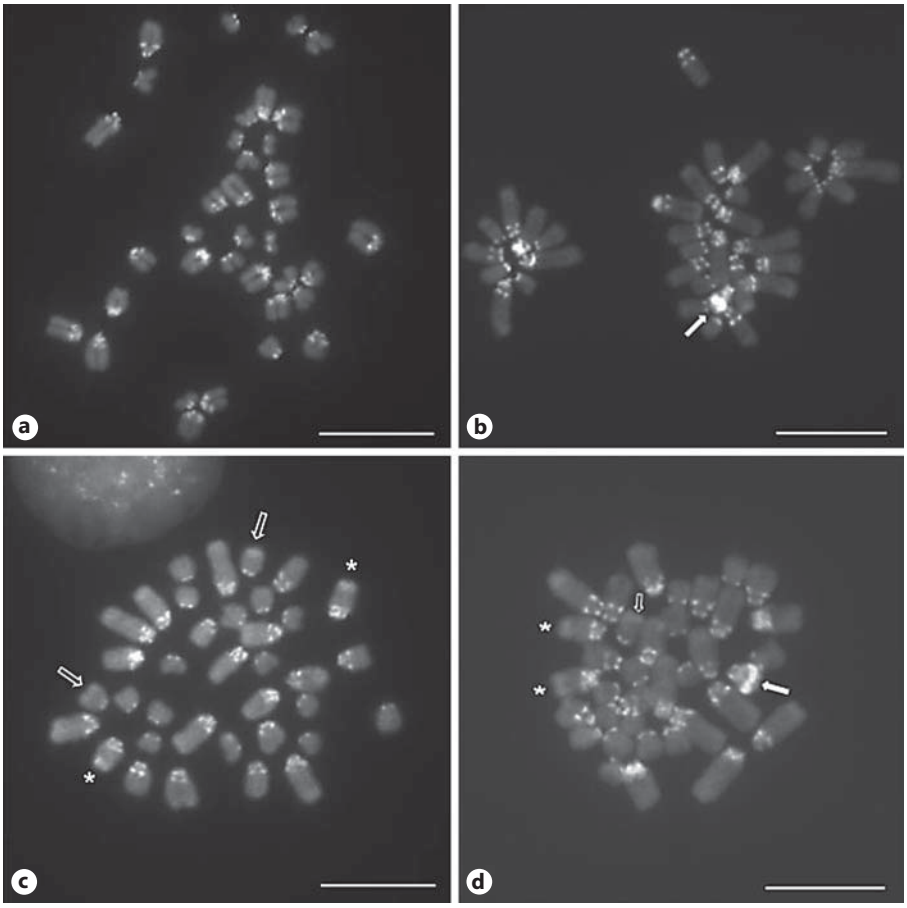


Fig. 4. Metaphase plates of male (**a**, **c**) and female (**b**, **d**) *I. monticola* from Puerto de Vegarada, C-banded and stained with DAPI (**a**, **b**) and CMA_3 (**c**, **d**). Asterisks in **c** and **d** indicate CMA_3-positive signals associated with NORs. Empty and filled arrows point to Z and W sex chromosomes, respectively. Scale bars = 10 μm.

The differences in the pattern of heterochromatin distribution between sexes clearly revealed the presence of a cytologically differentiated ZW sex chromosome pair. The W chromosome was easily recognizable in female metaphases, being one of the smallest chromosomes of the karyotype (fig. 2b) and almost completely heterochromatic, with only a small euchromatic region located in an interstitial position (fig. 3b). The heterochromatin of the W chromosome was intensely stained with both DAPI and CMA_3 (figs. 3d, f, 4b, d). C-banding also allowed the identification of the Z chromosome, present in 2 copies in males and in a single copy in females. This element was as large as the chromosomes of the 9th or 10th pair and differed only slightly from the autosomes in bearing a brighter, CMA_3-positive, telomeric C-band (figs. 2a, 3e, 4c).

Chromosomal Mapping of the 18S-5.8S-28S rRNA Genes

Ag-NOR banding agreed with CMA_3 evidence and showed active NORs on the secondary constriction in the subtelomeric regions of chromosome pair 6 (figs. 2, 5a, b).

Fluorescent hybridization signals of the 18S-5.8S-28S rRNA genes were also coincident with Ag-NOR bands and did not reveal more inactive loci (fig. 5c, d).

Chromosomal Location of the $(TTAGGG)_n$ Sites

FISH with a telomeric probe $(TTAGGG)_n$ produced discrete fluorescent signals at the telomeres of all chromosomes (fig. 5e, f). Additionally, bright hybridization signals were detected at interstitial sites (so-called interstitial telomeric sites, ITSs) in 5 large chromosome pairs in all the metaphase spreads examined. None of these ITSs were located on either the sex chromosomes or the NOR-bearing pairs.

Discussion

Chromosome Number and Karyotypes

In accordance with previously published results [Odierna et al., 1996], the karyotypes obtained from males and females of *I. monticola* showed a diploid chromosome

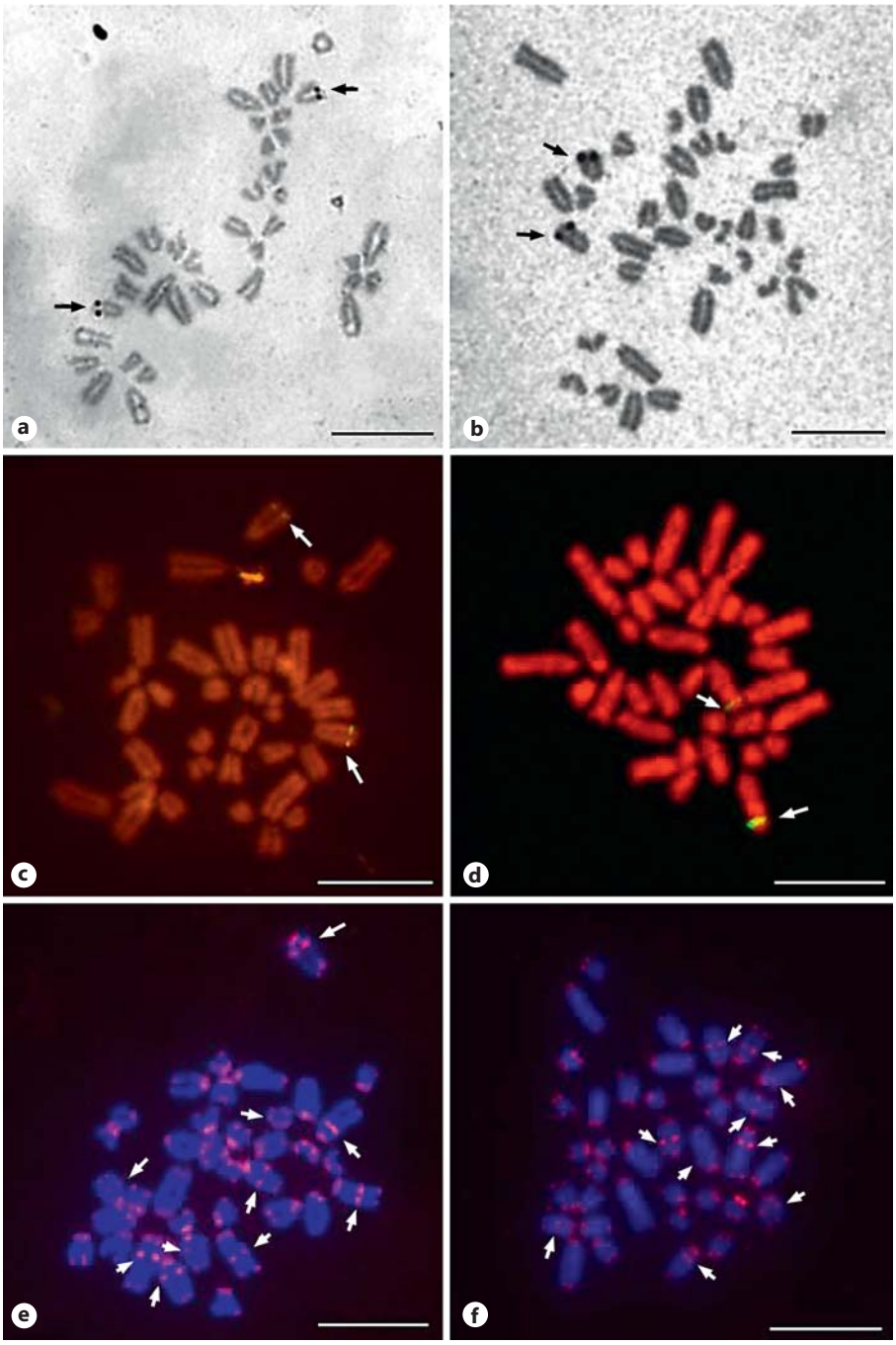


Fig. 5. Chromosomal localization of the 18S-5.8S-28S rRNA genes and $(TTAGGG)_n$ telomeric sequences in male (**a**, **c**, **e**) and female (**b**, **d**, **f**) *I. monticola*. Ag-NOR bands (**a**, **b**) and FISH signals (**c**, **d**) of the 18S-5.8S-28S rRNA genes (arrows). **e**, **f** Hybridization patterns of the telomeric probe $(TTAGGG)_n$. Arrows point to interstitial telomeric sites. Scale bars = 10 μm.

complement of 2n = 36 acrocentric elements, which is common to all the species assigned to the 'Iberian group' of the genus *Iberolacerta*, namely *I. galani*, *I. martinezricai*, *I. cyreni*, and the said *I. monticola.*

In contrast with chromosome morphology, the pattern of heterochromatin distribution is not so conservative between these taxa [Odierna et al., 1996], and each species displays its own heterochromatin profile. In general, all the *Iberolacerta* species – with the only exception of *I. bonnali* – show prominent C-bands at the centromeres of almost all the acrocentric chromosome pairs. The presence of centromeric heterochromatin is a widespread character in lacertids [Olmo et al., 1986, 1993; Odierna et al., 1996], and it has been suggested that it may

play a role in centromere structure and function [e.g. Capriglione et al., 1998].

However, the composition of the highly repetitive DNA sequences that constitute this centromeric heterochromatin is not necessarily conserved between the different *Iberolacerta* species, as indicated by the fact that these DAPI-positive C-bands are also brightly stained by CMA_3 in *I. monticola* and *I. galani* [Arribas et al., 2006], but are CMA_3-negative in *I. martinezricai* [Arribas and Odierna, 2004].

Moreover, the C-banding technique revealed the presence of additional DAPI- and CMA_3-positive heterochromatin in the pericentromeric regions of the 10 larger chromosome pairs. These interstitial heterochromatic regions have not been previously detected by C-banding in any of the *Iberolacerta* species, although they are probably correlated with the pericentromeric bands generated on the 6 larger chromosome pairs of *I. monticola* after the digestion of heterochromatin with the endonuclease *Alu*I [Odierna et al., 1996]. This *Alu*I banding pattern shows the variation in sequence composition between the *Alu*I-sensitive heterochromatin located at the centromeres and the pericentromeric *Alu*I-resistant heterochromatin present at least on 6 chromosome pairs.

Even though satellite DNAs in constitutive heterochromatin are usually composed of AT-rich elements [e.g. King and Cummings, 1997; Plohl et al., 2008], the faint C-bands revealed at the telomeres in the 12 larger chromosome pairs of *I. monticola* were only visible after CMA_3 staining, and therefore, a high GC content can be postulated. GC-rich satellites have been reported for some animal species [Meneveri et al., 1995; Malykh et al., 2001; Barragán et al., 2002; Petrović et al., 2009], and in Squamate reptiles, a telomeric GC-rich satellite has been described for the skink *Eumeces schneideri* [Giovannoti et al., 2009b]. The compartmentalization of GC-rich elements in telomeric heterochromatin could be related to the hypothesized role of short guanine stretches in telomere maintenance and stability [Muniyappa et al., 2000], as well as in promoting chromosome rearrangements through recombination between satellite and telomeric sequences [e.g. Hartmann and Scherthan, 2004].

The presence of telomeric heterochromatin blocks in some chromosome pairs of *I. monticola* and in all chromosomes of *I. galani* [Arribas et al., 2006] constitutes a cytogenetic marker that further discriminates the karyotypes of both species from *I. martinezricai*, where all chromosomes are devoid of telomeric C-bands [Arribas and Odierna, 2004].

On the whole, C-banding data gathered so far in the genus *Iberolacerta* reveal extensive heterogeneity in the amount and distribution of the heterochromatic fraction, even between species so closely related as *I. martinezricai*, *I. monticola* and *I. galani*. However, the karyological affinities unveiled between *I. monticola* and *I. galani* are not consistent with molecular data [Arribas et al., 2006; Remón et al., 2013], which indicate that *I. monticola* is the sister taxon to the clade formed by *I. galani* and *I. martinezricai* (online suppl. fig. 1). In the light of the phylogeny, it seems likely that the C-banding patterns of *I. monticola* and *I. galani* represent the ancestral condition for this lineage; thus, the particular differences in heterochromatin distribution and composition reported for *I. martinezricai* constitute a derived character that, similarly to other cytogenetic traits (e.g. NOR location, see below) or osteological autapomorphies distinctive of this taxon [Arribas and Odierna, 2004], could have become fixed after the species divergence, due to a random genetic drift in small populations. In conclusion, our findings support the idea that, even if C-banding patterns in lacertid lizards can be useful to identify species' diagnostic characters, they may not accurately reflect the phylogenetic relationships among taxa [Olmo et al., 1986].

Ribosomal Loci

As previously reported in *I. monticola* [Odierna et al., 1996], silver-staining documented a single NOR site in a subtelomeric position of chromosome pair 6. Such NOR location at the telomeres of a large chromosome pair (L-type after Olmo et al. [1993]) appears to be ubiquitous among lacertids [Olmo et al., 1993], and it is also the plesiomorphic condition for the genus *Iberolacerta*, where only *I. cyreni* and *I. martinezricai* differ in showing a NOR in an interstitial position on a medium-sized chromosome pair (M-type after Olmo et al. [1993]) [Odierna et al., 1996; Arribas and Odierna, 2004].

FISH with the 28S-5.8S-18S rDNA probe, carried out for the first time in this genus, confirmed the presence of the ribosomal clusters at the sites identified by silver-staining and did not show additional transcriptionally inactive loci. In addition, the bright CMA_3 signal associated with the NOR site highlighted the GC-richness in rDNA base composition, as reported for a wide variety of organisms [e.g. Sumner, 1990 and references therein].

Telomeric Repeats

Hybridization signals of the $(TTAGGG)_n$ probe were located at the telomeres of all chromosomes and at interstitial positions on 5 large chromosome pairs.

ITSs have been observed in many vertebrate species [e.g. Meyne et al., 1990; Lee et al., 1993; Nanda and Schmid, 1994; Garagna et al., 1997; Ventura et al., 2006], including several families of Squamate reptiles [Meyne et al., 1990; Schmid et al., 1994; Pellegrino et al., 1999; Bertolotto et al., 2001; Srikulnath et al., 2009]. They usually consist of large arrays of telomeric-like repeats commonly located in pericentromeric regions, within or at the margins of constitutive heterochromatin.

A large body of evidence indicates that ITSs may be remnants of chromosomal rearrangements that occurred during chromosome evolution [for a review, see Lin and Yan, 2008; Ruiz-Herrera et al., 2008]. Likewise, the ITSs detected in *I. monticola* could be the result of chromosome reorganization events, such as tandem fusions of ancestral acrocentric chromosomes, paracentric inversions involving the telomeric sequences or pericentric inversions in ancestral sub-/metacentric chromosomes. The high intensity of the ITS signals, generally larger than those detected at the telomeric ends, suggests that the retained $(TTAGGG)_n$ sequences have also been amplified. In this regard, it is interesting to point out that karyotype evolution in lacertids is thought to be characterized by a progressive translocation of microchromosomes to macrochromosomes [Olmo et al., 1986; Odierna et al., 1987]. In fact, the basic diploid number of *Iberolacerta* (2n = 36) differs from the common lacertid karyotype in that it lacks a pair of microchromosomes [Olmo et al., 1993]. Moreover, ITSs have been associated with fragile sites and recombination hotspots [recently reviewed in Bolzán, 2012] that may confer greater flexibility for karyotype change by providing potential new sites for telomere formation [Meyne et al., 1990].

However, the presence of ITSs in the karyotype is not always related to structural chromosome changes. Preexisting ITSs, including the short stretches of telomeric hexamers that are presumably inserted during the repair of double strand breaks [Nergadze et al., 2004, 2007], could be subsequently spread and expanded at different intrachromosomal regions by common mechanisms of repetitive DNA amplification, such as unequal crossing-over or sequence conversion [Wiley et al., 1992; Vermeesch et al., 1996; Garagna et al., 1997; Nanda et al., 2008]. For instance, a process of heterochromatin association and unequal exchange has been proposed to explain the dispersion and amplification of ITSs embedded within heterochromatin to new chromosomal locations in lemur and rodent species [Go et al., 2000; Rovatsos et al., 2011].

Therefore, further studies of the occurrence of ITSs and comparative karyological analyses, such as chromosome painting, between lacertids and closely related lizard families are required to elucidate the origin of these nontelomeric sites and clarify their association with karyotype evolution in this lineage.

Sex Chromosomes

Populations of *I. monticola* from the locality of Puerto de Vegarada, in the Cantabrian Mountain range, were first reported to lack differentiated sex chromosomes [Odierna et al., 1996]. In the present study, however, a heteromorphic ZW chromosome pair was consistently identified in the female specimens analyzed from this same population. The discrepancy between those observations and our results could be just due to experimental artifacts. For instance, the higher degree of chromosome condensation in metaphase spreads obtained by scraping techniques from tissues (former work) in comparison with chromosomes obtained from cell cultures (present study) could hamper the detection of the small-sized W chromosome by C-banding.

The presence of a cytologically distinguishable ZZ/ZW system was also confirmed in specimens from 2 other Cantabrian populations, as well as from the population of Eume, at the northwesternmost edge of the species' range. All 4 studied populations are currently isolated, and according to recent molecular analysis [Remón et al., 2013], their independent evolution began roughly between 1.5 and 0.9 mya, possibly as a consequence of climatic fluctuations during the Pleistocene. Even so, the sex chromosome pairs of any of these populations are highly similar in terms of relative size and in the amount and distribution of heterochromatin, albeit they could exhibit some differentiation at finer scales hardly evidenced by C-banding and fluorochrome staining. Therefore, a closer examination with more sensitive cytogenetic methods would be required to investigate the presence of subtle differences in DNA content of sex chromosomes between genetically divergent populations of *I. monticola*.

Likewise, the sex chromosome pair detected in *I. monticola* closely resembles that of other *Iberolacerta* species for which sex chromosomes have been described, i.e. *I. horvathi*, *I. cyreni* and *I. galani* [Capula et al., 1989; Odierna et al., 1996; Arribas et al., 2006]. All of them possess a highly heteromorphic ZW pair, in which the W chromosome is smaller than the Z and completely or almost completely heterochromatic. Nevertheless, greater similarities are found between *I. monticola* and *I. galani*. In particular, the presence of a bright telomeric heterochromatic block in the Z chromosome is a feature that appears to be exclusive of both species. Even if the nature of the

sequences responsible for the heteromorphism in the sex chromosome pair is not known, reverse fluorochrome staining revealed at least certain differences in molecular composition, since heterochromatin in the Z chromosome resulted only positive after CMA_3 staining (similarly to the weak C-bands at the ends of some autosomal pairs), while W chromosome heterochromatin was completely stained with both CMA_3 and DAPI.

In general, the properties of sex chromosomes in *I. monticola* and the remaining *Iberolacerta* species may be concordant with the evolutionary model proposed for other lacertids [Olmo et al., 1987; Odierna et al., 1993]: the initial step of sex chromosome differentiation would be the accumulation of repetitive sequences on either homologue, leading to the formation of 2 heterochromatic areas, a proximal and a distal, as observed in the W chromosome of *I. monticola*. This may subsequently be followed by structural rearrangements, such as deletions of heterochromatic regions not involved in sex determination, originating a heteromorphic sex chromosome pair in which the W is distinctly smaller than the Z. In this context, it would be of interest to verify whether the W chromosome of *I. galani*, reported to be totally imbibed with heterochromatin [Arribas et al., 2006], certainly lacks the intercalary euchromatic region observed in the W chromosome of *I. monticola* and thus represents a more advanced stage of sex chromosome differentiation.

Despite the common features of the ZW pair of these *Iberolacerta* species, it is likely that not all of the sex chromosome systems in this genus followed the same evolutionary pathway: multiple sex chromosome systems ($Z_1Z_1Z_2Z_2$ male and Z_1Z_2W female), with W chromosomes at different degrees of heterochromatinization, have been found in *I. bonnali* and *I. aurelioi* [Odierna et al., 1996]. In addition, homomorphic and cytologically undetectable sex chromosomes are presumably present in *I. aranica* and *I. martinezricai* (online suppl. fig. 1) [Odierna et al., 1996; Arribas and Odierna, 2004]. Moreover, variation in the degree of sex chromosome differentiation is found among species that diverged no more than 2.5 mya *(I. monticola*, *I. galani* and *I. martinezricai)*.

Such interspecific variability in the stage of degeneration of the W chromosomes, with no clear phylogenetic correlation, is representative of the remarkable heterogeneity of sex chromosome systems reported for lacertid lizards (Chromorep: A reptile chromosomes database) [Olmo et al., 1986, 1987; Odierna et al., 1993], which suggests that in this family, as in many reptile lineages, sex chromosomes can have multiple independent origins even in closely related taxa [e.g. Ezaz et al., 2009].

Thus, considering that degradation of W chromosome and dosage compensation would evolve more slowly in ZW taxa, as compared with XY taxa [Naurin et al., 2010], and bearing in mind the advanced state of degeneration of the W chromosome in the basal *Iberolacerta* species, *I. horvathi* [Capula et al., 1989], it seems probable that the presence of a heteromorphic ZZ/ZW pair is the ancestral condition for this genus. Accordingly, it could be hypothesized that the seemingly undifferentiated sex chromosomes in *I. martinezricai* and *I. aranica* might represent neo-sex chromosomes resulting from recent turnover events (e.g. the appearance of a new sex-determining gene on an autosome or the transposition of a sex-determining gene to a new chromosomal location), which would have replaced the preexisting heteromorphic ZW pair. Nonetheless, the putative absence of heteromorphic sex chromosomes in both species should be further investigated in detail.

Future comparative cytogenetic analyses, along with the application of high-resolution molecular cytogenetic techniques, will therefore be necessary to deepen the knowledge about the degree and patterns of sex chromosome differentiation and the transitions between simple ZW and multiple Z_1Z_2W systems in the genus *Iberolacerta*, which ultimately would shed light on the mechanisms underlying sex chromosome evolution and the plasticity of sex determination systems in lacertid lizards.

Acknowledgements

This research was supported by grants REN2003-02931/GLO (Ministerio de Ciencia y Tecnología, Spain), PGIDIT03RFO-10301PR and PGIDIT06RFO10301PR (Xunta de Galicia, Spain), awarded to H.N., and by a grant PR IN 2009 'Ricerche di citogenetica molecolare sui sistemi di determinazione del sesso nei rettili squamati (sottordine Sauria)' provided by the Ministry of Education, University and Research, Italy, to V.C.B. The funding to V.R. was provided by a 'FPU' fellowship from the Ministerio de Educación, Cultura y Deporte, Spain.

References

Arribas OJ, Carranza S: Morphological and genetic evidence of the full species status of *Iberolacerta martinezricai* (Arribas, 1996). Zootaxa 634:1–24 (2004).

Arribas OJ, Odierna G: Karyological and and osteological data supporting the specific status of *Iberolacerta* (*cyreni*) *martinezricai* (Arribas, 1996). Amphibia-Reptilia 25:359–368 (2004).

Arribas OJ, Carranza S, Odierna G: Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240:1–55 (2006).

Barragán MJL, Martínez S, Marchal JA, Bullejos M, Díaz de la Guardia R, Sánchez Bullejos A: Highly repeated DNA sequences in three species of the genus *Pteropus* (Megachiroptera, Mammalia). Heredity 88:366–370 (2002).

Bertolotto CEV, Rodrigues MT, Yonenaga-Yassuda Y: Banding patterns, multiple sex chromosome system and localization of telomeric (TTAGGG)n sequences by FISH on two species of *Polychrus* (Squamata, Polychrotidae). Caryologia 54:217–226 (2001).

Bolzán AD: Chromosomal aberrations involving telomeres and interstitial telomeric sequences. Mutagenesis 27:1–15 (2012).

Capriglione T, De Santo MG, Odierna G, Olmo E: An alphoid-like satellite DNA sequences is present in the genome of a lacertid lizard. J Mol Evol 46:240–244 (1998).

Capula M, Lapini L, Capanna E: The karyotype of *Lacerta horváthi* (Reptilia, Sauria, Lacertidae). Genetica 79:11–16 (1989).

Carranza S, Arnold EN, Amat F: DNA phylogeny of *Lacerta (Iberolacerta)* and other lacertine lizards (Reptilia: Lacertidae): did competition cause long-term mountain restriction? Syst Biodivers 2:57–77 (2004).

Charlesworth D, Mank JE: The birds and the bees and the flowers and the trees: lessons from genetic mapping of sex determination in plants and animals. Genetics 186:9–31 (2010).

Charlesworth D, Charlesworth B, Marais G: Steps in the evolution of heteromorphic sex chromosomes. Heredity 95:118–128 (2005).

Crochet PA, Chaline O, Surget-Groba Y, Debain C, Cheylan M: Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae). Mol Phylogenet Evol 30:860–866 (2004).

Ezaz T, Sarre SD, O'Meally D, Marshall Graves JA, Georges A: Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet Genome Res 127:249–260 (2009).

Galán P: Declive y extinciones puntuales en poblaciones de baja altitud de *Lacerta monticola cantabrica*. Bol Asoc Herpetol Esp 10:47–51 (1999).

Galán P, Vila M, Remón N, Naveira HF: Caracterización de las poblaciones de *Iberolacerta monticola* en el Noroeste ibérico mediante la combinación de datos morfológicos, ecológicos y genéticos. Munibe 25:34–43 (2007).

Garagna S, Ronchetti E, Mascheretti S, Crovella S, Formenti D, et al: Non-telomeric chromosome localization of (TTAGGG)n repeats in the genus *Eulemur*. Chromosome Res 5:487–491 (1997).

Giovannotti M, Caputo V, O'Brien PC, Lovell FL, Trifonov V, et al: Skinks (Reptilia: Scincidae) have highly conserved karyotypes as revealed by chromosome painting. Cytogenet Genome Res 127:224–231 (2009a).

Giovannotti M, Nisi Cerioni P, Caputo V, Olmo E: Characterisation of a GC-rich telomeric satelliteDNA in *Eumeces schneideri* Daudin (Reptilia, Scincidae). Cytogenet Genome Res 125:272–278 (2009b).

Go Y, Rakotoarisoa G, Kawamoto Y, Randrianjafy A, Koyama N, Hirai H: PRINS analysis of the telomeric sequence in seven lemurs. Chromosome Res 8:57–65 (2000).

González-Tizón A, Martínez-Lage A, Rego I, Ausió J, Méndez J: DNA content, karyotype, and chromosomal location of 18S-5.8S-28S ribosomal loci in some species of bivalve molluscs from the Pacific Canadian coast. Genome 43:1065–1072 (2000).

Hartmann N, Scherthan H: Characterization of ancestral chromosome fusion points in the Indian muntjac deer. Chromosoma 112:213–220 (2004).

Howell WM, Black DA: Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 3:1014–1015 (1980).

in den Bosch HAJ, Odierna G, Aprea G, Barucca M, Canapa A, et al: Karyological and genetic variation in Middle Eastern lacertid lizards *Lacerta laevis* and *Lacerta kulzeri* complex: a case of chromosomal allopatric speciation. Chromosome Res 11:165–178 (2003).

King LM, Cummings MP: Satellite DNA repeat sequence variation is low in three species of burying beetles in the genus *Nicrophorus* (Coleoptera: Silphidae). Mol Biol Evol 14:1088–1095 (1997).

Kupriyanova LA, Melashchenko O: The common Eurasian lizard *Zootoca vivipara* (Jacquin, 1787) from Russia: sex chromosomes, subspeciation, and colonization. Russ J Herpetol 18:99–104 (2011).

Lee C, Sasi R, Lin CC: Interstitial localization of telomeric DNA sequences in the Indian muntjac chromosomes: further evidence for tandem chromosome fusions in the karyotypic evolution of the Asian muntjacs. Cytogenet Cell Genet 63:156–159 (1993).

Lin KW, Yan J: Endings in the middle: current knowledge of interstitial telomeric sequences. Mutat Res 658:95–110 (2008).

Malykh AG, Zhurov VG, Saifitdinova AF, Deryusheva SE, Gaginskaya ER: Structural and functional characterization of a centromeric highly repetitive DNA sequence of *Fringilla coelebs* L. (Aves: Passeriformes). Mol Biol 35:331–335 (2001).

Mayer W, Arribas O: Phylogenetic relationships of the European lacertid genera *Archaeolacerta* and *Iberolacerta* and their relationships to some other 'Archaeolacertae' (sensu lato) from Near East, derived from mitochondrial DNA sequences. J Zool Syst Evol Res 41:157–161 (2003).

Meneveri R, Agresti A, Rocchi M, Marozzi A, Ginelli E: Analysis of GC-rich repetitive nucleotide sequences in great apes. J Mol Evol 40:405–412 (1995).

Meyne J, Baker RJ, Hobart HH, Hsu TC, Ryder OA, et al: Distribution of non-telomeric sites of the (TTAGGG)n telomeric sequence in vertebrate chromosomes. Chromosoma 99:3–10 (1990).

Muniyappa K, Anuradha S, Byers B: Yeast meiosis-specific protein Hop1 binds to G4 DNA and promotes its formation. Mol Cell Biol 20:1361–1369 (2000).

Nanda I, Schmid M: Localization of the telomeric (TTAGGG)n sequence in chicken *(Gallus domesticus)* chromosomes. Cytogenet Cell Genet 65:190–193 (1994).

Nanda I, Fugate M, Steinlein C, Schmid M: Distribution of (TTAGGG)n telomeric sequences in karyotypes of the *Xenopus* species complex. Cytogenet Genome Res 122:396–400 (2008).

Naurin S, Hansson B, Bensch S, Hasselquist D: Why does dosage compensation differ between XY and ZW taxa? Trends Genet 26:15–20 (2010).

Nergadze SG, Rocchi M, Azzalin CM, Mondello C, Giulotto E: Insertion of telomeric repeats at intrachromosomal break sites during primate evolution. Genome Res 14:1704–1710 (2004).

Nergadze SG, Santagostino MA, Salzano A, Mondello C, Giulotto E: Contribution of telomerase RNA retrotranscription to DNA double-strand break repair during mammalian genome evolution. Genome Biol 8:R260 (2007).

Odierna G, Olmo E, Cobror O: Taxonomic implications of NOR-localisation in lacertid lizards. Amphibia-Reptilia 8:373–381 (1987).

Odierna G, Kupriyanova LA, Capriglione T, Olmo E: Further data on sex chromosomes of Lacertidae and a hypothesis on their evolutionary trend. Amphibia-Reptilia 14:1–11 (1993).

Odierna G, Aprea G, Arribas OJ, Capriglione T, Caputo V, Olmo E: The karyology of the Iberian rock lizards. Herpetologica 52:542–550 (1996).

Odierna G, Heulin B, Guillaume CP, Vogrin N, Aprea G, et al: Further analysis of the karyological variations existing within and between oviparous and viviparous forms of *Lacerta (Zootoca) vivipara:* evolutionary and biogeographic implications. Ecography 24:332–340 (2001).

Olmo E, Odierna G, Cobror O: C-band variability and phylogeny of Lacertidae. Genetica 71:63–74 (1986).

Olmo E, Odierna G, Capriglione T: Evolution of sex-chromosomes in lacertid lizards. Chromosoma 96:33–38 (1987).

Olmo E, Odierna G, Capriglione T: The karyology of Mediterranean lacertid lizards, in Valakos ED, Boehme W, Pérez-Mellado V, Maragou P (eds): Lacertids of the Mediterranean Region: A Biological Approach, pp 61–84 (Hellenic Zoological Society, Athens 1993).

Pellegrino KCM, Rodrigues MT, Yonenaga-Yassuda Y: Chromosomal evolution in the Brazilian lizards of genus *Leposoma* (Squamata, Gymnophthalmidae) from Amazon and Atlantic rain forests: banding patterns and FISH of telomeric sequences. Hereditas 131:15–21 (1999).

Petrović V, Pérez-García C, Pasantes JJ, Šatović E, Prats E, Plohl M: A GC-rich satellite DNA and karyology of the bivalve mollusc *Donax trunculus*: a dominance of GC-rich heterochromatin. Cytogenet Genome Res 124:63–71 (2009).

Plohl M, Luchetti A, Meštrović N, Mantovani B: Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. Gene 409:72–82 (2008).

Remón N, Galán P, Vila M, Arribas O, Naveira H: Causes and evolutionary consequences of population subdivision of an Iberian mountain lizard, *Iberolacerta monticola*. PLoS One 8:e66034 (2013).

Roiha H, Miller JR, Woods LC, Glover DM: Arrangements and rearrangements of sequences flanking the two types of rDNA insertion in *D. melanogaster*. Nature 290:749–753 (1981).

Rovatsos MT, Marchal JA, Romero-Fernández I, Fernández FJ, Giagia-Athanosopoulou EB, Sánchez A: Rapid, independent, and extensive amplification of telomeric repeats in pericentromeric regions in karyotypes of arvicoline rodents. Chromosome Res 19:869–882 (2011).

Ruiz-Herrera A, Nergadze SG, Santagostino M, Giulotto E: Telomeric repeats far from the ends: mechanisms of origin and role in evolution. Cytogenet Genome Res 122:219–228 (2008).

Schmid M, Haaf T, Geile B, Sims S: Chromosome banding in Amphibia. VIII. An unusual XY/XX sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). Chromosoma 88:69–82 (1983).

Schmid M, Feichtinger W, Nanda I, Schakowski R, Visbal Garcia R, et al: An extraordinarily low diploid chromosome number in the reptile *Gonatodes taniae* (Squamata, Gekkonidae). J Hered 85:255–260 (1994).

Schweizer D: Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58:307–324 (1976).

Srikulnath K, Matsubara K, Uno Y, Thongpan A, Suputtitada S, et al: Karyological characterization of the butterfly lizard (*Leiolepis reevesii rubritaeniata*, Agamidae, Squamata) by molecular cytogenetic approach. Cytogenet Genome Res 125:213–223 (2009).

Sumner AT: A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306 (1972).

Sumner AT: Chromosome Banding (Unwin Hyman, London 1990).

Ventura K, Silva MJJ, Fagundes V, Christoff AU, Yonenaga-Yassuda Y: Non-telomeric sites as evidence of chromosomal rearrangement and repetitive (TTAGGG)n arrays in heterochromatic and euchromatic regions in four species of *Akodon* (Rodentia, Muridae). Cytogenet Genome Res 115:169–175 (2006).

Vermeesch JR, De Meurichy W, Van Den Berghe H, Marynen P, Petit P: Differences in the distribution and nature of the interstitial telomeric (TTAGGG)n sequences in the chromosomes of the Giraffidae, okapai *(Okapia johnstoni)*, and giraffe *(Giraffa camelopardalis)*: evidence for ancestral telomeres at the okapi polymorphic rob(5;26) fusion site. Cytogenet Cell Genet 72:310–315 (1996).

Wiley JE, Meyne J, Little ML, Stout JC: Interstitial hybridization sites of the (TTAGGG)n telomeric sequence on the chromosomes of some North American hylid frogs. Cytogenet Cell Genet 61:55–57 (1992).

ANNEX II

RESEARCH ARTICLE

Isolation and Characterization of Two Satellite DNAs in Some Iberian Rock Lizards (Squamata, Lacertidae)

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART B
MOLECULAR AND DEVELOPMENTAL EVOLUTION

MASSIMO GIOVANNOTTI[1], VERÓNICA ROJO[2], PAOLA NISI CERIONI[1], ANA GONZÁLEZ-TIZÓN[2], ANDRÉS MARTÍNEZ-LAGE[2], ANDREA SPLENDIANI[1], HORACIO NAVEIRA[2], PAOLO RUGGERI[1], ÓSCAR ARRIBAS[3], ETTORE OLMO[1], AND VINCENZO CAPUTO BARUCCHI[1,4]*

[1]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy
[2]Departamento de Biología Celular y Molecular, Universidade da Coruña, La Coruña, Spain
[3]Avda. Francisco Cambó 23, Barcelona, Spain
[4]Consiglio Nazionale delle Ricerche, Istituto di Scienze Marine Sezione Pesca Marittima, Ancona, Italy

ABSTRACT

Satellite DNAs represent a large portion of all high eukaryotic genomes. They consist of numerous very similar repeated sequences, tandemly arranged in large clusters up to 100 million base pairs in length, usually located in the heterochromatic parts of chromosomes. The biological significance of satDNAs is still under discussion, but most of their proposed functions are related to heterochromatin and/or centromere formation and function. Because information about the structure of reptilian satDNA is far from exhaustive, we present a molecular and cytogenetic characterization of two satDNA families in four lacertid species. Two families of tandemly repeated DNAs, namely *Taq*I and *Hin*dIII satDNAs, have been cloned and sequenced from four species belonging to the genus *Iberolacerta*. These satDNAs are characterized by a monomer length of 171–188 and 170–172 bp, and by an AT content of 60.5% and 58.1%, respectively. FISH experiments with *Taq*I satDNA probe produced bright signals in pericentromeric regions of a subset of chromosomes whereas all the centromeres were marked by *Hin*dIII probe. The results obtained in this study suggest that chromosome location and abundance of satDNAs influence the evolution of these elements, with centromeric families evolving tenfold faster than interstitial/pericentromeric ones. Such different rates render different satellites useful for phylogenetic investigation at different taxonomic ranks. *J. Exp. Zool. (Mol. Dev. Evol.) 322B:13–26, 2014.* © 2013 Wiley Periodicals, Inc.

How to cite this article: Giovannotti M, Rojo V, Nisi Cerioni P, González-Tizón A, Martínez-Lage A, Splendiani A, Naveira H, Ruggeri P, Arribas Ó, Olmo E, Caputo Barucchi V. 2014. Isolation and characterization of two satellite DNAs in some Iberian rock lizards (Squamata, Lacertidae). J. Exp. Zool. (Mol. Dev. Evol.) 322B:13–26.

J. Exp. Zool. (Mol. Dev. Evol.) 322B:13–26, 2014

Satellite DNAs (satDNAs) form a substantial part of eukaryotic genomes and consist of tandemly repeated DNA sequences typically arranged in large clusters of hundreds or thousands of copies usually located in the heterochromatic regions of chromosomes, mainly in the regions close to the centromeres and telomeres. The biological significance of satDNAs remains intriguing and challenging. The sequence conservation of some satellites over long evolutionary times, the presence of differentially expressed transcripts in several species and interactions with centromeric-specific proteins (e.g., the histone H3 variant CENH3) suggest a biological role for some satellites, although this is not fully understood (see Plohl et al., 2008; Plohl, 2010).

A satDNA family could arise in a phylogenetically short period by explosive amplification (Bachmann and Sperlich, '93) and afterwards its repeats could follow a gradual mode of sequence evolution during a long evolutionary time (Bachmann and Sperlich, '93). The processes by which satDNA families arise are not well known. A set of molecular-exchange mechanisms has been proposed to account for its origin by amplification of a tandem array of multi-copy sequences. These mechanisms include unequal crossing-over (Smith, '76), transposition (Miller et al., 2000), or extrachromosomal rolling-circle replication and reintegration of tandem arrays into the genome (Feliciello et al., 2006). A recently originated tandem array is initially homogeneous in sequence because of the multi-copy amplification of the same repeat. In the course of time, random mutations would accumulate and the repeats would diverge. However, the nonallelic repeats of a satDNA family do not evolve independently, but concertedly leading to near homogeneity for species-specific mutations (Bachmann and Sperlich, '93; Rudd et al., 2006). This phenomenon, known as concerted evolution, is achieved by a number of genomic mechanisms, mainly unequal crossing-over, biased gene conversion, slippage replication, and amplification by rolling-circle (Dover, '82; Walsh, '87; Charlesworth et al., '94). However, the rates of sequence change (homogenization and fixation) vary for each satDNA family or even for the same satDNA family within different lineages. Levels of sequence variation among repeats would depend on factors such as mutation rate, inter- and intrachromosomal recombination rates, copy number, array size and structure, chromosomal distribution, chromosomal structure, population size, divergence time, and reproductive mode; it is also subject to random genetic drift and possibly natural selection (Strachan et al., '85; Stephan and Cho, '94; Luchetti et al., 2003; Navajas-Pérez et al., 2005; Dawe and Henikoff, 2006; Kuhn et al., 2007). The relative importance of each factor remains controversial.

In this context, very little information exists on satDNA array size, composition and long-range organization, especially in reptiles (see Giovannotti et al., 2009). An exception is represented by Lacertidae, a species rich family of squamate reptiles, widespread in the Palaearctic region (Sindaco and Jeremcenko, 2008). This family comprises the subfamilies Gallotiinae and Lacertinae, with the latter comprising two monophyletic tribes, the Eremiadini of Africa and arid southwest and central Asia, and the Lacertini of Europe (Arnold et al., 2007). So far, five satDNA families have been described for the genome of the Lacertinae subfamily: the pLCS (190 bp in length) is shared by the genera *Algyroides*, *Teira*, *Lacerta*, and *Podarcis* (Capriglione et al., '89, '91; Capriglione, 2000); the pLHS (140 bp) is specific for *Podarcis* only (Capriglione et al., '94; Capriglione, 2000); the pGPS (185 bp) is present in the genome of *Podarcis* and in species belonging to the genera *Archaeolacerta*, *Algyroides*, *Lacerta*, and *Zootoca* (Capriglione et al., '98), so that its appearance would precede the divergence within the Lacertinae subfamily; the CLsat family is described for the Caucasian genus *Darevskia* (145–147 bp, Ciobanu et al., 2003; Grechko et al., 2006); the Agi160 is restricted to the genus *Lacerta* (138–184 bp, Ciobanu et al., 2004; Grechko et al., 2005). These satDNA families revealed several common features, such as the same range of monomer lengths (140–190 bp), AT content (tendency toward AT enrichment 50–65%) and homopolymeric (A_{3-4} and T_{3-4}) stretches (Capriglione et al., '91; Ciobanu et al., 2001, 2004). All these features were also found in other nonreptilian satDNAs (see King and Cummings, '97).

The genus *Iberolacerta* (see Arribas, '99) has a disjunct range in mountain areas of western Europe: a portion comprises central Portugal, central and northern Spain and Pyrenees; another part embraces western Alps and northern Dinaric chain. Until recently the rock-lizard populations endemic to the Iberian Peninsula were considered to represent a single species, *Lacerta monticola* Boulenger, 1905 (see Salvador, '85), that has recently been split into the following taxa: *Iberolacerta aranica*, *I. aurelioi*, and *I. bonnali* restricted to the Pyrenees and *I. cyreni*, *I. galani*, *I. martinezricai*, and *I. monticola*, in the central-western parts of Iberian Peninsula (see Arribas et al., 2006). An additional species is represented by the east-Alpine and Dynaric species *I. horvathi*. This classification was based on (i) morphological (biometry, scalation), ostelogical, and karyological data; (ii) on the use of

Grant sponsor: Ministry of Education, University and Research (Italy) ("Ricerche di citogenetica molecolare sui sistemi di determinazione del sesso nei rettili squamati, sottordine Sauria"); grant number: PRIN2009/20093HYH97; grant sponsor: Ministerio de Ciencia y Tecnología, Spain; grant number: REN2003-02931/GLO; grant sponsor: Xunta de Galicia, Spain; grant numbers: PGIDIT03RFO10301PR, PGIDIT06RFO10301PR; grant sponsor: "FPU" fellowship; grant number: AP2008-02686; grant sponsor: Ministerio de Educación (Spain) (Beca de Formación de Profesorado Universitario).

Conflicts of interest: None.

*Correspondence to: Vincenzo Caputo Barucchi, Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy. E-mail: v.caputo@univpm.it

Received 8 April 2013; Revised 29 July 2013; Accepted 31 July 2013

DOI: 10.1002/jez.b.22530

Published online 6 September 2013 in Wiley Online Library (wileyonlinelibrary.com).

molecular tools, namely nuclear (c-mos) and mitochondrial DNA (12S and cytochrome *b*), and (iii) on the construction of phylogenetic trees ranking the different allopatric populations based on the degree of genetic divergence, with *I. horvathi* as the most basal species (for a revision see Arribas et al., 2006). Another conceptual framework influencing the species subdivision of these largely allopatric lizards is the phylogenetic species concept, according to which species are segments of a phylogenetic lineage beyond nodes, irrespective of the degree of reproductive isolation (for a criticism see Mace, 2004). Considering the well-known usefulness of satDNAs in facing phylogenetic issues (i.e., Martinsen et al., 2009), the aim of the present paper was to isolate and characterise satDNA in some lacertid species in order to (i) increase the knowledge of this genomic elements in an important amniote group for which data on occurrence, genomic distribution, and evolutionary rates are limited to a handful of species; (ii) use the satDNAs isolated to verify the robustness of the proposed phylogenetic reconstruction for some *Iberolacerta* taxa on the light of independent molecular markers.

MATERIALS AND METHODS

Samples

Two males and two females of *Iberolacerta monticola* (from Fragas do Eume, A Capela, Galicia, Spain) and two males and two females of *I. galani* (from A Ponte, Pena Trevinca, A Veiga, Galicia, Spain) were used to make metaphase chromosomes and to extract genomic DNA. In addition, genomic DNA was extracted from seven ethanol preserved specimens of *I. cyreni* from three different Iberian locations (Navacerrada, Sierra de Guadarrama, Segovia-Madrid, Spain; Pico Zapatero, Sierra de la Paramera, Ávila; Puerto de Peña Negra, Sierra de Villafranca, Ávila, Spain) and one of *I. martinezricai* (Puerto El Portillo, Salamanca, Spain). Permissions for field work and experimental procedures were issued by the competent Spanish authorities: Xunta de Galicia (for *I. monticola* and *I. galani*) (permission number 79/2008) and Junta de Castilla y León (for *I. cyreni* and *I. martinezricai*) (permission numbers: 20051630007003/2005, 20061630024599/2006, 2007167004130/2007, 20081630020386/2008, 20092390004760/2009). Finally, genomic DNA of *Lacerta bilineata*, *Podarcis muralis*, *P. siculus*, and *Timon lepidus*, was extracted from ethanol preserved tissues of voucher specimens belonging to one of the authors (Vincenzo Caputo Barucchi).

Isolation and Characterization of Satellite DNAs

Genomic DNA was extracted from whole blood, using standard protocols with proteinase K digestion followed by phenol/chloroform extraction (see Sambrook et al., '89). Fifteen restriction endonucleases (*Alu*I, *Apa*I, *Ava*II, *Bam*HI, *Bcn*I, *Bgl*I, *Bgl*II, *Dra*I, *Eco*RV, *Hin*dIII, *Msp*I, *Rsa*I, *Sma*I, *Taq*I, *Xba*I) (Fermentas International, Inc., Burlington, ON, USA) were screened and about 8 μg of *I. monticola* and *I. galani* purified genomic DNA were utilized for each digestion. Electrophoresis on 2% agarose gel of the digested DNA revealed a band of about 170 bp for *Hin*dIII and 190 bp for *Taq*I, corresponding to the monomeric unit of repetitive DNA (Fig. 1A), whereas no clear bands were produced by the remaining 13 endonucleases. The 170 and 190 bp fragments were excised from agarose gel, purified with Pure Link Quick Gel Extraction Kit (Invitrogen, Carlsabad, CA, USA) and cloned in the pCR®-blunt vector with Zero Blunt PCR Cloning Kit (Invitrogen) following the manufacturer's recommendations. Ten clones of each *I. monticola* satellite DNAs (*Hin*dIII and *Taq*I satDNAs henceforth) and 13 (*Hin*dIII) and 16 (*Taq*I) of *I. galani* satDNAs were sequenced on an ABI PRISM 3730XL (Applied Biosystems, Foster City, CA, USA) automatic sequencer.

Digoxigenin-labeled probes were produced by PCR amplification of single clones and used in Southern hybridization experiments to verify that the elements isolated were tandemly arranged, as expected for satDNAs. In these experiments, *Hin*dIII and *Taq*I digested genomic DNAs from *I. monticola* and other lizards (*I. cyreni*, *I. galani*, *I. martinezricai*, *Lacerta bilineata*, *Podarcis muralis*, *P. siculus*, *Timon lepidus*) were used in order to assess the presence of these repetitive elements in other genera of this family. The hybridization with the digoxigenin-labeled satDNA probes was performed at 50°C overnight with the Sure Blot CHEMI Hybridization and Detection Kit (EMD Millipore Co., Billerica, MA, USA) following the manufacturer's recommendations. The hybridization was detected with the same kit.

The genomic abundance of satDNAs was estimated by quantitative dot blot analysis. Dilutions of genomic DNA and clones containing *Hin*dIII and *Taq*I satDNAs used as a standard were blotted onto a nylon membrane with BIO-DOT® microfiltration apparatus (Bio-Rad Laboratories, Hercules, CA, USA), following manufacturer's recommendations. In order to avoid errors due to the differences in the hybridization kinetics, sonicated salmon sperm DNA was used as a carrier and added to each sample up to a final amount of 0.5 μg DNA/sample (see Cafasso et al., 2003). Hybridization was performed overnight at 45°C. The same clones as those used as a standard were employed to produce digoxigenin-labeled probes. The detection protocol was carried out with the same protocol as the one used for Southern hybridization.

From the sequences of the monomeres of *I. monticola* and *I. galani*, *Hin*dIII and *Taq*I satDNAs two pairs of primers (*Hin*dIII-F: 5′-TGAGTGTTTTACAGTTGAAAAGCT-3′; *Hin*dIII-R: 5′-CATTGTGTTATTTGAGCGCAA-3′; *Taq*I-F: 5′-ATTCTGACCCTGGGGGTTAG-3′; *Taq*I-R: 5′-CATATTTAAAGAAATCAGGCCTCG-3′) were designed and used for isolation of these satellites from the genomes of the other two *Iberolacerta* species. PCR products from the amplification of *Iberolacerta* genomic DNAs with above primers were run on 2% agarose gel, the band corresponding to the amplified monomers excised from the gel, purified with Pure Link Quick Gel Extraction Kit (Invitrogen) and cloned in the pCR®-blunt vector with Zero Blunt PCR Cloning Kit (Invitrogen)

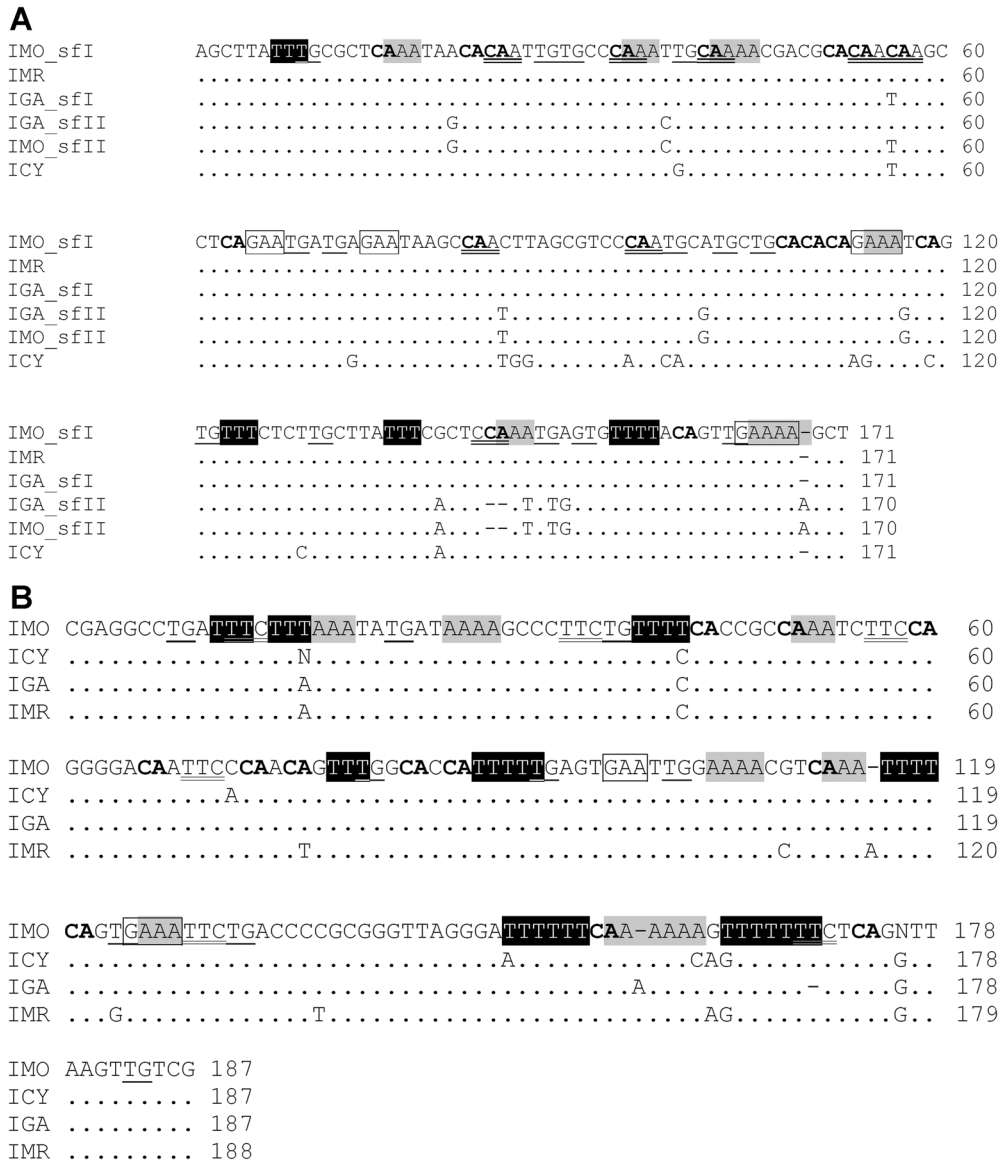


Figure 1. Comparisons of consensus sequences of *Hind*III (a) and *Taq*I (b) between the four *Iberolacerta* species analysed. Repeated motifs are highlighted. sfI: HindIII satDNA subfamily I; sfII: HindIII satDNA subfamily II. Dots refer to nucleotide identity and dashes indicate indels.

following manufacturer's recommendations. Clones of *Hind*III and *Taq*I satDNAs were sequenced on an ABI PRISM 3730XL (Applied Biosystems) automatic sequencer. These sequences were then aligned in CLUSTAL W (Larkin et al., 2007), using default parameters. The visual inspection of sequence alignments was carried out to check for the presence of shared nucleotide changes, which could serve as diagnostic positions to define subsets (subfamilies) within each satDNA family. A GenBank search was performed in order to compare *Hind*III and *Taq*I satDNAs with other satDNAs in the database.

Maximum parsimony (MP), neighbor joining (NJ), maximum likelihood (ML), and Bayesian analyses (BA) were used to infer the phylogenetic relationships among sequences of each satDNA. MP consensus trees (50% majority rule) were constructed with PAUP* version 4.0b10 (Swofford, 2002) using the heuristic search method with 1,000 random-addition-sequence replicates, tree-bisection-reconnection (TBR) branch swapping and holding 100 trees at each cycle of the stepwise-addition procedure. To increase the number of informative characters, gaps were coded as binary (presence/absence) characters.

NJ analyses were performed in MEGA version 5 (Tamura et al., 2011). The NJ trees were based on distances obtained by the maximum composite likelihood method, with pairwise deletion and 1,000 bootstrap replicates. ML analyses were conducted in MetaPIGA v.2.1.3 (http://www.metapiga.org) (Helaers and Milinkovitch, 2010) using the metapopulation genetic algorithm (metaGA) with probability consensus pruning among four populations of four individuals each. The best-fitting nucleotide substitution models [Jukes–Cantor (JC) for *Hin*dIII satDNA and Hasegawa–Kishino–Yano plus Gamma (HKY + G) for *Taq*I satDNA] were selected based on the Likelihood Ratio Test implemented in this software. Branch support values that approximate the posterior probability distribution of the corresponding branches were estimated by performing a minimum of 100 replicated metaGA searches that were stopped when the mean relative error (MRE) among 10 consecutive consensus trees remained below 5%. BA were carried out using the software MrBayes v.3.2.1 (Ronquist and Huelsenbeck, 2003). As in the MP analyses, gaps were coded as binary characters and included as a separate data partition in the matrix. A binary model (lset coding = variable) was applied to the coded gaps, whereas the previously selected models of sequence evolution, JC and HKY + G, were applied to the DNA partitions of *Hin*dIII and *Taq*I satDNAs, respectively. The analyses included two separate concurrent Monte Carlo Markov Chain (MCMC) runs, each composed of four chains (one cold, three heated). Each Markov chain was started from a random tree and run for up to 10^6 generations, sampling every 500 generations. Stationarity was assessed using the software Tracer v.1.5 (Rambaut and Drummond, 2009). Samples obtained during the first 25% generations were discarded as burn-in, and the remaining data were used to generate a majority-rule consensus tree where the percentage of samples recovering any particular clade of the consensus tree represented the clade's posterior probability.

Intraspecific nucleotide diversity (π) was estimated using DnaSP v. 5 (Librado and Rozas, 2009). Net average genetic distances between groups were calculated under the appropriate substitution model for each satDNA family (see above) with MEGA v. 5. Rates of *Hin*dIII and *Taq*I satDNAs evolution were determined according to the divergence times estimated for the four *Iberolacerta* species here investigated by Arribas et al. (2006).

The occurrence of genetic differentiation between the four species analyzed was assessed with the analysis of molecular variance (AMOVA) (Excoffier et al., '92) calculating Φ-statistics. This test was performed at two hierarchical levels to test how satDNAs sequence variability was distributed within species and among species, for both *Hin*dIII and *Taq*I satDNAs. The test was based on pair wise genetic distances between clones and performed as implemented in ARLEQUIN 2.000 (Schneider et al., 2000), using 1,000 permutations.

The repeats of the analyzed species were compared using satDNA Analyzer version 1.2 (Navajas-Pérez et al., 2007). This program allows the discrimination between shared and nonshared polymorphic sites. The program identifies polymorphic sites shared between two species when the same polymorphism is found in both species. When this occurs, we assume that these are ancestral sites that appeared before the split between the two species (Navajas-Pérez et al., 2005). By contrast, nonshared polymorphic sites are autapomorphies, representing different transitional stages in the process of intraspecific sequence homogenization and interspecific divergence. Under the assumption that concerted evolution is a time dependent process, the expected stages of transition during the spread of a variant repeat unit toward its fixation can be defined according to the model of Strachan et al. ('85). This is a method of partitioning the variation by analyzing the patterns of variation at each nucleotide site considered independently among all the repeats of a repetitive family when comparing a pair of species (Strachan et al., '85; Navajas-Pérez et al., 2007). This method examines the distribution of nucleotide sites among six stages (Classes I–VI) in the spread of variant repeats through the family and the species. Briefly, the Class I site represents complete homogeneity across all repeat units sampled from a pair of species, whereas Classes II, III, and IV represent intermediate stages in which one of the species shows a polymorphism. The frequency of the new nucleotide variant at the site considered is low in Class II and intermediate in Class III, while Class IV represents sites in which a mutation has replaced the progenitor base in most members of the repetitive family in the other species. Class V represents diagnostic sites in which a new variant is fully homogenized and fixed in all the members of one of the species while the other species retains the progenitor nucleotide. A Class VI site represents an additional step over the stage of Class V (new variants appear in some of the members of the repetitive family at a site fully divergent between the two species). The statistical significance (*P*-value) of the variation in the relative proportions of Strachan transitions stages among different interspecific comparisons was evaluated using chi-square heterogeneity tests that were performed in the interactive online calculator available at http://www.quantpsy.org/chisq/chisq.htm (Preacher, 2001).

Chromosome Analysis

For metaphase preparations, about 50 μl of blood were taken from *I. monticola* and *I. galani* individuals with a sterile heparinized syringe and cultured in CO_2 incubators using the culture conditions indicated by Ezaz et al. (2005). Metaphase preparations were obtained by exposing cell cultures to 75 ng/ml of Demecolcine (Sigma-Aldrich Co., St Louis, MO, USA) for 4 hr before harvesting (Ezaz et al., 2005). Cells were hypotonized in KCl 0.75 M for 30 min at 37°C, prefixed by adding several drops of freshly prepared methanol:acetic acid fixative (3:1), then fixed through three changes of fixative. Suspensions of fixed cells were dropped onto microscope slides and air dried at room temperature.

Fluorescence in situ hybridization (FISH) experiments were performed on metaphase preparations using (i) a telomeric probe

(TTAGGG)n produced by PCR according to Ijdo et al. ('91), and (ii) the probes obtained by PCR amplification of *Taq*I and *Hin*dIII satDNA clones. Telomeric and *Taq*I probes were also used in two-color FISH experiments. The probes were labeled by PCR either with biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). Slide pretreatment, denaturation, hybridization, post-hybridization washes, and detection were performed according to Schwarzacher and Heslop-Harrison (2000). The *Hin*dIII satDNA and telomeric probes were evidenced with fluorescein iso-thyocianate (FITC) and tetramethyl rhodamine iso-thyocianate (TRITC), respectively. Chromosomes were observed with a Nikon Eclipse 800 epifluorescence microscope and the images were captured and processed with a Leica CytoVision version 7.2 system.

In order to define the relationships between satDNAs and the constitutive heterochromatin, C-banding was performed on metaphase plates following Sumner ('72). The relations between AT-rich heterochromatic regions and satDNAs were determined by staining C-banded metaphases with 4′,6-diamidino-2-phenylindole (DAPI) (Schweizer, '76).

RESULTS

Isolation and Characterization of Satellite DNAs

The digestion of *I. monticola* and *I. galani* genomic DNA with *Hin*dIII and *Taq*I restriction enzymes revealed bands corresponding to a monomer of a repetitive element of about 170 and 190 bp, respectively (not shown). PCR amplification using primers designed by aligning *I. monticola* and *I. galani* sequences of both satDNAs was successful in individuals representing the other two lineages of *Iberolacerta* recognized as distinct species (*I. martinezricai*, *I. cyreni*). The length of the 45 clones sequenced for *Hin*dIII ranged between 170 and 172 bp, whereas the length of the 42 clones sequenced for *Taq*I ranged between 171 and 188 bp (Table 1). Sequences of both satDNAs were deposited in GenBank (*Hin*dIII accession numbers: from KF453637 to KF453681; *Taq*I accession numbers: from KF453682 to KF453723). When *Hin*dIII and *Taq*I satDNA sequences were subjected to a BLASTN search, no significant similarities with sequences deposited in databases were found.

Southern blot analysis revealed hybridization of both satDNA probes onto *Iberolacerta monticola* digested genomic DNA with a ladder-like pattern, indicating the tandem arrangement of repeating units which is typical of satDNAs. A strong hybridization signal was also produced on the other three *Iberolacerta* species whit both *Hin*dIII and *Taq*I probes; this latter probe also produced a clear signal on the other lizards tested, whereas no signal appeared when *Hin*dIII probe was hybridized on representatives of the genera *Lacerta*, *Podarcis*, and *Timon* (not shown).

Quantitative dot blot analysis revealed that *Hin*dIII satDNA represents around 10% of *I. monticola* and *I. galani*, and 5% of *I. cyreni* and *I. martinezricai* genomes. *Taq*I satDNA represents 5% of *I. cyreni*, *I. galani*, and *I. monticola* genomes, and 2.5% in *I. martinezricai* (data not shown). The estimation of the number of repeats was not possible because the genome size of these lizards is not known.

The consensus sequences of the two satDNAs were very similar in the four *Iberolacerta* species, with an AT average content of 58.4% for *Hin*dIII and 60.3% for *Taq*I, indicating an enrichment in AT (Table 1). Both satellites repeats are characterized by the occurrence of short motifs such A and T stretches and dinucleotides steps TG and CA, with more numerous and longer A (T) stretches in *Taq*I satDNA (Fig. 1), as expected from its higher AT content. Within *Hin*dIII satDNA, two monomer variants or subfamilies (I and II) were detected in *I. galani* and *I. monticola* (Fig. 1A). The consensus sequences of subfamily I in both species were virtually identical to the consensus of *I. martinezricai*, whereas subfamily II showed several (nine) randomly distributed diagnostic nucleotide substitutions, as well as three exclusive indels located in the terminal region of the monomer. Both

Table 1. Summary of repeat features and π values.

	*Hin*dIII				*Taq*I			
Species	*n*	%AT	Repeat length	π	*n*	%AT	Repeat length	Nucleotide diversity (π)
I. cyreni	11	57.0	171	0.0055 ± 0.0022	9	60.2	186–187	0.0384 ± 0.0058
I. galani	13	58.9	170–171	0.0358 ± 0.0033	16	60.1	186–187	0.0475 ± 0.0070
I. galani (sfI)	6	59.4	171	0.0175 ± 0.0031				
I. galani (sfI)	7	58.5	170	0.0101 ± 0.0020				
I. monticola	10	59.0	170–171	0.0187 ± 0.0035	10	60.8	171–188	0.0569 ± 0.0062
I. monticola (sfI)	9	59.0	171	0.0062 ± 0.0019				
I. monticola (sfI)	1	58.8	170	–				
I. martinezricai	10	58.7	171–172	0.0105 ± 0.0052	7	60.1	187–188	0.0428 ± 0.0114

Number of monomeric repeats sequenced (*n*), nucleotide composition of repeats (AT), length of repeats (expressed in base pairs), and nucleotide diversity (π) ± SE for both satDNAs for each *Iberolacerta* species investigated. sfI: HindIII satDNA subfamily I; sfII: HindIII satDNA subfamily II

monomer variants were present in similar proportions in the sequence data set of *I. galani*, but only one out of ten sequences in *I. monticola* belonged to subfamily II (Table 1).

The phylogenetic tree obtained from the Bayesian analysis of *Hin*dIII satDNA is shown in Figure 2. The four different phylogenetic analyses (NJ, MP, ML, and BA) yielded very similar topologies, with some minor incongruences. Two major clades were recovered with maximum support, one harbouring *I. cyreni* clones and the other the sequences of the remaining three *Iberolacerta* species. Within this second cluster, monomers of subfamily II constitute a well-supported clade sister to that formed by sequences belonging to subfamily I, [with the exception of two clones from *I. galani* (IGA_32 and IGA_39) that share some private nucleotide substitutions]. Within subfamily I, relationships between most monomers were poorly resolved and they were not grouped according to the species of origin.

The Bayesian tree constructed using the sequences of *Taq*I satDNA was largely unresolved, regardless of the phylogenetic method employed, showing that this satellite cannot discriminate effectively the four *Iberolacerta* species here investigated (Fig. 3).

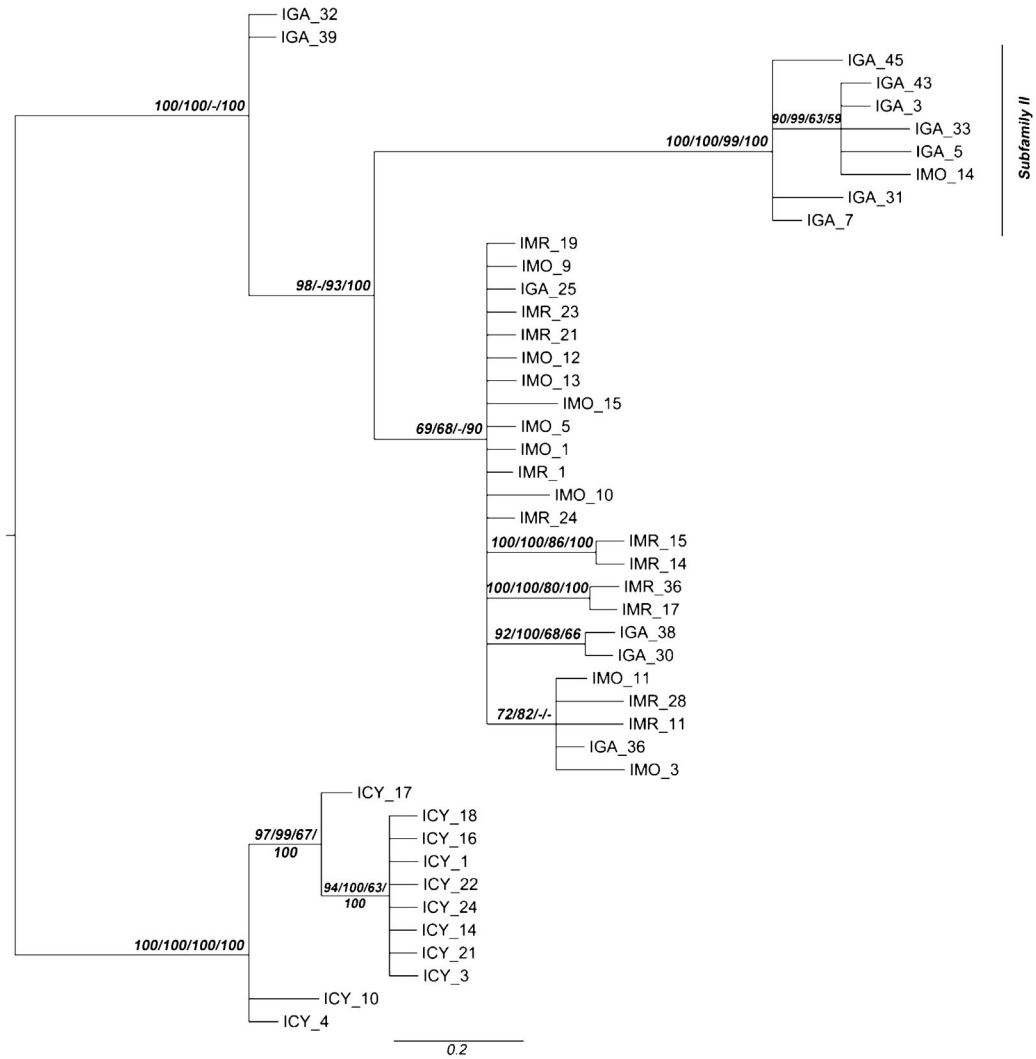


Figure 2. Bayesian phylogenetic tree depicting the the phylogenetic relationships between the 45 monomeric units of *Hin*dIII satDNA sequenced. Support values obtained by four different methods of analysis are shown at each node; from left to right: Bayes posterior probability (100×), metaGA branch support values (100×), NJ-bootstrap (%), and equally MP trees (%). A hyphen was inserted whenever a particular method did not support the Bayesian topology. Numbers after the species names are experimental number for clone identification. ICY: *Iberolacerta cyreni*; IGA: *Iberolacerta galani*; IMO: *Iberolacerta monticola*; IMR: *Iberolacerta martinezricai*.

Even though several well-supported subclusters including conspecific monomers were recognized, the number of diagnostic mutations shared by these sequences was too low to be considered species-specific *Taq*I satDNA subfamilies (not shown).

The π values indicated that intraspecific sequence heterogeneity is higher for *Taq*I satDNA (from 3.84% in *I. cyreni* to 5.69% in *I. monticola*) than for *Hin*dIII satDNA (from 0.55% in *I. cyreni* to 3.58% in *I. galani*) (Table 1). Interspecific mean net distances are low and similar for both satellites when *I. cyreni* is excluded from the analysis of *Hin*dIII satDNA (from 0.04% between *I. monticola* subfamily I and *I. martinezricai* to 5.60% between *I. galani* subfamily II and *I. martinezricai* for *Hin*dIII, and from 0.90% between *I. galani* and *I. martinezricai* to 1.30% between *I. monticola* and *I. galani* for *Taq*I satDNA) (Tables 2 and 3). Pair wise comparisons of *Hin*dIII satDNA involving *I. cyreni* and the other *Iberolacerta* analyzed, showed distance values substantially higher, between 8.40% and 13.90% (Table 2).

In addition, higher levels of sequence divergence were obtained in the comparisons between subfamilies I and II of *Hin*dIII satDNA in *I. galani* (4.5%) than in the comparisons between monomeric repeats belonging to subfamily I in different species (from 0.04% to 0.4%) (Table 2).

The evolutionary rate of these two satellites was then calculated based on sequence divergence between *I. cyreni* and the other three species, that were considered as a single taxonomic unit not being discriminated by either satellite. The values found are 1.2% for *Hin*dIII and 0.14% for *Taq*I, indicating an evolutionary rate almost 10-fold faster for the former.

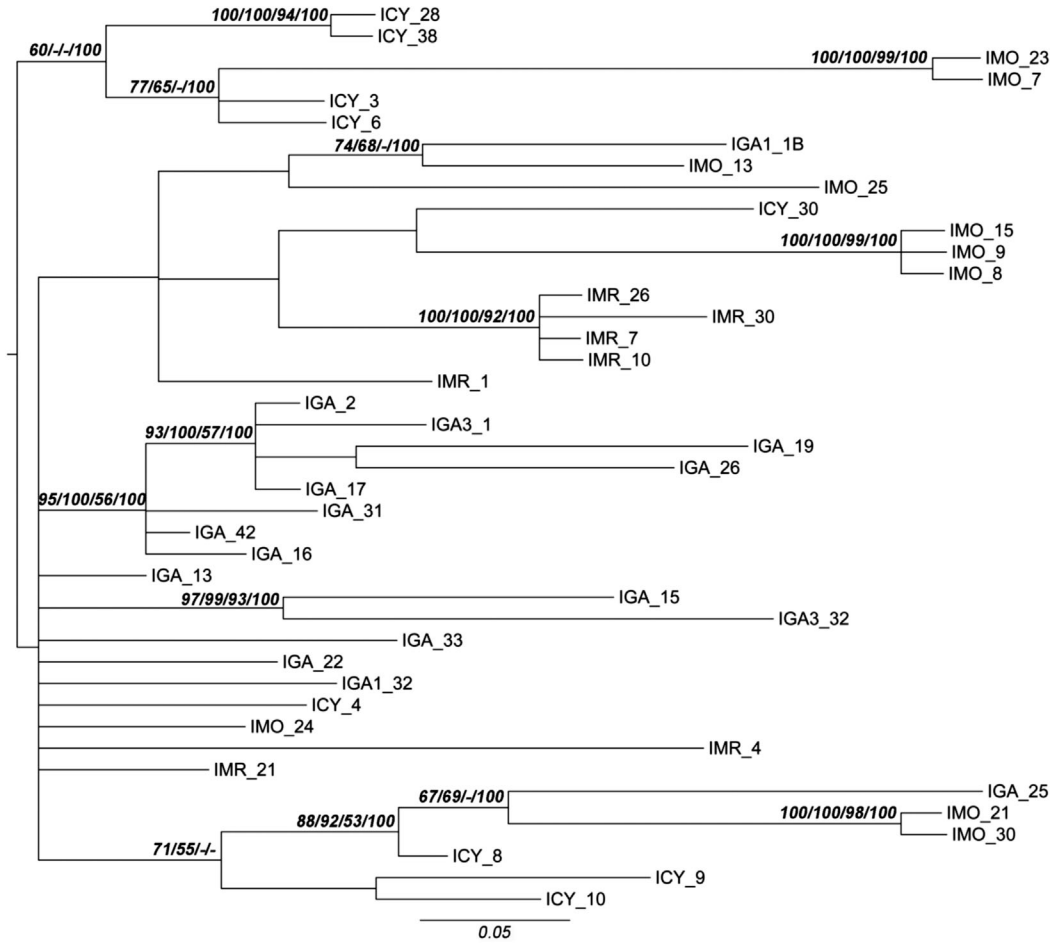


Figure 3. Bayesian phylogenetic tree depicting the the phylogenetic relationships between the 42 monomeric units of *Taq*I satDNA sequenced. Support values obtained by four different methods of analysis are shown at each node; from left to right: Bayes posterior probability (100×), metaGA branch support values (100×), NJ-bootstrap (%) and equally MP trees (%). A hyphen was inserted whenever a particular method did not support the Bayesian topology. Numbers after the species names are experimental number for clone identification. ICY: *Iberolacerta cyreni*; IGA: *Iberolacerta galani*; IMO: *Iberolacerta monticola*; IMR: *Iberolacerta martinezricai*.

Table 2. Interspecific and intersubfamily comparative analysis of *Hin*dIII repeats

Species comparison	SP (%)	Strachan sites II–III (%)	Strachan sites IV–VI (%)	Genetic distance
*Hin*dIII				
I. cyreni versus *I. galani* (sfI)	0 (0%)	4 (2.3%)	15 (8.8%)	0.0838 ± 0.0232
I. cyreni versus *I. galani* (sfII)	0 (0%)	5 (2.9%)	21 (12.3%)	0.1388 ± 0.0326
I. cyreni versus *I. monticola* (sfI)	0 (0%)	5 (2.9%)	16 (9.4%)	0.1025 ± 0.0265
I. cyreni versus *I. martinezricai*	0 (0%)	5 (2.9%)	15 (8.8%)	0.0996 ± 0.0258
I. galani (sfI) versus *I. monticola* (sfI)	1 (0.59%)	7 (4.1%)	1 (0.59%)	0.0038 ± 0.0025
I. galani (sfI) versus *I. martinezricai*	2 (1.2%)	6 (3.5%)	1 (0.59%)	0.0034 ± 0.0026
I. monticola (sfI) versus *I. martinezricai*	2 (1.2%)	4 (2.3%)	0 (0%)	0.0004 ± 0.0005
I. galani (sfII) versus *I. monticola* (sfI)	0 (0%)	8 (4.7%)	9 (5.3%)	0.0545 ± 0.0190
I. galani (sfII) versus *I. martinezricai*	0 (0%)	8 (4.7%)	9 (5.3%)	0.0555 ± 0.0192
I. galani (sfI) versus *I. galani* (sfII)	0 (0%)	6 (3.5%)	10 (5.8%)	0.0447 ± 0.0160

The table reports number and percentage of shared polymorphic sites (SP); variable nucleotide sites classified according to Strachan et al. ('85); net genetic distances (Jukes–Cantor method) in pair wise comparisons of species. sfI: *Hin*dIII satDNA subfamily I; sfII: *Hin*dIII satDNA subfamily II.

The poor phylogenetic differentiation of these species based on the sequences of the satDNAs here isolated was confirmed by AMOVA analysis. When this test was performed on the *Hin*dIII sequences, most of the percentage of the molecular variation was distributed among species (69.60%; Φ_{ST} 0.69596, $P < 0.0001$) whereas the percentage of variation within species was much lower, but still significant (30.40%; Φ_{ST} 0.69596, $P < 0.001$) (Table 4). The variance among species became much lower (32.07%; Φ_{ST} 0.32072, $P < 0.001$) and the one within populations became the preponderant variance component (67.93%; Φ_{ST} 0.32072, $P < 0.001$) when the sequences of *I. cyreni* were excluded from the analysis (Table 4). This result can be explained by the fact that *I. cyreni* was recovered as a distinct cluster with a high support in the phylogeny based on *Hin*dIII sequences, whereas the other three cannot be discriminated by this molecular marker. The AMOVA test carried out on *Taq*I satDNA sequences produced results very similar to those obtained with *Hin*dIII sequences after excluding *I. cyreni*, with a preponderant variance component distributed within species (82.69%; Φ_{ST} 0.17314, $P < 0.001$), confirming that this satDNA cannot effectively discriminate between these *Iberolacerta* species (Table 4). These results emerged also by analyzing the pattern of variation at each nucleotide position considered independently among all *Hin*dIII repeats (Table 2). Indeed, when comparing *I. cyreni* with the other species, a high percentage of Strachan sites belonging to the categories IV, V, and VI were found (average = 9.9%), while 5.1% of sites per repeat were Strachan transition stages (II + III), and no shared polymorphic sites were observed. Conversely, for *Taq*I satDNA sites of the classes IV–VI were very few (average = 0.5%) in all the comparison, while 20.7% of the sites represented Strachan stages II–III and an average of 4.1% were polymorphic sites (Table 3). According to the chi-square heterogeneity test, these differences in the relative proportions of Strachan transition stages between *Hin*dIII and *Taq*I satDNAs are highly significant ($P < 0.001$).

The relatively high degree of genetic differentiation detected in the analysis of sequence divergence between *Hin*dIII subfamily II

Table 3. Interspecific comparative analysis of *Taq*I repeats.

Species comparison	SP (%)	Strachan sites II–III (%)	Strachan sites IV–VI (%)	Genetic distance
*Taq*I				
I. cyreni versus *I. galani*	8 (4.3%)	51 (27.3%)	3 (1.6%)	0.0099 ± 0.0040
I. cyreni versus *I. monticola*	9 (4.8%)	25 (13.4%)	1 (0.5%)	0.0113 ± 0.0040
I. cyreni versus *I. martinezricai*	7 (3.7%)	25 (13.4%)	2 (1.1%)	0.0109 ± 0.0039
I. galani versus *I. monticola*	10 (5.3%)	43 (23%)	1 (0.5%)	0.0130 ± 0.0057
I. galani versus *I. martinezricai*	5 (2.7%)	57 (30.5%)	3 (1.6%)	0.0089 ± 0.0037
I. monticola versus *I. martinezricai*	7 (3.7%)	31 (16.6%)	2 (1.1%)	0.0114 ± 0.0040

The table reports number and percentage of shared polymorphic sites (SP); variable nucleotide sites classified according to Strachan et al. ('85); net genetic distances (maximum composite likelihood method) in pair wise comparisons of species.

Table 4. AMOVA analysis.

Source of variation	Variance components	Percentage of variation
Among species	4.21275	69.60
	1.07719	32.07
	1.14218	17.31
Within species	1.84035	30.40
	2.28152	67.93
	5.45487	82.69

The test was carried on *Hin*dIII satDNA sequences including the four species selected for this study (first line of each hierarchical level), and removing *Iberolacerta cyreni* from the analysis (second line of each hierarchical level). The test on *Taq*I satDNA sequences included all four of the *Iberolacerta* investigated (third line of each hierarchical level). Φ-statistics were highly significant in all comparisons ($P < 0.001$).

and subfamilies I from *I. galani*, *I. monticola*, and *I. martinezricai* was also evident in the comparisons of Strachan transition stages among these groups (Table 2). No shared polymorphisms were found and the number of sites falling in classes IV and V (between 5% and 6%) was significantly larger ($P < 0.001$) than the average frequency of these "differentiated sites" in the comparisons among subfamilies I in different species.

Chromosome Analysis

FISH experiments with *Hin*dIII satDNA probe on metaphase chromosomes of *I. galani* and *I. monticola* revealed that this repetitive element is widespread in the genome of these species, occurring at centromeres of all the 36 chromosomes of the diploid complement (Fig. 4A,B), with no differences between males and females. The occurrence of "bouquet" figures where chromosomes are linked together at the level of centromeres seems to indicate that this satDNA is involved in the interchromosome connection during mitosis (Fig. 4B). FISH with *Taq*I satDNA probe produced bright signals in interstitial position in a subset of 18 chromosomes in *I. galani* and 20 in *I. monticola*. No differences between males and females were detected with this probe either (Fig. 4C,D). Results of FISH experiments are consistent with the genomic abundance of *Hin*dIII and *Taq*I satDNAs as showed by quantitative dot blot analysis for these two species, with the former around twofold more abundant than the latter.

FISH with a telomeric probe (TTAGGG)n produced a fluorescent signal at telomeres of all the chromosomes. Besides telomeric signals, also interstitial telomeric sites (ITS) were marked in about five chromosome pairs. When a two-color FISH with both telomeric and *Taq*I satellite probes were performed, the fluorescent signals of ITS resulted distally located to the satellite ones (Fig. 4D).

C-banding, performed in order to assess the relationships between the isolated satellites and constitutive heterochromatin, revealed that in *Iberolacerta* the chromosomal distribution of *Hin*dIII satDNA overlaps the centromeric heterochromatic blocks, whereas *Taq*I probe colocalizes with pericentromeric heterochromatin (Fig. 4E,F).

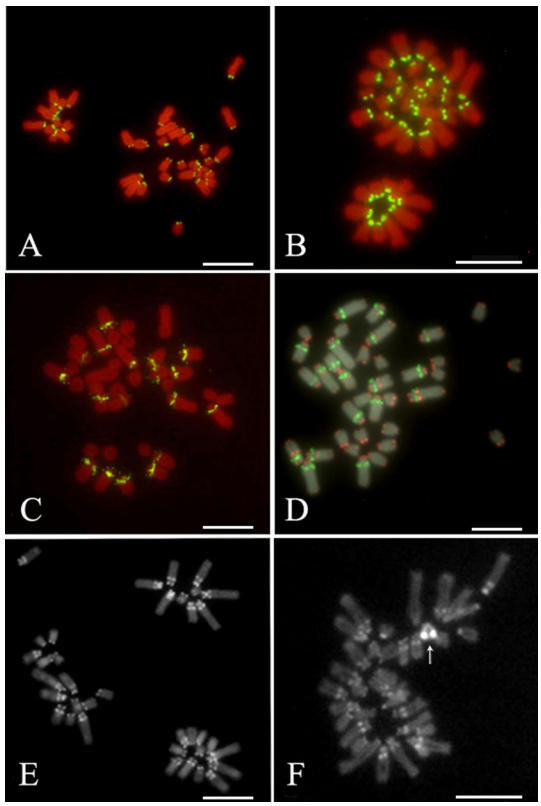


Figure 4. FISH with *Hin*dIII probe onto metaphases from females of *Iberolacerta galani* (**A**) and *I. monticola* (**B**). FISH with *Taq*I probe onto a metaphase of *I. galani* female (**C**). Two-color FISH with telomeric (red) and *Taq*I (green) probes on a metaphase of *I. monticola* female (**D**). C-banding on *I. monticola* male (**E**) and *I. galani* female (**F**) metaphases. The W chromosome of *I. galani* is indicated by an arrow.

DISCUSSION

Satellite DNAs represent rapidly evolving genomic elements, and therefore, even among most closely related species, they usually differ in nucleotide sequence, copy number, and/or composition of satellite families (Csink and Henikoff, '98). However, some satDNA families evolve more slowly than others and occur in several closely related species with different degrees of sequence similarity (Bachmann and Sperlich, '93; Mantovani et al., '97; Watabe et al., '97). Some satDNAs seem to be rather ancient and are widely distributed among higher taxa (Modi et al., 2004; Robles et al., 2004). Consequently, some satDNAs may be valuable

taxonomic identification tools while others might be useful for phylogenetic analyses at higher taxonomic levels. In the present study, we compared sequences of two different satDNA families (*Hin*dIII and *Taq*I) in four closely related lacertid species, allopatrically distributed in mountain areas of the Iberian Peninsula. These satDNAs seem to evolve at different rates in the studied lizards, with *Hin*dIII showing a 10-fold faster evolutionary rate than *Taq*I. Indeed, Southern blot analysis using *Iberolacerta* satellite probes revealed a clear hybridization pattern also in other lizard genera (namely, *Lacerta*, *Podarcis*, and *Timon*) only for *Taq*I repeats, whereas *Hin*dIII seems to be restricted to the genus *Iberolacerta*. However, a significant level of genetic divergence was detected only in comparisons involving *I. cyreni* when *Hin*dIII satDNA was considered. For this satDNA, analysis of turnover dynamics indicate the effectiveness of the molecular drive process, after species split, in the spreading of new sequence variants leading to intraspecific homogeneity (0.56% of sequence variation within *I. cyreni*) and interspecific divergence (around 9% of sequence divergence between *I. cyreni* and the other species), an evolutionary pattern known as concerted evolution (Dover, '82). The fact that the other species are scarcely differentiated at *Hin*dIII repeats can be interpreted in two alternative ways: (i) it may represent the outcome of the relatively recent (approximately 2 mya, Arribas et al., 2006) and rapid succession of speciation events within this group. In fact, previous molecular analyses based on nuclear and mitochondrial markers also failed to resolve the phylogenetic relationships or even track lineage splitting at this taxonomic level (Mayer and Arribas, 2003; Carranza et al., 2004; Crochet et al., 2004; Arribas et al., 2006; Arnold et al., 2007); (ii) the specific status for these three taxa might not have been reached yet. Indeed, estimation of divergence times among these three *Iberolacerta* species are similar to those recorded for different populations of the lizard *Podarcis muralis* that diverged genetically in separate refuges during glaciations, currently not showing evidence for reproductive isolation (Giovannotti et al., 2010).

The deep divergence observed between *I. cyreni* and the other *Iberolacerta* species here investigated with *Hin*dIII satellite is in good accordance with the molecular phylogenies published so far (Mayer and Arribas, 2003; Carranza et al., 2004; Crochet et al., 2004; Arribas et al., 2006; Arnold et al., 2007). This analysis showed that this species was the most diverged clade of the tree, with an estimated splitting time of about 7.5 million years. The relatively scarce representation of transitional stages (only 5% of the nucleotide positions) might suggest that the concerted evolution mechanisms have led to sequence differentiation between *I. cyreni* and the other species, probably due to the efficiency of the molecular-exchange homogenizing mechanisms among chromosomes.

The occurrence of two different types of monomeric variants or subfamilies was described for *Hin*dIII satDNA sequences. These subfamilies were defined according to a set of particular nucleotide substitutions or indels, in two of the four species examined. However, given the almost simultaneous speciation processes between *I. monticola*, *I. galani*, and *I. martinezricai*, it seems unlikely that subfamily II constitutes a specific variant of *I. monticola* and *I. galani*. An interspecific analysis of the pattern of nucleotide change was not possible for subfamily II due to the lack of a representative number of sequences in *I. monticola* or *I. martinezricai*. Even so, our results show that both subfamilies are presumably evolving independently, as indicated by the substantially high percentage of transitions stages IV and V between the monomers of subfamily II (*I. galani*) and the sequences of subfamily I, either belonging to *I. galani*, *I. monticola*, or *I. martinezricai*. The coexistence and divergent evolution of satellite subfamilies in the genomes of these species could be in agreement with the Nijman and Lenstra model (2001), in which mutations inhibiting the interactions of repeat units in a satellite family would lead to sequence diversification and the independent amplification or contraction of concurrent sequence variants. Nevertheless, a more extensive survey of *Hin*dIII satDNA will be the subject of further studies, in order to assess the presence and abundance of both monomeric variants in other *Iberolacerta* species, as well as to elucidate the processes driving the evolution of this satellite family.

Conversely to *Hin*dIII sequences, the tandem arrays of *Taq*I show a low sequence change rate when comparing *I. cyreni* with the other *Iberolacerta*. In fact, we detected a low rate of sequence change (0.1% per Myr), a rate 10-fold lower than that estimated for *Hin*dIII sequences (about 1.2% per Myr) and only 1.1% of Strachan stages IV–VI compared to 18% of II–III stages. In addition, we also observed some shared polymorphic sites and a comparatively higher intraspecific heterogeneity, suggesting that most of the intraspecific variability in each species is ancestral, originated prior to the separation of these lineages; moreover, the high number of transitional stages of differentiation (Strachan stages II–III) suggest that after the allopatric isolation, processes of concerted evolution were less efficient than in the *Hin*dIII repeats. In addition, contrarily to *Hin*dIII, Southern hybridization with *Taq*I probe produced a clear signal also in other lacertid genera, like *Lacerta*, *Podarcis*, and *Timon*, also suggesting a strong conservation of this satellite DNA family.

Various factors were invoked to explain different evolutionary turnover rates between satDNA families, like interchromosomal and intrachromosomal recombination rates, copy number, array size and structure, chromosomal distribution, chromosomal structure, population size, divergence time and reproductive mode. Moreover, evolutionary conservation of satDNA repeats might be a likely indication of functional constraints and natural selection (see Plohl et al., 2008). Unfortunately, very few examples are found in the literature with both fast-evolving and slow-evolving satDNAs found within the same species. For instance, in the genus *Dolichopoda*, a comparison among three satDNA families showed a trend of sequence variability and copy number

being positively correlated, and a trend of sequence variability and length of repeats being negatively correlated (Martinsen et al., 2009). Like in *Dolichopoda*, it seems that also in the studied lizards an increase in copy number is linked to a trend of sequence homogenization. In fact, it was observed that *Hin*dIII repeats represent between 5% and 10% of the *Iberolacerta* genome, while *Taq*I satDNA between 2.5% and 5%. The different chromosome localization of the two satellites may also play a role in the different rate of sequence homogenization recorded for the two satDNA families. First of all, it should be noted that *Hin*dIII repeats are centromerically located on all the acrocentric chromosomes of *I. galani* and *I. monticola* karyotypes. In fact, it is reported that satellite DNAs at centromeres of acrocentric chromosomes show greater homology and a higher rate of homogenization than in noncentromeric locations or nonacrocentric chromosomes (Jantsch et al., '90; Bandyopadhyay et al., 2001). It has been hypothesized that homogenization occurs through physical association and crossing-over between nonhomologous chromosomes (Ohno et al., '61). Indeed, acrocentric chromosomes associate at the heterochromatic regions during meiotic prophase and somatic interphase (Schmid et al., '83; Tuck-Muller et al., '84; Kuznetsova et al., 2007) and we also observed typical "bouquet" figures, where chromosomes are linked together at the level of centromeres (Fig. 4B). This process may be the most important mechanisms for spontaneous chromosomal mutation, concerted evolution, and homogenization of satellite subfamilies of DNA among acrocentric chromosomes (Maeda and Smithies, '86).

Conversely, *Taq*I repeats are pericentromerically located on a lower number of chromosomes (10 pairs in *I. monticola* and 9 in *I. galani*). In this case, we could explain the low homogenization rate within single species in terms of primary rate of the homogenization process. That is, it is possible that the exchange between nonhomologous chromosomes having *Taq*I sequences is limited. The *Taq*I repeats are indeed restricted to a subset of chromosomes in these species and located in a pericentromeric position less prone to physical association: this could reduce interchromosomal exchange and homogenization, thus determining a lower rate of interspecific divergence and a higher degree of intraspecific repeat heterogeneity. Similar considerations were reported for satDNAs of *Rumex*, where repeats in nonrecombining Y chromosomes show low rates of concerted evolution and intraspecific variability increase with no interspecific divergence (Navajas-Pérez et al., 2009; see also Kuhn et al., 2008), and to explain the lower mutation rate of satDNAs in sturgeons as compared to sparids. In fact, the more symmetrical karyotypes of these latter fishes would represent no physical barrier to interchromosomal exchange (de la Herrán et al., 2001a,b). However, also these AT-rich pericentromeric repeats could represent chromosome sites favoring spontaneous rearrangements. Indeed, we observed that the majority of the *Taq*I repeats are flanked by interstitial telomeric sequences that would insert in these chromosome points during the repair of double strand breaks (see Bolzán and Bianchi, 2006). These unstable sequences might explain the high rate of Robertsonian translocation observed in Pyrenean *Iberolacerta* (Odierna et al., '96).

In conclusion, our study suggests the effect of differential location and repeat copy number in the evolution of satDNAs, revealing features that could also improve the use of this genomic component as a molecular marker in phylogenetic analyses. Moreover, these results indicate that some molecular markers should be used cautiously in species identification when divergence times are shallow among the taxa compared.

ACKNOWLEDGMENTS

Special thanks are given to Pedro Galán for providing the samples of *Iberolacerta monticola* and *I. galani* used in this study. All authors declare that no conflict of interests exists for this manuscript.

LITERATURE CITED

Arnold EN, Arribas O, Carranza S. 2007. Systematics of the palaearctic and oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa 1430:1–86.

Arribas O. 1999. Taxonomic revision of the Iberian "Archaeolacertae" 2: diagnosis, morphology, and geographic variation of "*Lacerta*" *aurelioi* Arribas, 1994 (Squamata: Sauria: Lacertidae). Herpetozoa 11:155–180.

Arribas O, Carranza S, Odierna G. 2006. Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240:1–55.

Bachmann L, Sperlich D. 1993. Gradual evolution of a specific satellite DNA family in *Drosophila ambigua*, *D. tristis*, and *D. obscura*. Mol Biol Evol 10:647–659.

Bandyopadhyay R, McQuillan C, Page SL, Choo KHA, Shaffer LG. 2001. Identification and characterization of satellite III subfamilies to the acrocentric chromosomes. Chromosome Res 9:223–233.

Bolzán AD, Bianchi MS. 2006. Telomeres, interstitial telomeric repeat sequences, and chromosomal aberration. Mutat Res 612:189–214.

Cafasso D, Cozzolino S, Chinali G, De Luca P. 2003. An unusual satellite DNA from *Zamia paucijuga* (Cycadales) characterised by two different organisations of the repetitive unit in the plant genome. Gene 311:69–77.

Capriglione T. 2000. Repetitive DNA as a tool to study the phylogeny of cold-blooded vertebrates. Chromosomes Today 13:183–194.

Capriglione T, Olmo E, Odierna G, Smith DI, Miller OJ. 1989. Genome composition and tandemly repetitive sequence at some centromeres in the lizard *Podarcis s. sicula* Raf. Genetica 79:85–91.

Capriglione T, Cardone A, Odierna G, Olmo E. 1991. Evolution of a centromeric satellite DNA and phylogeny of lacertid lizards. Comp Biochem Physiol B 100:641–645.

Capriglione T, Cardone A, Odierna G, Olmo E. 1994. Further data on the occurrence and evolution of satellite DNA families in the lacertid genome. Chromosome Res 2:327–330.

Capriglione T, De Santo MG, Odierna G, Olmo E. 1998. An alphoid-like satellite DNA sequence is present in the genome of lacertid lizards. J Mol Evol 46:240–244.

Carranza S, Arnold EN, Amat F. 2004. DNA phylogeny of *Lacerta* (*Iberolacerta*) and other lacertine lizards (Reptilia: Lacertidae): did competition cause long-term mountain restriction? Syst Biodivers 2:57–77.

Charlesworth B, Sniegowski P, Stephan W. 1994. The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371:215–220.

Ciobanu D, Roudykh IA, Grechko VV, Kramerov DA. 2001. Molecular mechanisms of satellite DNA evolution in a group of closely related Caucasian rock lizards. In: Piruzian E, Volkova L, editors. International Symposium Molecular Mechanisms of Genetic Processes and Biotechnology, Moskow [Thesis]. p 26.

Ciobanu D, Grechko VV, Darevsky IS. 2003. Molecular evolution of satellite DNA CLsat in lizards from the genus *Darevskia* (Sauria: Lacertidae): correlation with species diversity. Russ J Genetics 39:1527–1541.

Ciobanu DG, Grechko VV, Darevsky IS, Kramerov DA. 2004. New satellite DNA in *Lacerta* s. str. lizards (Sauria: Lacertidae): evolutionary pathways and phylogenetic impact. J Exp Zool 302B:505–516.

Crochet PA, Chaline O, Surget-Groba Y, Debain C, Cheylan M. 2004. Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae). Mol Phylogenet Evol 30:860–866.

Csink AK, Henikoff S. 1998. Something from nothing: the evolution and utility of satellite repeats. Trends Genet 14:200–204.

Dawe RK, Henikoff S. 2006. Centromeres put epigenetics in the drivers's seat. Trends Biochem Sci 31:662–669.

de la Herrán R, Fontana F, Lanfredi M, et al. 2001a. Slow rates of evolution and sequence homogenization in an ancient satellite DNA family of sturgeons. Mol Biol Evol 18:432–436.

de la Herrán R, Ruiz Rejon, Ruiz C, Rejon M, Garrido-Ramos MA. 2001b. The molecular phylogeny of the Sparidae (Pisces, Perciformes) based on two satellite DNA families. Heredity 87:691–697.

Dover GA. 1982. Molecular drive: a cohesive mode of species evolution. Nature 299:111–117.

Excoffier L, Smouse P, Quattro J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Ezaz T, Quinn AE, Miura I, et al. 2005. The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. Chromosome Res 13:763–776.

Feliciello I, Picariello O, Chinali G. 2006. Intra-specific variability and unusual organization of the repetitive units in a satellite DNA from *Rana dalmatina*: molecular evidence of a new mechanism of DNA repair acting on satellite DNA. Gene 383:81–92.

Giovannotti M, Nisi Cerioni P, Caputo V, Olmo E. 2009. Characterization of a GC-rich telomeric satellite DNA in *Eumeces schneideri* Daudin (Reptilia, Scincidae). Cytogenet Genome Res 125:272–278.

Giovannotti M, Nisi Cerioni P, Caputo V. 2010. Mitochondrial DNA sequence analysis reveals multiple Pleistocene glacial refugia for *Podarcis muralis* (Laurenti, 1768) in the Italian Peninsula. Ital J Zool 77:277–288.

Grechko VV, Ciobanu DG, Darevsky IS, Kramerov DA. 2005. Satellite DNA of lizards of the genus *Lacerta* s. str. (the group *L. agilis*), the family Lacertidae. Dokl Biochem Biophys 400:44–47.

Grechko VV, Ciobanu DG, Darevsky IS, Kosushkin SA, Kramerov DA. 2006. Molecular evolution of satellite DNA repeats and speciation of lizards of the genus *Darevskia* (Sauria: Lacertidae). Genome 49:1297–1307.

Helaers R, Milinkovitch MC. 2010. MetaPIGA v2.0: maximum likelihood large phylogeny estimation using the metapopulation genetic algorithm and other stochastic heuristics. BMC Bioinformatics 11:379.

Ijdo JW, Wells RA, Baldini A, Reeders ST. 1991. Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. Nucleic Acids Res 19:4780.

Jantsch M, Hamilton B, Mayr B, Schweizer D. 1990. Meiotic chromosome behavior reflects levels of sequence divergence in *Sus scrofa domestica* satellite III DNA. Chromosoma 99:330–335.

King LM, Cummings MP. 1997. Satellite DNA repeat sequence variation is low in three species of burying beetles in the genus *Nicroporus* (Coleoptera: Silphidae). Mol Biol Evol 14:1088–1095.

Kuhn GCS, Franco FF, Manfrin MH, Moreira-Filho O, Sene FM. 2007. Low rates of homogenization of the DBC-150 satellite DNA family restricted to a single pair of microchromosomes in species from the *Drosophila buzzatii* cluster. Chromosome Res 15:457–469.

Kuhn GCS, Sene FM, Moreira-Filho O, Schwarzacher T, Heslop-Harrison JS. 2008. Sequence analysis, chromosomal distribution and long-range organization show that rapid turnover of new and old pBuM satellite DNA repeats leads to different patterns of variation in seven species of the *Drosophila buzzatii* cluster. Chromosome Res 16:307–324.

Kuznetsova IS, Enukashvily NI, Noniashvili EM, et al. 2007. Evidence for the existence of satellite DNA-containing connection between metaphase chromosomes. J Cell Biochem 101:1046–1061.

Larkin MA, Blackshields G, Brown NP, et al. 2007. ClustalW and ClustalX version 2.0. Bioinformatics 23:2947–2948.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Luchetti A, Cesari M, Carrara G, et al. 2003. Unisexuality and molecular drive: Bag320 sequence diversity in *Bacillus* taxa (Insecta Phasmatodea). J Mol Evol 56:587–589.

Mace GM. 2004. The role of taxonomy in species conservation. Philos Trans R Soc Lond B 359:711–719.

Maeda N, Smithies O. 1986. The evolution of multigene families: human haptoglobin genes. Annu Rev Genet 20:81–108.

Mantovani B, Tinti F, Bachmann L, Scali V. 1997. The Bag320 satellite DNA family in *Bacillus* stick insects (Phasmatodea): different rates of molecular evolution of highly repetitive DNA in bisexual and parthenogenic taxa. Mol Biol Evol 14:1197–1205.

Martinsen L, Venanzetti F, Johnsen A, Sbordoni V, Bachman L. 2009. Molecular evolution of the *pDo500* satellite DNA family in *Dolichopoda* cave crickets (Rhaphidophoridae). BMC Evol Biol 9:1–14.

Mayer W, Arribas O. 2003. Phylogenetic relationships of the European lacertid genera *Archaeolacerta* and *Iberolacerta* and their relationships to some other 'Archaeolacertae' (*sensu lato*) from Near East, derived from mitochondrial DNA sequences. J Zool Syst Evol Res 41:157–161.

Miller WJ, Nagel A, Bachmann J, Bachmann L. 2000. Evolutionary dynamics of the SGM transposon family in the *Drosophila obscura* species group. Mol Biol Evol 17:1597–1609.

Modi WS, Ivanov S, Gallagher DS. 2004. Concerted evolution and higher-order repeat structure of the 1.709 (satellite IV) family in bovids. J Mol Evol 58:460–465.

Navajas-Pérez R, de la Herrán R, Jamilena M, et al. 2005. Reduced rates of sequence evolution of Y-linked satellite DNA in *Rumex* (Polygonaceae). J Mol Evol 60:391–399.

Navajas-Pérez R, Ruiz Rejón M, Garrido-Ramos MA, Aznarte JL, Rubio-Escudero C. 2007. SatDNA Analyzer: a computing tool for satellite-DNA evolutionary analysis. Bioinformatics 23:767–768.

Navajas-Pérez R, Quesada del Bosque ME, Garrido-Ramos MA. 2009. Effect of location, organization, and repeat-copy number in satellite-DNA evolution. Mol Genet Genomics 282:395–406.

Nijman IJ, Lenstra JA. 2001. Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite repeats. J Mol Evol 52:361–371.

Odierna G, Aprea G, Arribas O, et al. 1996. The karyology of the Iberian rock lizards. Herpetologica 52:542–550.

Ohno S, Trujillo JM, Kaplan WD, Kinosita R. 1961. Nucleolus-organizer in the causation of chromosomal abnormalities. Lancet 2:123–126.

Plohl M. 2010. Those mysterious sequences of satellite DNAs. Periodicum 112:403–410.

Plohl M, Luchetti A, Meštrovi N, Mantovani B. 2008. Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. Gene 409:72–82.

Preacher KJ. 2001. Calculation for the chi-square test: an interactive calculation tool for chi-square tests of goodness of fit and independence [Computer software]. Available online at: http://quantpsy.org [Accessed February 13, 2013].

Rambaut A, Drummond AJ. 2009. Tracer version 1.5 [computer program]. Available online at: http://beast.bio.ed.ac.uk.

Robles F, de la Herrán R, Ludwig A, et al. 2004. Evolution of ancient satellite DNAs in sturgeon genomes. Gene 338:133–142.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Rudd MK, Wray GA, Willard HF. 2006. The evolutionary dynamics of alpha-satellite. Genome Res 16:88–96.

Salvador A. 1985. Guia de campo de los anfibios y reptiles de la Peninsula Iberica, islas Baleares y Canarias. León, Spain: Santiago García Ed.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmid M, Grunert D, Haaf T, Engel W. 1983. A direct demonstration of somatically paired heterochromatin of human chromosomes. Cytogenet Cell Genet 36:554–561.

Schneider S, Roessli D, Excoffier L. 2000. Arlequin, version 2.000: a software for population genetic data analysis. Geneva, Switzerland: Genetics and Biometry Laboratory. University of Geneva.

Schwarzacher T, Heslop-Harrison P. 2000. Practical in situ hybridization. New York, NY: Springer-Verlag.

Schweizer D. 1976. Fluorescent chromosome banding in plants: applications, mechanisms and implications for chromosome structure. In: Davies DR, Hopwood AP, editors. The plant genome. Proc 4th John Innes Symposium. Norwich, UK: John Innes Charity. p 61–72.

Sindaco R, Jeremcenko VK. 2008. The reptiles of the Western Paleartic. Latina, Italy: Edizioni Belvedere.

Smith GP. 1976. Evolution of repeated DNA sequences by unequal crossover. Science 191:528–535.

Stephan W, Cho S. 1994. Possible role of natural selection in the formation of tandem repetitive noncoding DNA. Genetics 136:333–341.

Strachan T, Webb D, Dover G. 1985. Transition stages of molecular drive in multiple-copy DNA families in *Drosophila*. EMBO J 4:1701–1708.

Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306.

Swofford DL. 2002. PAUP* Phylogenetic Analysis Using Parsimony (*and other methods), Version4.0b10a. Sunderland, MA: Sinauer Associates.

Tamura K, Peterson D, Peterson N, et al. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739.

Tuck-Muller CM, Bordson BL, Varela M, Bennett JW. 1984. NOR associations with heterochromatin. Cytogenet Cell Genet 38:165–170.

Walsh JB. 1987. Persistence of tandem arrays: implications for satellite and simple-sequence DNAs. Genetics 115:553–567.

Watabe H, Bachmann L, Haring E, Sperlich D. 1997. Taxonomic and molecular studies on *Drosophila sinobscura* and *D. hubeiensis*, two sibling species of the *D. obscura* group. J Zool Syst Evol Res 35:81–94.

ANNEX III

Chromosome Res
DOI 10.1007/s10577-015-9489-1

ORIGINAL ARTICLE

Evolutionary dynamics of two satellite DNA families in rock lizards of the genus *Iberolacerta* (Squamata, Lacertidae): different histories but common traits

Verónica Rojo · Andrés Martínez-Lage · Massimo Giovannotti · Ana M. González-Tizón · Paola Nisi Cerioni · Vincenzo Caputo Barucchi · Pedro Galán · Ettore Olmo · Horacio Naveira

Abstract Satellite DNAs compose a large portion of all higher eukaryotic genomes. The turnover of these highly repetitive sequences is an important element in genome organization and evolution. However, information about the structure and dynamics of reptilian satellite DNA is still scarce. Two satellite DNA families, HindIII and TaqI, have been previously characterized in four species of the genus *Iberolacerta*. These families showed different chromosomal locations, abundances, and evolutionary rates. Here, we extend the study of both satellite DNAs (satDNAs) to the remaining *Iberolacerta* species, with the aim to investigate the patterns of variability and factors influencing the evolution of these repetitive sequences. Our results revealed disparate patterns but also common traits in the evolutionary histories of these satellite families: (i) each satellite DNA is made up of a library of monomer variants or subfamilies shared by related species; (ii) species-specific profiles of satellite repeats are shaped by expansions and/or contractions of different variants from the library; (iii) different turnover rates, even among closely related species, result in great differences in overall sequence homogeneity and in concerted or non-concerted evolution patterns, which may not reflect the phylogenetic relationships among taxa. Contrasting turnover rates are possibly related to genomic constraints such as karyotype architecture and the interspersed organization of diverging repeat variants in satellite arrays. Moreover, rapid changes in copy number, especially in the centromeric HindIII satDNA, may have been associated with chromosomal rearrangements and even contributed to speciation within *Iberolacerta*.

Keywords Concerted evolution · FISH · *Iberolacerta* · Library model · Satellite DNA · Squamate reptiles

Abbreviations

Cy3	Cyanine 3
dNTP	Deoxyribonucleotide triphosphate

Responsible Editors: Maria Assunta Biscotti, Pat Heslop-Harrison and Ettore Olmo.

Electronic supplementary material The online version of this article (doi:10.1007/s10577-015-9489-1) contains supplementary material, which is available to authorized users.

V. Rojo · A. Martínez-Lage · A. M. González-Tizón · H. Naveira (✉)
Grupo de Investigación en Bioloxía Evolutiva, Departamento de Bioloxía Celular e Molecular, Universidade da Coruña, E-15071 A Coruña, Spain
e-mail: horacio.naveira.fachal@udc.es

M. Giovannotti · P. N. Cerioni · V. C. Barucchi · E. Olmo
Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, via Brecce Bianche, 60131 Ancona, Italy

V. C. Barucchi
Consiglio Nazionale delle Ricerche, Istituto di Scienze Marine Sezione Pesca Marittima, Largo Fiera della Pesca, 60125 Ancona, Italy

P. Galán
Grupo de Investigación en Bioloxía Evolutiva, Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía, Universidade da Coruña, E-15071 A Coruña, Spain

Published online: 18 September 2015

FCA	Factorial correspondence analysis
FISH	Fluorescence in situ hybridization
FITC	Fluorescein iso-thyocianate
Mya	Million years ago
π	Nucleotide diversity
satDNA	Satellite DNA

Introduction

Satellite DNAs (satDNAs) represent one of the major classes of repetitive sequences in almost all eukaryotic genomes. They consist of tandemly repeated non-coding DNA sequences, typically arranged in large clusters of hundreds or thousands of copies usually located in the heterochromatic regions of chromosomes, close to the centromeres and telomeres (Charlesworth et al. 1994). Several satDNA families of independent origin are commonly found in the genome of a species or group of species, and they usually differ in nucleotide sequence, monomer length, and complexity, as well as in evolutionary history (Ugarković and Plohl 2002; Kuhn et al. 2008, 2010). The biological function of these sequences is not yet fully understood, although numerous reports point out the role of certain satellites in centromeric condensation, chromosome organization, or chromosome pairing (see Plohl et al. 2008). A growing field of research is also addressing the role of satDNA transcripts in the formation and maintenance of heterochromatin and even in regulation of gene expression (Ugarković 2009; Pezer et al. 2012). In addition, several examples support the hypothesis that the rapid evolution of satDNAs can act as a driver of population and species divergence (Ugarković and Plohl 2002; Feliciello et al. 2015).

Despite their biological significance, satDNAs are still the least understood genomic component, underrepresented in outputs of most genome projects (Plohl et al. 2012). A common feature of many of them is that, even though monomers can be present in many thousand copies per genome, sequence divergence between repeats of the same family is often very low, usually less than 15 % (Plohl et al. 2008). The non-independent or concerted evolution of repeat units is postulated to be a consequence of a two-step process called molecular drive, consisting of the gradual spread of a sequence variant (1) through a genome (homogenization) and (2) through a species (fixation) (Dover 1982). Sequence homogenization is due to diverse molecular mechanisms of nonreciprocal transfer, such as unequal crossing-over, gene conversion, rolling circle replication and reinsertion, and transposon-mediated exchange (Stephan 1986; Dover 2002), while fixation results from random chromosomal assortment in sexual reproduction, depending thus on population factors. This process results in rapid divergence of satellite sequences in reproductively isolated groups of organisms, and in this case, satDNAs can be used as phylogenetically informative markers (Plohl et al. 2012).

Accumulation of mutations in satellite families is not the only way to alter specific profiles of satellite repeats in short evolutionary periods. In addition to sequence changes, satDNAs are permanently altered in copy number by expanding and contracting arrays of satellite monomers (Ugarković and Plohl 2002; Plohl et al. 2012). Because usually more than one satellite family exists in a genome, fluctuations in their copy numbers can change very efficiently and rapidly any profile of genomic satDNA. The library model of satDNA evolution explains the occurrence of species-specific satellite profiles as a result of differential amplifications and/or contractions within a collection, or library, of satellite sequences shared by related species (Fry and Salser 1977; Meštrović et al. 1998; Ugarković and Plohl 2002). Not only distinct satDNAs but also monomer variants or subfamilies from a single family can be distributed in genomes in the form of a library (Cesari et al. 2003).

SatDNAs have been extensively studied in insects (Palomeque and Lorite 2008) and mammals (Enukashvily and Ponomartsev 2013), and less so in other taxa, although there are several exceptions. Squamata, by far the largest reptile order, is one of them (see, for example, Giovannotti et al. 2009, 2013; Chaiprasertsri et al. 2013). It includes the Lacertidae, a widespread species-rich group restricted to the Palearctic region, formed by two subfamilies, Gallotiinae and Lacertinae (Arnold et al. 2007; Sindaco and Jeremčenko 2008). So far, five satDNA families have been described in Lacertinae, with different taxonomic distributions. Three satellite families are genus-specific, namely, pLHS in *Podarcis* (Capriglione et al. 1994; Capriglione 2000), CLsat in *Darevskia* (Ciobanu et al. 2003; Grechko et al. 2006), and Agi160 in *Lacerta* (Ciobanu et al. 2004; Grechko et al. 2005). The other two families, on the contrary, are broadly distributed in Lacertinae: pLCS, shared by *Algyroides*, *Teira*, *Lacerta*, and *Podarcis*

(Capriglione et al. 1989, 1991; Capriglione 2000), and pGPS, present in *Podarcis*, *Archaeolacerta*, *Algyroides*, *Lacerta*, and *Zootoca* (Capriglione et al. 1998).

In a previous work (Giovannotti et al. 2014) we isolated two new satDNA families in the lacertid genus *Iberolacerta*, a monophyletic group of rock lizards mainly distributed in highland areas of Western Europe. This genus comprises eight species, which can be subdivided into three main units: (1) *I. horvathi*, occurring in the Eastern Alps and the north of the Dinaric Chains; (2) the subgenus *Pyrenesaura*, which includes the three species found in the Pyrenees, (*I. aranica*, *I. aurelioi*, and *I. bonnali*); and (3) the four species included in the "Iberian group" (*I. cyreni*, *I. martinezricai*, *I. galani*, and *I. monticola*), with disjunct distributions in central and northern mountain ranges of the Iberian Peninsula. Previous cytogenetic surveys of the *Iberolacerta* species (Capula et al. 1989; Odierna et al. 1996; Arribas and Odierna 2004; Arribas et al. 2006; Rojo et al. 2014) showed them to possess a diploid number of 2n=36, and a similar karyotypic macrostructure, with all chromosomes acrocentric. Only the karyotypes of the three Pyrenean species differ from this formula, with reduced diploid numbers that range from 2n=24 to 26 in males and from 2n=23 to 26 in females, and many biarmed chromosomes that probably evolved from the ancestral acrocentric complement through a series of Robertsonian fusions (Odierna et al. 1996).

According to the most recently published phylogeny (Arribas et al. 2014), speciation within *Iberolacerta* started *ca.* 13.5 million years ago (Mya; 95 % credibility interval 11.6–15.6), with the split between the clades formed by *I. horvathi* and the Iberian group, on one side, and by the Pyrenean species, on the other. This event was most likely quickly followed by the separation of *I. horvathi*, which took place approximately 11.5 Mya (9.6–13.7). Within the Iberian group, *I. cyreni* split earlier (7.3–8.5 Mya), while the speciation events within the clade formed by *I. martinezricai*, *I. galani*, and *I. monticola* occurred considerably later, at the beginning of the Pleistocene, 2.1–2.9 Mya. The three Pyrenean species probably originated in rapid succession *ca.* 3.8 Mya (2.7–4.9), although this phylogenetic analysis suggests that *I. bonnali* split first, shortly before the separation between *I. aranica* and *I. aurelioi*, 3.3 Mya (2.3–4.3). Notwithstanding minor uncertainties still remaining, the mapping of satDNA differences on that species tree is likely to provide valuable information about the time and mode of evolution of these repetitive sequences. In our previous work (Giovannotti et al. 2014), we analyzed two unrelated satDNA arrays in the Iberian clade of *Iberolacerta*: (1) the centromeric HindIII family, which comprises two subfamilies (I and II) and represents 5–10 % of the genome and (2) the TaqI family, which shows only interstitial loci and represents 2.5–5 % of the genome. The nucleotide sequences of the two families were presumably evolving at different rates, almost tenfold higher for centromeric than for instertitial repeats, after comparing *I. cyreni* vs. the other, relatively closer, species of the Iberian clade. In agreement with this conclusion, the HindIII family seems to be specific to the genus *Iberolacerta* (Capriglione et al. 1989, 1991, 1998; Capriglione 2000), whereas the TaqI satDNA has also been detected in representatives of three other genera of the subfamily Lacertinae (*Lacerta*, *Podarcis*, and *Timon*).

Here, we extend the study of both satDNAs to the remaining *Iberolacerta* species, and increase our dataset for HindIII satDNA, to further investigate the occurrence of two divergent subfamilies in the genomes of all these taxa. The results obtained offer a more complete portrait of the intra- and interspecific variability of these highly repetitive sequences and their genomic organization and chromosomal distribution, with the ultimate objective of contributing to assess the relative strength of the processes that determine their structure and mode of evolution.

Material and methods

Animals

Genomic DNA was isolated from a total of 20 specimens, representing all eight *Iberolacerta* species. The number of specimens per species and their geographical origin are given in Supplementary Table 1. In addition, one male and one female of *I. horvathi* and one female of *I. bonnali* were used to make metaphase chromosomes.

DNA extraction, PCR, cloning and sequencing

Genomic DNA was extracted from ethanol preserved tissues using standard protocols with proteinase K digestion followed by phenol/chloroform extraction (see Sambrook et al. 1989). Two primer pairs designed in our previous work (HindIII-F: 5′-

TGAGTGTTTTACAGTTGAAAAGCT-3′; HindIII-R: 5′-CATTGTGTTATTTGAGCGCAA-3′; TaqI-F: 5′-ATTCTGACCCTGGGGGTTAG-3′; TaqI-R: 5′-CATATTTAAAGAAATCAGGCCTCG-3′) were used for isolation of both satellite families from the genomes of *I. horvathi*, *I. bonnali*, *I. aranica*, and *I. aurelioi*. An additional primer pair was designed to specifically amplify HindIII-subfamily II in all eight *Iberolacerta* species (Hind_sfII-F: 5′-CTCTTGCTTATTTCGCTCCAAATGA-3′; Hind_sfII-R: 5′-ATTTCTGTGTGCAGCATGCATTGG-3′). PCR reactions were performed in a final volume of 25 μl containing ~25 ng of genomic DNA, 0.625 U of *Taq* DNA polymerase and 1× PCR buffer (Roche Diagnostics), 5 nmol of each dNTP (Roche Diagnostics), and 20 pmol of each primer. The general reaction conditions were as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at the following temperatures (HindIII-F/HindIII-R, 55 °C; TaqI-F/TaqI-R, 47 °C; Hind_sfII-F/Hind_sfII-R, 58 °C) for 30 s, extension at 72 °C for 30–60 s, and a final extension at 72 °C for 7 min. The obtained PCR products were run on 1.5 % agarose gels; DNA in bands of interest was eluted using Pure Link Quick Gel Extraction Kit (Invitrogen) and cloned in the T&A cloning vector with T&A cloning kit (Yeastern Biotech) following manufacturer's recommendations. Positive clones were selected through PCR amplification using the M13 forward and M13 reverse primers. Bidirectional sequencing with the M13 primers was performed on an ABI PRISM 3730XL (Applied Biosystems) automatic sequencer.

Sequence analysis

The newly sequenced repeats were analyzed together with the previously reported sequences of the HindIII and TaqI satDNA families from *I. cyreni*, *I. monticola*, *I. galani*, and *I. martinezricai* (DDBJ/EMBL/GenBank accession numbers for HindIII: from KF453637 to KF453681; accession numbers for TaqI: from KF453682 to KF453723) (Giovannotti et al. 2014). Multiple sequence alignment was performed with MUSCLE (Edgar 2004), using default parameters, as implemented in Geneious version 8.0.5 (Kearse et al. 2012). After visual inspection of alignments, sequences were classified into different sets according to shared nucleotide changes and indels.

Intraspecific nucleotide diversity (π) was estimated using DnaSP v. 5 (Librado and Rozas 2009). Net average genetic distances between groups were calculated using the Maximum Composite Likelihood model (Tamura et al. 2004) in MEGA v. 6.0 (Tamura et al. 2013). Sequence variability among satellite repeats was further investigated by performing a factorial correspondence analysis (FCA), carried out with Genetix v. 4.05.2 (Belkhir et al. 2004). For this analysis, we constructed a matrix with all the sequences, where the nucleotide present at each diagnostic position was coded with a unique integer (100, 120, 140, or 160).

For the subsequent phylogenetic analysis, a consensus sequence was obtained for each sequence set by choosing the most frequent nucleotide at each position, except when a combination of dinucleotides of the three pairs CpG, CpA, and TpG was present at the same doublet position. In that case, the CpG dinucleotide was chosen as the consensus unless the T or A nucleotides were present in >70 % of the sequences. A phylogenetic network of the consensus sequences was constructed with TCS v. 1.21 (Clement et al. 2000) using the statistical parsimony algorithm under the 95 % parsimony criterion (Templeton et al. 1992).

Chromosome analysis

Metaphase chromosome spreads were prepared as described previously (Giovannotti et al. 2014). As for *I. horvathi*, individuals of this species were induced to autotomize their tail tips, the tissues were collected in the field following the protocol by Waters et al. (2008) and transferred to the laboratory for the establishment of primary cell cultures. For fluorescence in situ hybridization (FISH) experiments, we developed species-specific probes obtained by PCR amplification of HindIII and TaqI satDNA clones. The probes were labeled either with Cy3, using a PCR labeling kit (Jena Bioscience), or with FITC, using the Platinum Bright 495 labeling kit (KREATECH Biotechnology). Slide pretreatment, denaturation, hybridization, post-hybridization washes, and detection were performed according to Schwarzacher and Heslop-Harrison (2000). Images were captured using the epifluorescence microscopes (Nikon Microphot-FXA; Leica Leitz

DMRBE) equipped with monochrome cameras (Nikon DS-Qi1Mc; JAI CV-M4+CL). The NIS-Elements D 3.10 (Nikon Instruments) and Leica CytoVision version 7.2 (Leica Microsystems) softwares were used to process the images and reconstruct the karyotypes.

Results

Isolation and characterization of satellite DNAs

PCR amplification using primers specific for HindIII and TaqI satDNA was successful in all tested species and produced a ladder-like banding pattern, which is typical for satellite DNA. PCR products included complete monomers and multimers (from dimers up to hexamers), flanked by partial monomer sequences. Only clones with complete repeat units were sequenced and, for further analyses, multimers were separated into individual monomers. A total of 187 new sequences were obtained for HindIII, whereas 109 clones were sequenced for TaqI. Comparison of these new sequences with the HindIII and TaqI monomers isolated from *I. cyreni*, *I. monticola*, *I. galani*, and *I. martinezricai* in our previous study (Giovannotti et al. 2014) indicated that all of them belong to the same satDNA families. Altogether, our dataset comprises 232 HindIII and 151 TaqI monomers from all eight *Iberolacerta* species, which are likely to reflect the overall variability of the two satellite families in the genus.

Both HindIII and TaqI satDNAs are characterized by an AT bias (average AT content of 58.9 and 59.1 %, respectively) and by the occurrence of short repeat motifs such as A and T stretches, dinucleotide TG and CA, and trinucleotide CAA and TTC (Supplementary Figs. 1a, b). The size of HindIII repeats ranged between 169 and 172 bp, with the exception of two monomers with lengths of 151 bp (IAR_99b) and 161 bp (ICY_209c) (Table 1). TaqI repeats showed a broader range of length variation, from 155 to 191 bp (Table 1). Several indels varying in size from 1 to 31 bp are the causes of the repeat length variation in this satDNA family.

After alignment, monomers within each satDNA family were classified into subfamilies, according to the state of diagnostic positions, characterized by nucleotide substitutions or indels shared by at least 90 % of all the members grouped in the same subfamily. The subfamilies were designated with Roman numerals following the nomenclature previously used in Giovannotti et al. (2014) for HindIII subfamilies I and II. Additional diagnostic positions further divided each subfamily into several sequence groups and subgroups, denoted by a Latin letter and a numeral, respectively, after the subfamily name (Table 2).

Sequence variability within HindIII satDNA

Within HindIII satDNA, we found a total of 30 diagnostic positions, which identified three subfamilies—namely HI, HII, and HIII—and 27 sequence groups (Table 2a and Supplementary Fig. 1a). Their abundances ranged from 1.3 to 17 % (3–39 representatives) of the examined sequences. Figure 1a overlies data on the abundance and distribution of HindIII sequence groups onto a phylogenetic tree for *Iberolacerta* derived from mitochondrial markers (Arribas et al. 2014). As evidenced in this figure, sequence groups were not equally represented in the different species. The Pyrenean species (*I. aurelioi*, *I. aranica*, and *I. bonnali*) harbor a wide diversity of HindIII repeats, mainly belonging to subfamilies HI and HII. Only 12 monomers were retrieved from *I. horvathi*, and they are all members of subfamily HI. Similarly, subfamily HI is also the most abundant variant of the HindIII family in the Iberian species *I. martinezricai*, *I. monticola*, and *I. galani*. A strikingly different profile of HindIII repeats was found in *I. cyreni*, also an Iberian species, which is characterized by the presence of several private sequence groups belonging to subfamily HIII and one exclusive sequence group within subfamily HI.

The coexistence of more than one subfamily explains the higher nucleotide diversity values (π) in species such as *I. bonnali* (4.91%) or *I. aurelioi* (3.96%), in comparison with the values obtained for those species in which all their HindIII repeats belonged to a single subfamily, i.e., *I. horvathi* (1.16%) and *I. martinezricai* (1.51%) (Table 1). Interestingly, despite their different abundances, mean π values for each subfamily were roughly similar (from 2.30 % in subfamily HII to 2.54 % in subfamily HIII).

The factorial correspondence analysis (FCA) based on diagnostic positions highlighted the differentiation among the three HindIII subfamilies, lending further support to our classification. Altogether, the three main axes of variation explain 96.53 % of the observed variation (Fig. 2a). The most informative is axis 1 (69.70 %), which identifies two main clusters,

Table 1 Summary of repeat features of HindIII and TaqI satDNA

	HindIII				TaqI			
Species	Subfamily	n	Repeat length	Nucleotide diversity (π)	Subfamily	n	Repeat length	Nucleotide diversity (π)
I. monticola	All combined	34		0.0151±0.0018	All combined	10		0.0600±0.0089
	HI	30	171	0.0142±0.0023	TI	10	171–188	0.0600±0.0089
	HII	4	170	0.0177±0.0060				
I. galani	All combined	31		0.0331±0.0040	All combined	16		0.0489±0.0001
	HI	23	171	0.0148±0.0019	TI	16	186–188	0.0489±0.0001
	HII	8	169–170	0.0211±0.0082				
I. martinezricai	All combined	33		0.0151±0.0018	All combined	7		0.0541±0.0103
	HI	33	171–172	0.0151±0.0018	TI	7	187–188	0.0541±0.0103
I. cyreni	All combined	40		0.0356±0.0037	All combined	9		0.0406±0.0001
	HI	7		0.0180±0.0030	TI	9	186–187	0.0406±0.0001
	HIII	33	161–171	0.0240±0.0029				
I. horvathi	All combined	12		0.0116±0.0028	All combined	33		0.1218±0.0079
	HI	12	171	0.0116±0.0028	TI	31	167–191	0.1184±0.0083
					TII	2	189 - 191	0.0699±0.0349
I. aurelioi	All combined	25		0.0396±0.0034	All combined	20		0.0976±0.0086
	HI	14	171	0.0290±0.0048	TI	1	187	
	HII	11	170	0.0262±0.0026	TII	19	177–188	0.0908±0.0074
I. aranica	All combined	22		0.0355±0.0043	All combined	34		0.1209±0.0070
	HI	7	151–171	0.0265±0.0055	TI	14	175–190	0.1082±0.0126
	HII	15	170	0.0164±0.0028	TII	20	177–190	0.0960±0.0059
I. bonnali	All combined	35		0.0491±0.0050	All combined	22		0.1204±0.0096
	HI	17	171	0.0257±0.0027	TI	17	155–188	0.1060±0.0102
	HII	15	169–170	0.0230±0.0076	TII	5	177–190	0.0983±0.0156
	HIII	3	171	0.0195±0.0033				
All species combined	HI	143		0.0241±0.0015	TI	105		0.1342±0.0060
	HII	53		0.0230±0.0018	TII	46		0.0961±0.0044
	HIII	36		0.0254±0.0029				
	TOTAL	232		0.0539±0.0020	TOTAL	151		0.1567±0.0038

Number of monomeric repeats sequenced (n), length of repeats (expressed in base pairs), and nucleotide diversities (π)±S.E. for both satDNAs for each *Iberolacerta* species investigated

corresponding to subfamily HIII repeats of *I. cyreni* and *I. bonnali* on one side, and to subfamilies HI and HII on the other. Axis 2, which accounts for 24.60 % of the observed variation, separates subfamilies HI and HII. Finally, axis 3, with 2.23 % of the observed variation, probably corresponds to sequence heterogeneity within each subfamily. The clustering of HindIII repeats revealed by the FCA matches the estimates of interspecies and inter-subfamilies net genetic distances, shown in Table 3a. Monomers of subfamily HIII are the most divergent, with average genetic distances of 7.50 and 9.90 % from subfamily HI and HII, respectively. These values are substantially higher than the average distance between subfamilies HI and HII (around 4.0 %). When *I. cyreni* is excluded from the analysis, pairwise interspecies genetic distances within each subfamily are all very low and uncorrelated with relative divergence times between species, with average values of 1.0 % within subfamily HIII, 0.34 % within subfamily HII, and 0.33 % within subfamily HI. Net genetic distances

Table 2 Nucleotide differences among the consensus sequences of the different groups of (a) HindIII subfamilies HI, HII, and HIII, and (b) TaqI subfamilies TI and TII. The second row refers to base positions relative to the alignment shown in Supplementary Fig. 1a (HindIII) and 1b (TaqI). The general consensus sequence of each satDNA was used as reference. Dots indicate identity with this reference sequence

a)

	1	2	3	4	6	7	8	10	11	12	13	14	15	17	18	19	20
Positions	14	15	21	27	38	39	56	73	83	84	85	86	87	95	98	99	101
Consensus	T	C	A	T	T	T	C	A	A	A	T	T	T	C	T	G	A
HI_A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HI_B	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
HI_C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
HI_D1	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
HI_D2	.	.	G	.	.	.	T	.	.	.	C	.	.	.	.	.	.
HI_E	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.
HI_F	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
HI_G	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.
HI_H	.	.	.	C	.	.	.	.	.	C	C	.	.	.	.	.	.
HI_I	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
HI_J	.	G	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.
HI_K	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
HI_L	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.
HI_M	.	.	.	C	.	G	T	.	.	.	.	.	G	.	.	.	.
HII_A	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.
HII_B	.	.	.	C	.	G	T	.	C	.	.	.	.	.	.	.	G
HII_C	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.
HII_D	.	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	.
HII_E	A	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	.
HII_F	.	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	.
HII_G	.	.	G	.	C	.	T	.	C	.	.	.	.	.	.	.	G
HII_H	.	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	G
HIII_A	.	.	.	C	.	G	T	G	C	.	.	.	G	A	C	A	.
HIII_B	.	.	.	C	.	G	T	G	C	.	.	.	G	A	C	A	.
HIII_C	.	.	.	C	.	G	T	G	C	.	.	.	G	A	C	A	.
HIII_D	.	.	.	C	.	G	T	G	C	.	.	G	G	A	C	A	.
HIII_E	.	.	.	C	.	G	T	G	C	.	.	G	G	A	C	A	.

b)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Positions	1	5	6	9	14	16	17	18	19	20	21	22	31	33	43	54	56	58	59	60	61	66	71	72
Consensus	C	G	C	G	C	T	A	A	C	C	T	A	A	C	C	T	T	C	C	A	G	G	C	G
TI_A1	.	.	.	.	.	.	C	.	G	G	.	.	.	.	T	.	C	.	.	G	C	.	T	.
TI_A2	.	.	.	.	.	.	C	.	G	G	.	.	.	.	T	.	C	.	.	G	C	.	T	.
TI_B1	.	.	.	.	.	.	C	.	G	G	.	.	.	.	T	.	C	.	.	.	C	.	T	.
TI_B2	.	.	.	.	.	.	C	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TI_C1	G	.	T	T	.	.	C	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.

Table 2 (continued)

TI_C2	.	.	.	.	.	.	C	.	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
TI_D	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
TI_E	.	.	.	.	.	.	G	.	.	G	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.
TI_F1	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
TI_F2	.	.	.	.	.	.	G	.	.	.	C	.	G	.	.	.	.	.	.	.	.	.	.	.	.
TI_G1	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TI_G2	.	.	.	C	.	.	G	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
TI_H1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	C	
TI_H2	.	.	.	.	.	.	T	.	A	A	.	.	G	.	T	.	.	.	.	.	.	C	.	C	
TI_I1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	T	.	T	C	.	A	
TI_I2	.	.	.	.	.	.	T	.	A	A	.	.	G	.	.	.	.	.	T	.	T	C	.	A	
TI_J1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	A	
TI_J2	.	.	.	.	.	.	T	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	A	
TI_K	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	C	
TI_L1	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	C	
TI_L2	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	C	.	A	
TI_L3	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	.	.	.	.	.	.	.	.	A	
TII_A	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	
TII_B1	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	
TII_B2	.	.	.	.	T	C	.	T	.	.	.	.	G	.	.	C	.	T	.	.	.	.	T	.	
TII_C1	.	.	.	.	T	T	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	
TII_C2	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	
TII_D	.	.	.	.	T	C	.	T	.	G	.	.	.	G	.	C	A	T	.	.	.	.	T	.	
TII_E1a	.	.	.	.	T	C	.	T	.	.	.	G	.	.	.	C	.	T	.	.	.	.	T	.	
TII_E1b	.	.	.	.	T	C	.	T	.	.	.	G	.	.	.	C	.	T	.	.	.	.	T	.	
TII_E2	T	.	.	.	T	C	.	T	.	.	.	G	.	.	.	C	.	T	.	.	.	.	T	.	
TII_F	.	.	.	.	T	C	.	T	.	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	
TII_G1	.	.	.	.	T	C	.	T	.	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	
TII_G2a	T	.	T	.	T	C	.	T	.	.	.	.	.	A	.	C	.	T	.	.	.	.	T	.	
TII_G2b	T	.	T	.	T	C	T	T	.	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	
TII_G3	.	T	.	.	T	C	.	T	G	.	.	.	.	G	.	C	.	T	.	.	.	.	T	.	
TII_G4	T	A	.	.	T	C	.	T	.	.	.	.	.	.	.	C	.	T	.	.	.	T	T	.	

a)

	21	22	23	24	26	27	28	29	30	31	32	33	35
Positions	113	114	117	119	129	140	144	145	147	149	150	151	165
Consensus	G	A	T	A	T	G	C	A	A	G	A	G	-
HI_A	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_B	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_C	.	.	.	.	.	.	.	.	.	.	.	.	-
HI_D1	.	.	.	.	.	.	.	.	.	.	.	.	-

Table 2 (continued)

HI_D2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_F	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_J	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_K	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_L	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HI_M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HII_A	.	.	.	C	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	A	.
HII_B	.	.	.	G	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	A	.
HII_C	.	.	.	G	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	C	.	.	.	A	.
HII_D	.	.	.	G	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	C	.	.	.	A	.
HII_E	.	.	.	G	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	A	.
HII_F	.	.	.	G	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	A	.
HII_G	.	.	.	G	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	A	.
HII_H	.	.	.	G	.	.	.	.	.	A	.	-	-	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	A	.
HIII_A	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HIII_B	A	.	T	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HIII_C	A	.	G	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HIII_D	A	.	G	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
HIII_E	A	.	G	.	.	C	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
b)																															
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50					
Positions	83	93	101	110	114	117	120	121	123	125	126	127	139	142	150	157	163	167	168	169	170	175	181	184	187	188					
Consensus	G	T	T	G	G	T	T	G	T	A	A	A	C	G	T	C	A	A	G	T	T	G	T	G	G	T					
TI_A1	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	T	.	.	.	T	G	.	.	.					
TI_A2	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	T	.	.	.	T	G	.	C	.					
TI_B1	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	G	.	.	.					
TI_B2	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	G	.	.	.					
TI_C1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.					
TI_C2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.					
TI_D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.					

Table 2 (continued)

TI_E	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	A	A	T	.	.	.	.			
TI_F1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			
TI_F2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			
TI_G1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			
TI_G2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			
TI_H1	.	.	.	.	A	.	C	A	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			
TI_H2	.	.	.	.	A	.	C	A	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			
TI_I1	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			
TI_I2	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			
TI_J1	.	.	.	.	A	.	C	A	.	.	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.			
TI_J2	.	.	.	.	A	.	C	A	.	.	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.			
TI_K	.	.	.	.	A	.	C	A	.	.	.	.	.	C	.	.	.	.	.	.	.	T	G	.	.	.			
TI_L1	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			
TI_L2	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			
TI_L3	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			
TII_A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G			
TII_B1	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	A	.	.	G			
TII_B2	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	A	.	.	G			
TII_C1	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G			
TII_C2	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G			
TII_D	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-	-	-	.	.	.	.	C	.	G			
TII_E1a	.	G	.	.	.	C	.	.	.	G	G	.	.	.	.	.	.	T	C	.	.	.	.	.	.	G			
TII_E1b	.	.	C	.	.	C	.	.	.	G	G	.	.	.	.	.	.	T	C	.	.	.	.	.	.	G			
TII_E2	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G			
TII_F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G			
TII_G1	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.			
TII_G2a	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	G			
TII_G2b	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	G			
TII_G3	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	G			
TII_G4	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	A	.	.	G			

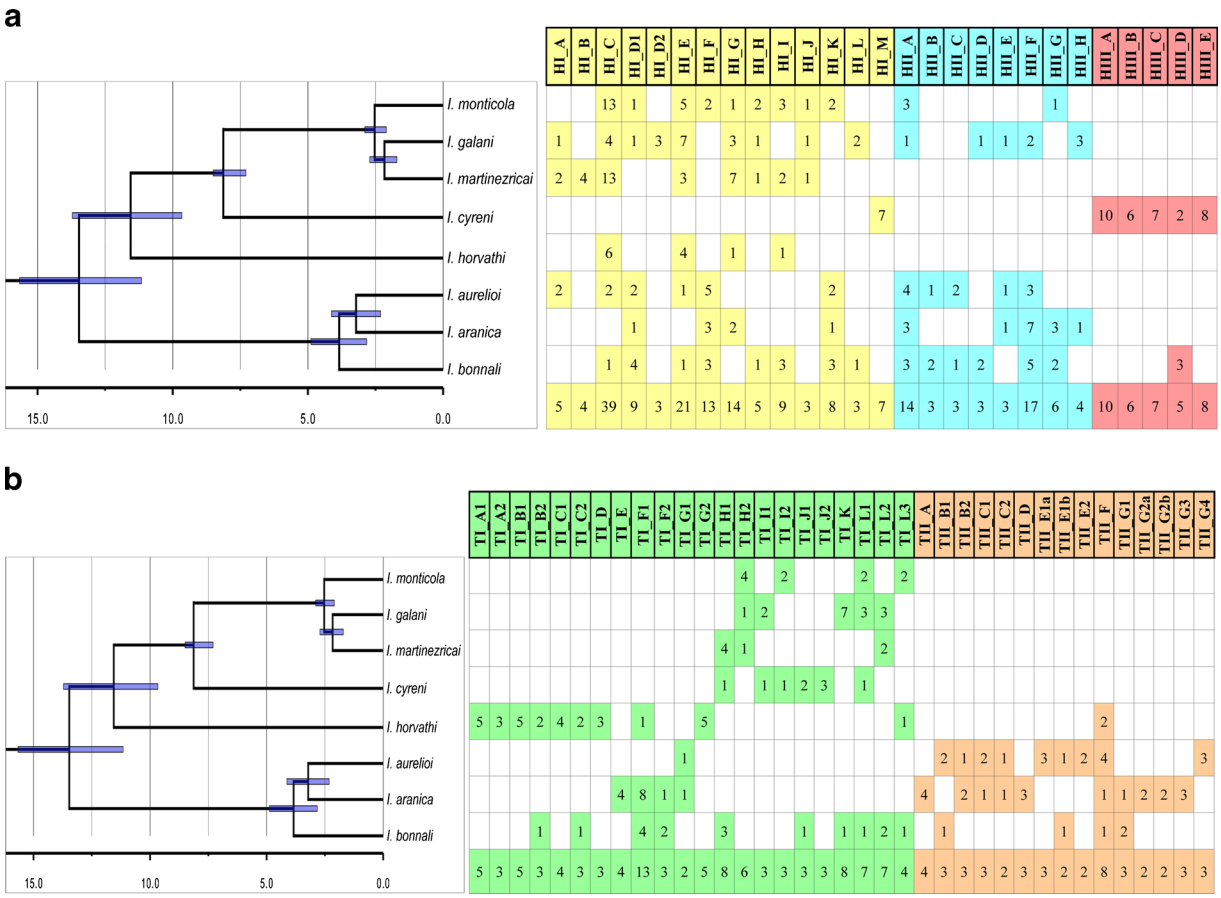


Fig. 1 Distribution and abundance of HindIII (**a**) and TaqI (**b**) subfamilies in *Iberolacerta* coupled to a Bayesian tree obtained from two mitochondrial loci (Cyt *b*, cytochrome *b*; CR, control region) (adapted from Arribas et al. 2014). *Node bars* indicate 95 % credibility intervals (regions of highest posterior density) for the corresponding divergence time (in million years). *Numbers in the table* indicate the number of repeats of each subfamily retrieved from each species. Colors identify different subfamilies

between HI repeats involving *I. cyreni* are always considerably higher (from 2.0 % between *I. cyreni* and *I. aranica* to 3.40 % between *I. cyreni* and *I. horvathi*).

Sequence variability within TaqI satDNA

From the alignment of TaqI sequences, we identified a total of 50 diagnostic positions, which defined two main subfamilies—namely TI and TII—and 37 sequence groups, whose abundances ranged from 1.3 to 8.5 % (2–13 representatives) of the examined sequences (Table 2b and Supplementary Fig. 1b).

In general, the species of the Iberian clade were characterized by the presence of TaqI repeats belonging only to subfamily TI (Fig. 1b), with a substantial proportion of private sequence groups (four groups, comprising 15 out of 42 sequences). Conversely, subfamily TII is essentially characteristic of the subgenus *Pyrenesaura*, although it has been residually observed also in *I. horvathi*. This subfamily appears to be the most abundant variant in the genomes of *I. aranica* and, above all, *I. aurelioi*, which show both species-specific and shared sequence groups. The sampled loci from *I. bonnali* and *I. horvathi* contain mostly T1 repeats. However, the clustering pattern of TI repeats differs markedly between the two species: while all the monomers retrieved from *I. bonnali* were grouped together with monomers from other species, *I. horvathi* shows the highest proportion of species-specific repeats (25 out of 33), allocated to six private sequence groups.

As expected from the distribution of subfamilies TI and TII in the genomes of the *Iberolacerta* species, intraspecific nucleotide diversity values

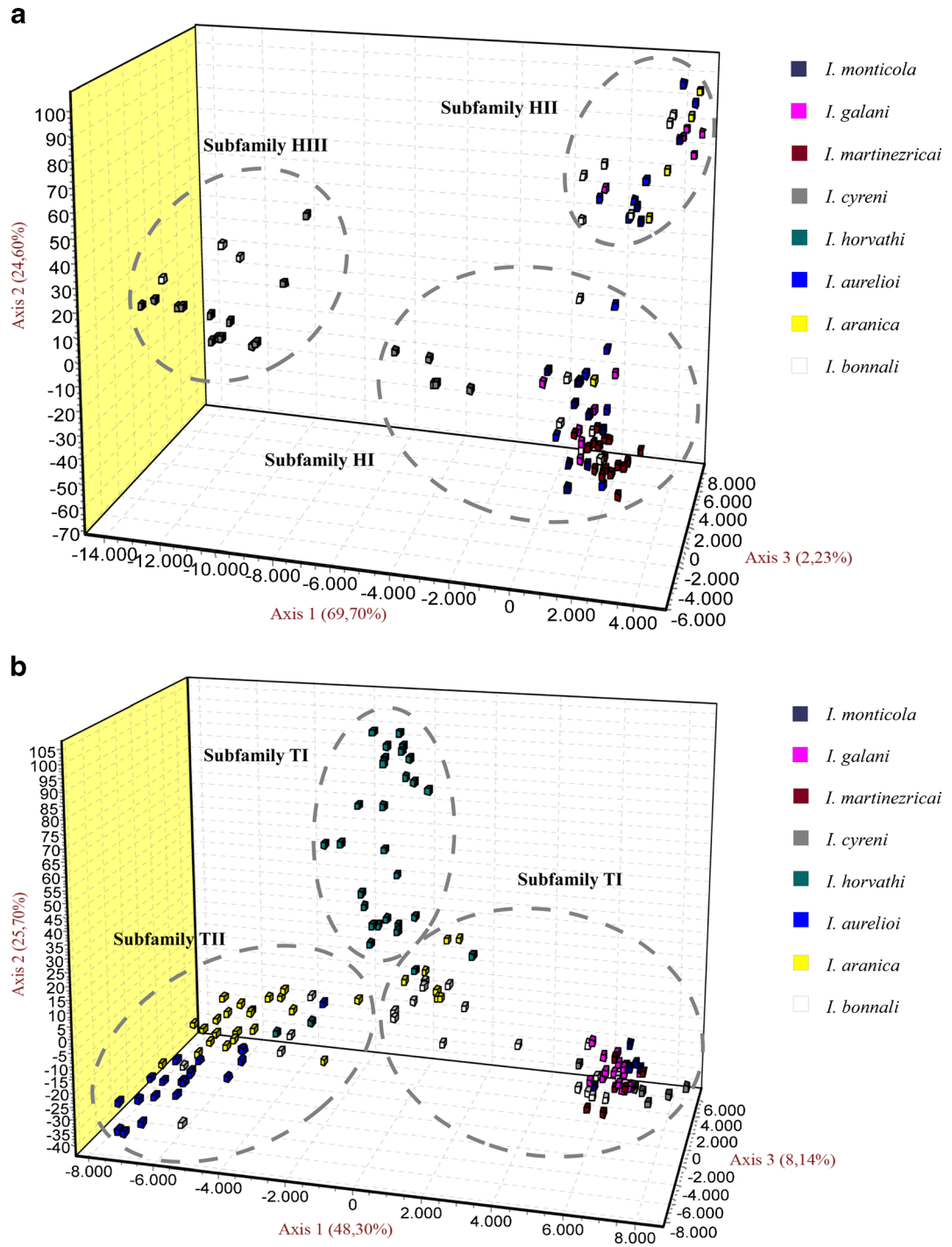


Fig. 2 Three-dimensional representation of a factorial correspondence analysis based on monomeric sequences of HindIII (**a**) and TaqI (**b**) satDNAs

are higher for *I. horvathi* and the Pyrenean species, which harbor both types of TaqI repeats in their genomes (Table 1). When each subfamily is analyzed separately, π values within subfamily TI are two- to threefold greater in these species than in the species of the Iberian clade (from 4.06 % in *I. cyreni* to 11.84 % in *I. horvathi*). High

Table 3 Interspecific and inter-subfamily net genetic distances for HindIII (a) and TaqI (b) repeats. Standard error estimates are shown above the diagonal. Color codes represent the different types of HindIII and TaqI subfamilies. Asterisks in b indicate those values obtained in comparisons involving IAU_TI, represented by only one sequence

a

	IGA HI	IMR HI	IAU HI	IHO HI	IMO HI	IBN HI	IAR HI	ICY HI	IGA HII	IBN HII	IAU HII	IAR HII	IMO HII	ICY HIII	IBN HIII
IGA_HI		0.001	0.003	0.001	0.000	0.002	0.003	0.011	0.017	0.016	0.016	0.017	0.016	0.023	0.026
IMR_HI	0.001		0.004	0.001	0.000	0.003	0.004	0.013	0.018	0.017	0.017	0.017	0.017	0.024	0.026
IAU_HI	0.006	0.008		0.005	0.003	0.000	0.001	0.011	0.015	0.013	0.013	0.014	0.012	0.023	0.025
IHO_HI	0.001	0.001	0.009		0.000	0.003	0.005	0.015	0.019	0.018	0.017	0.018	0.018	0.026	0.028
IMO_HI	0.000	0.001	0.005	0.000		0.002	0.003	0.012	0.017	0.016	0.016	0.017	0.016	0.024	0.026
IBN_HI	0.003	0.005	0.000	0.006	0.003		0.001	0.010	0.016	0.014	0.013	0.015	0.012	0.022	0.025
IAR_HI	0.005	0.006	0.001	0.008	0.004	0.001		0.010	0.016	0.014	0.013	0.015	0.013	0.021	0.024
ICY_HI	0.026	0.028	0.023	0.034	0.029	0.022	0.020		0.022	0.018	0.019	0.021	0.018	0.016	0.020
IGA_HII	0.047	0.048	0.039	0.050	0.046	0.042	0.041	0.065		0.001	0.004	0.001	0.007	0.032	0.030
IBN_HII	0.041	0.044	0.031	0.047	0.042	0.034	0.032	0.051	0.002		0.000	0.001	0.002	0.029	0.030
IAU_HII	0.038	0.042	0.026	0.044	0.038	0.029	0.028	0.051	0.007	0.000		0.002	0.001	0.029	0.030
IAR_HII	0.044	0.046	0.033	0.048	0.043	0.036	0.035	0.061	0.001	0.000	0.002		0.003	0.031	0.031
IMO_HII	0.039	0.043	0.024	0.045	0.038	0.027	0.026	0.049	0.012	0.004	0.000	0.010		0.028	0.030
ICY_HIII	0.077	0.080	0.074	0.086	0.080	0.073	0.068	0.043	0.115	0.102	0.101	0.112	0.100		0.005
IBN_HIII	0.088	0.090	0.086	0.097	0.091	0.085	0.080	0.063	0.104	0.103	0.105	0.106	0.108	0.011	

b

	IHO_TI	IBN_TI	IAR_TI	IAU_TI	IMR_TI	ICY_TI	IGA_TI	IMO_TI	IAR_TII	IAU_TII	IBN_TII	IHO_TII
IHO_TI		0.009	0.022	0.025	0.018	0.018	0.025	0.019	0.022	0.019	0.021	0.029
IBN_TI	0.033		0.004	0.027	0.006	0.006	0.004	0.008	0.020	0.021	0.015	0.019
IAR_TI	0.024	0.011		0.024	0.015	0.015	0.015	0.015	0.017	0.017	0.012	0.017
IAU_TI	0.100*	0.107*	0.084*		0.033	0.033	0.032	0.032	0.028	0.030	0.026	0.031
IMR_TI	0.066	0.014	0.050	0.152*		0.002	0.002	0.002	0.026	0.027	0.023	0.024
ICY_TI	0.070	0.016	0.051	0.154*	0.004		0.003	0.004	0.026	0.027	0.023	0.024
IGA_TI	0.064	0.014	0.049	0.147*	0.005	0.008		0.005	0.025	0.027	0.023	0.024
IMO_TI	0.061	0.016	0.047	0.146*	0.003	0.007	0.007		0.026	0.028	0.024	0.024
IAR_TII	0.062	0.075	0.056	0.122*	0.112	0.116	0.110	0.115		0.003	0.004	0.006
IAU_TII	0.066	0.075	0.056	0.128*	0.117	0.121	0.113	0.119	0.007		0.005	0.005
IBN_TII	0.044	0.054	0.036	0.104*	0.095	0.097	0.091	0.097	0.001	0.000		0.004
IHO_TII	0.055	0.057	0.046	0.120*	0.089	0.090	0.084	0.089	0.013	0.008	0.002	

π values were also obtained for subfamily TII in those species with a large number of monomers examined (9.08 % in *I. aurelioi* and 9.60 % in *I. aranica*).

The factorial analysis of TaqI monomers identified a main axis of variation (axis 1 at Fig. 2b, explaining 48.30 % of the observed variation), corresponding to the separation between three groups of repeats: (1) subfamily TII (i.e., essentially Pyrenesaura); (2) a subset of subfamily TI, including all the monomers of Iberian species and a few monomers of *I. bonnali*; and (3) a subset of subfamily TI, made up of monomers from *I. horvathi*, *I. aranica*, and *I. bonnali*. Axis 2 in the FCA, which accounts for 25.70 % of the total variation, separates a fourth group of repeats, comprising the remaining TI monomers of *I. horvathi*. Net genetic distances between repeats from the different species (Table 3b) give additional support to the FCA results. Leaving aside the comparisons involving the single monomer of TI in *I. aurelioi*, larger distances between T1 repeats correspond to pairs of the Iberian species with both *I. aranica* (4.70–5.10 %) and, above all, *I. horvathi* (6.10–7.0 %). As for the TII repeats, all the pairwise comparisons, involving the subgenus *Pyrenesaura* and *I. horvathi*, produce rather low values (0.0–1.30 %).

Organization of consecutive monomeric units

The cloning and sequencing of multimeric products allowed us to characterize the organization of consecutive monomeric repeats. In both satDNA families, and in all the species analyzed, we observed that adjacent monomers in a satellite array usually belong to different sequence groups and even to different subfamilies (for a list of all HindIII and TaqI composite arrays sampled in the *Iberolacerta* species, see Supplementary Tables 2 and 3, respectively).

Phylogenetic analysis

The statistical parsimony network obtained for HindIII satDNA showed a high degree of reticulation among the

members of subfamily HI (Fig. 3a). This pattern suggests that rearrangements due to recombination events are an important force generating new monomers in this subfamily —the most widespread among *Iberolacerta* species—, which occupies the central position of the parsimony network. Two sequence groups within this subfamily, HI_K and HI_M, branched into two separate lineages, corresponding to subfamilies HII and HIII, respectively. In contrast to subfamily HI, no evidence for recombination events has been found within subfamilies HII and HIII.

In the network of TaqI satDNA, all sequence groups converge on a group belonging to subfamily T1 (T1_ FI, Fig. 3b). The network shows a major separation of four clusters, connected to group TI_F1 by a few mutational steps. Three of them (T1_F2, T1_C2, and T1_G1, together with their related variants) include sequences only found in *I. horvathi* and in the subgenus *Pyrenesaura*. All sequence groups belonging to subfamily TII occupy a peripheral position within cluster G1. The extensive diversification within subfamily TII has been promoted, in some cases, by recombination events that created new monomer variants (e.g., TII_E1b or TII_G2a). Within the fourth cluster, the prolific lineage TI_L3 includes closely related sequence groups (separated by just one or two nucleotide changes), specific to the Iberian clade.

Chromosomal location of HindIII and TaqI satDNA families

FISH with HindIII satDNA probe on metaphase chromosomes of *I. monticola* and *I. galani* revealed that this repetitive element is present at centromeres of all the 36 chromosomes of the diploid complement (Fig. 4; Giovannotti et al. 2014). FISH on female metaphases

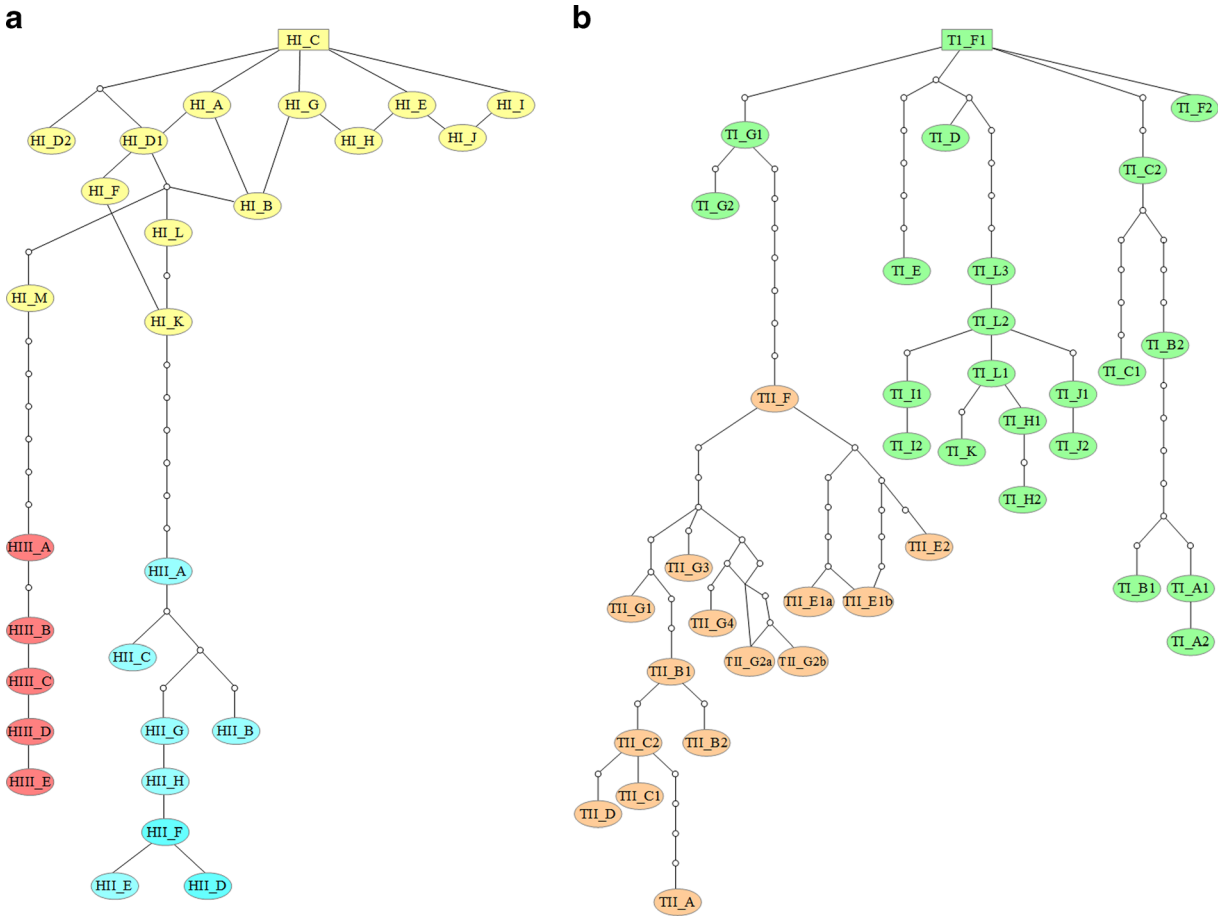


Fig. 3 Statistical parsimony network constructed from the consensus sequences of the different sequence groups of **a** HindIII satDNA and **b** TaqI satDNA

of *I. bonnali,* carried out in this work, showed hybridization signals in the centromeric regions of all the 23 chromosomes of the karyotype, although with variable signal strength in different chromosome pairs (Fig. 4). Moreover, the overall intensity of HindIII signals in *I. bonnali* was noticeably lower than in *I. monticola* and *I. galani*. No hybridization signals were observed in the chromosomes of *I. horvathi*.

FISH with TaqI satDNA probe in *I. monticola* and *I. galani* produced bright signals in interstitial position in a subset of 20 and 18 chromosomes, respectively (Fig. 5). In *I. bonnali*, similarly intense signals were detected interstitially on both arms of 10 meta-/submetacentric chromosomes. In some metaphases, an additional faint signal could be observed in a medium-sized chromosome pair (Fig. 5). In *I. horvathi*, strong hybridization signals were also observed in interstitial position but just in six chromosomes. However, after increased exposure times, 10 additional chromosomes appeared weakly labeled (Fig. 5).

Discussion

The turnover rate of a satDNA family is a complex feature that depends on many parameters, such as interchromosomal and intrachromosomal recombination rates, copy number and long-range organization of repeat units, genome location and distribution, putative functional interactions, reproductive mode, and population factors (Strachan et al. 1985; Dover 2002; Luchetti et al. 2003; Robles et al. 2004; Meštrović et al. 2006; Kuhn et al. 2008; Navajas-Pérez et al. 2009; Giovannotti et al. 2013). In consequence, sequence dynamics of satDNA families may differ not only among families but also, for a given family, among genomic regions (Kuhn et al. 2011), populations (Wei et al. 2014), species, or higher taxonomic groups (e.g., Macas et al. 2006; Kuhn et al. 2008; Martinsen et al. 2009; Plohl et al. 2010).

In agreement with Giovannotti et al. (2014), the results of the present work show that overall variability

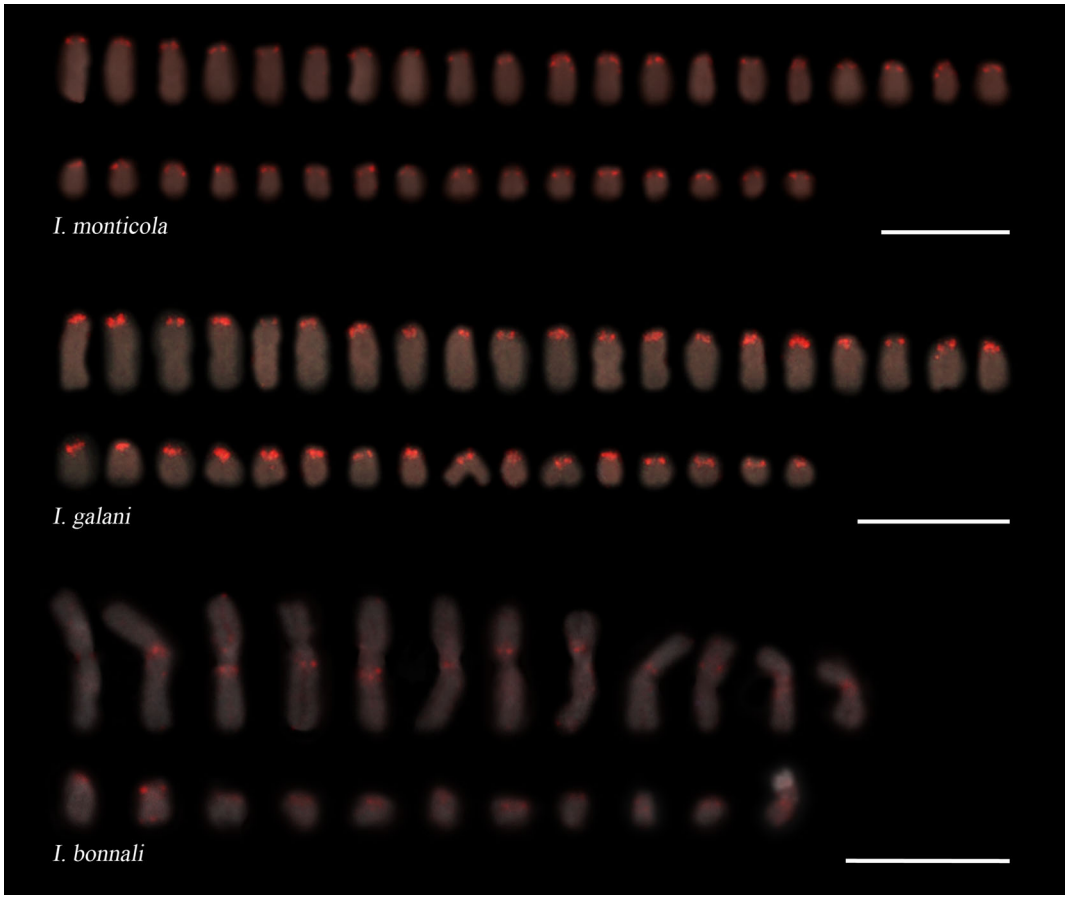


Fig. 4 Hybridization pattern of the HindIII probe in the karyotypes of *Iberolacerta monticola*, *I. galani* and *I. bonnali. Scale bar*=10 μm

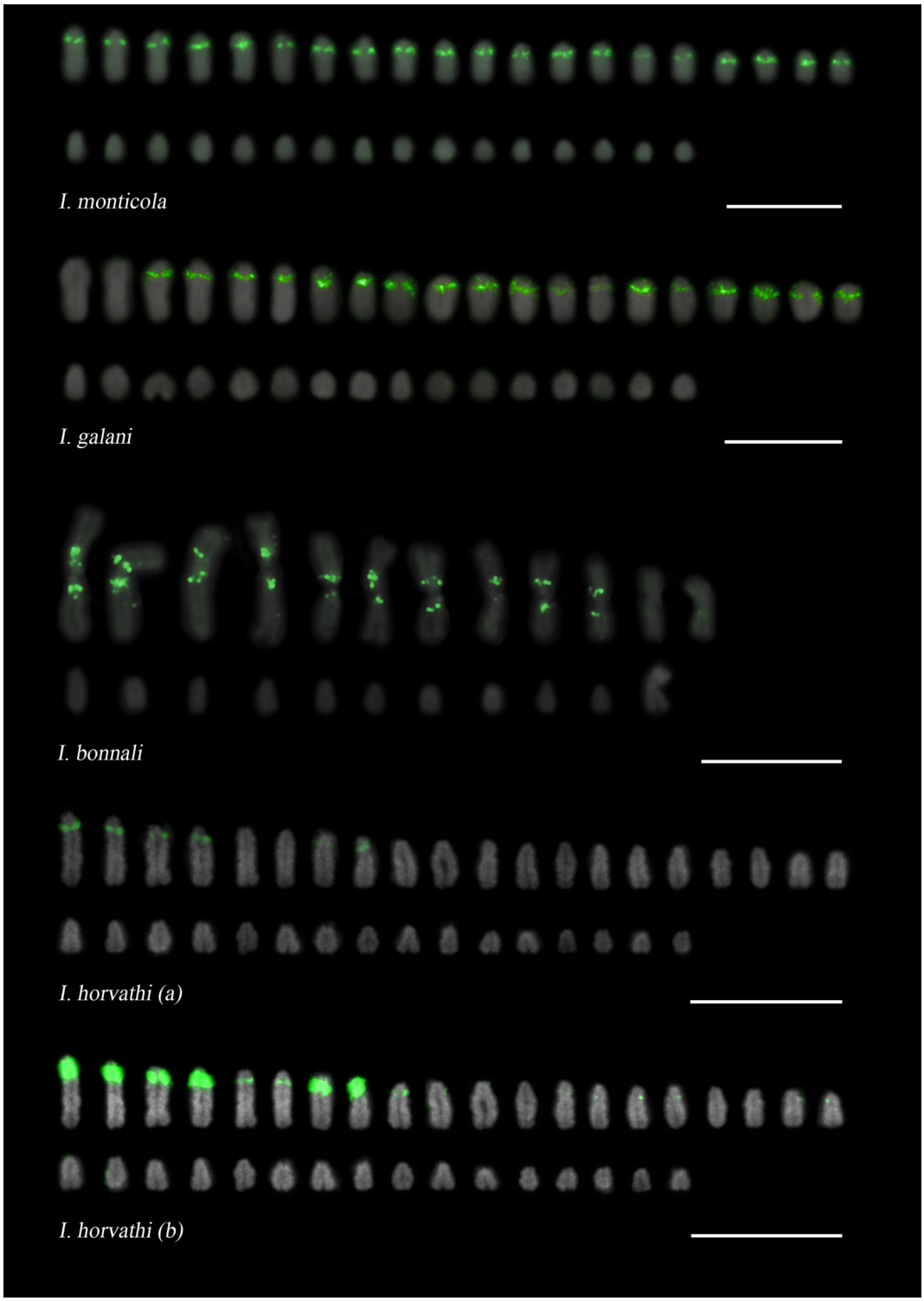


Fig. 5 Hybridization pattern of the TaqI probe in the karyotypes of *Iberolacerta monticola*, *I. galani*, *I. bonnali*, and *I. horvathi*. FISH signals on *I. horvathi* chromosomes are shown at standard (*a*) and increased (*b*) exposure times. *Scale bar*=10 μm

of TaqI repeats in the whole genus *Iberolacerta* is on average three times higher than the variability of HindIII repeats, which suggests a faster homogenization/fixation rate for the latter satDNA family. However, the detailed characterization of both satDNA families in all eight *Iberolacerta* species reveals that their evolutionary patterns are more complex than previously anticipated. The presence of HindIII_HI in all the species, and its central position in the phylogenetic network, suggests that this is the most ancestral variant of HindIII satDNA, from which subfamilies HII and HIII were derived. Interestingly, with the exception of *I. cyreni*, no intraspecific homogenization for any particular subfamily was detected in our study, and most different sequence groups of subfamilies HI and HII are widespread and shared by even distantly related species. Indeed, interspecific genetic distances within each subfamily are substantially lower than intraspecific genetic distances between repeats belonging to different subfamilies. On the contrary, *I. cyreni* shows a high proportion of private sequence groups belonging to subfamily HIII, and a well-differentiated subset of HI repeats, which explains the evidence of concerted evolution found for this species in our previous study. However, the finding of HIII repeats also in *I. bonnali* indicates that this subfamily is not exclusive of *I. cyreni*, but was already present in the common ancestral library of HindIII variants. Combining these data with the results of FISH experiments, the most parsimonious interpretation of HindIII satDNA evolution is that the diversification of HindIII repeats—which generated most of the extant variants—took place in the common ancestor of *Iberolacerta*, before species radiation, i.e., from 11.6 to 15.6 Mya (Arribas et al. 2014). In the ancestral species, HindIII satDNA might have been widely distributed in the centromeres of all chromosome pairs, with a subsequent decrease in copy number in *I. horvathi* and, at least, in the Pyrenean *I. bonnali*. In the latter species, and maybe also in the other two Pyrenean taxa, the reduced amounts of HindIII satDNA might obey to the possible involvement of this centromeric element in the Robertsonian fusions that originated the biarmed chromosomes characteristic of *Pyrenesaura* from the ancestral acrocentric karyotype, as has been suggested for other centromeric repeats in marsupials (Bulazel et al. 2007). Alternatively, HindIII could represent a minor satDNA family in the centromeres of the ancestral species, which was differentially amplified in the Iberian clade. In either case, the turnover of HindIII repeats in the different lineages mainly involved the same pool of "old" repeat variants. Long-term conservation of ancestral repeats could be a consequence of selective constraints imposed on functional motifs or structural features of satellite monomers (see, for example, Meštrović et al. 2006; Plohl et al. 2012), involved in any of the roles ascribed to satDNAs (reviewed in Ugarković 2009). Thus, even if we did not find any evidence of function in HindIII satDNA, selection may have favored the maintenance of some repeat variants and/or limited the diversification of this repetitive element. Nevertheless, the loss of HindIII repeats in *I. horvathi* and *I. bonnali* (or, alternatively, the amplification in the Iberian species) suggests that even if functional, a satellite family may be replaced by another in a relatively short evolutionary time.

Actually, and in contrast to the highly conserved function of the centromeres, the rapid evolution and extensive changes in copy number of satDNAs is a general characteristic of centromeric regions (Henikoff et al. 2001). The detection of recombinant sequences within subfamily HI suggests that mechanisms such as unequal crossovers between sister chromatids and gene conversion may have been an important source of new sequence variants in HindIII satDNA (e.g. Smith 1976; Talbert and Henikoff 2010). Moreover, unequal crossover occurring between highly homogeneous arrays can induce copy number alterations of satDNA repeats, such as those observed in the *Iberolacerta* species (Stephan 1986). This fast evolution of centromeric satDNAs can be linked to reproductive isolation and speciation (Bachmann et al. 1989; Bachmann and Sperlich 1993). For example, divergence of centromeric satDNA in *Drosophila* species can inhibit chromosome segregation in hybrids and thus directly cause hybrid incompatibilies and postzygotic isolation (Ferree and Barbash 2009). Likewise, the high copy number polymorphisms and rapid shifts in centromere sequence composition could have contributed and even triggered species radiation within *Iberolacerta*.

The TaqI satDNA family appears to have a very different evolutionary history from the HindIII family, and to evolve much faster in the lineage that leads to *I. horvathi*. According to the parsimony network, TaqI_TI, the most widespread subfamily among the analyzed species, would also be the most ancestral variant, from which subfamily TII was derived. Moreover, the phylogenetic distribution of the different sequence sets suggests that both subfamilies were

present in the common ancestor of *Iberolacerta*. Subsequently, subfamily TII spread in the Pyrenean species, whereas it was progressively lost in *I. horvathi* and maybe even completely removed from the genomes of the Iberian species. Altogether, TI repeats retrieved from *I. horvathi* show a general pattern of concerted evolution, with high interspecific distance values in all pairwise comparisons and a large subset of species-specific sequence groups. The allocation of these private groups (e.g., TI_A2 or TI_C1) in terminal clades of the statistical parsimony network indicates that they probably arose after the early separation of *I. horvathi* from the remaining species, about 11.5 Mya (9.6–13.7) (Arribas et al. 2014). The evolution of TaqI satDNA in *I. horvathi* was probably accompanied by a reduction in the abundance and chromosomal distribution, as inferred from the results of FISH experiments. TaqI satDNA also seems to evolve in concert in the Iberian clade but with a distinct pattern from that found in *I. horvathi*. In this case, the profile of TI repeats and the low levels of nucleotide diversity indicate that concerted evolution in the Iberian clade involved the preferential homogenization of a reduced subset of TaqI variants, all of which evolved from a single sequence lineage, TI_L3. After cladogenesis, however, the rate at which TI repeats evolved within the Iberian clade is presumably low, since TaqI sequences are poorly differentiated between the four taxa and we found almost no species-specific sequence sets.

In contrast with *I. horvathi* and the Iberian species, the turnover of TaqI satDNA seems to be remarkably slow in the Pyrenean *I. bonnali*. TaqI repeats from this species belong mainly to "old" sequence sets of subfamily TI, and lack species-specific diagnostic positions, which indicates that most of the variability found in *I. bonnali* obeys to synapomorphisms, and that TaqI repeats have been evolving with a low rate of sequence change after speciation. Conversely, the evolution of TaqI satDNA in the other two Pyrenean species, *I. aranica* and *I. aurelioi*, is characterized by the amplification of subfamily TII. Phylogenetic studies suggest that the three species of the Pyrenean clade originated in rapid succession, though *I. bonnali* probably split first, roughly 3.8 Mya (2.7–4.9) (Arribas et al. 2006, 2014). According to this phylogenetic reconstruction, the amplification of subfamily TII in the genomes of *I. aranica* and *I. aurelioi* may have occurred in a short time, after the separation of *I bonnali* and before the divergence of both species, *ca*. 3.3 Mya (2.3–4.3). A rapid expansion of subfamily TII agrees well with the high levels of intraspecific nucleotide diversity and interspecific sequence conservation observed for this subfamily in both species.

The different turnover rates of TaqI repeats among the Pyrenean species, *I. horvathi* and the Iberian species, could be related to differences in their karyotypes. It is possible that interchromosomal exchange and homogenization between the asymmetric meta-/submetacentric chromosomes of the Pyrenean species is more limited than in the species with all acrocentric chromosomes, more homogeneous in shape and size. Similar considerations have been proposed to explain the lower evolutionary rate of satDNAs in sturgeons as compared to sparids (de la Herrán et al. 2001). Limited interchromosomal exchange would lead to a progressive compartmentalization of satellite repeats, followed by a reduction in their interactions and, eventually, by a lack of homogenization of different sequence variants. However, this hypothesis is at least partially contradicted by our analysis of consecutive monomeric units, which revealed that, in both HindIII and TaqI satDNA families, adjacent repeats are not necessarily more similar than are repeats selected at random and that members of different sequence groups or even subfamilies can be interspersed in the same array.

In fact, this pattern of composite repeats may be a key factor explaining the disparate turnover rates of each satDNA family in different species. In eukaryotes, homologous recombination within or between chromosomes can be inhibited by only one mutation per 200 bp (Nijman and Lenstra 2001 and references therein). Likewise, mutations in new monomer variants would inhibit the interactions of repeat units, leading to sequence diversification, divergent evolution, and the formation of satDNA subfamilies. Accordingly, our estimates of intraspecific genetic distances between repeats belonging to different subfamilies suggest that each subfamily within HindIII and TaqI satDNAs is evolving independently. In this context, the intermixing between subfamilies HI and HII within HindIII arrays in most of the species analyzed, and between TaqI subfamilies TI and TII in the Pyrenean taxa, would strongly reduce recombination and homogenization within each subfamily, resulting in the pattern of non-concerted evolution observed

in our study. Conversely, the amplification of subfamily HIII in *I. cyreni*, and the preponderance of subfamily TI in *I. horvathi* and the Iberian species, allows a more efficient homogenization of HindIII and TaqI repeats, respectively, which translates into the overall patterns of concerted evolution observed for these satDNA families in the species mentioned above.

Taken together, our results on the dynamics of HindIII and TaqI satDNAs in *Iberolacerta* are congruent with proposed models of satDNA evolution and life history, intended to explain the considerable fluctuations in copy number and variability of satDNAs shared by related species (Nijman and Lenstra 2001; Plohl et al. 2010). They also support the idea that the "library model" may be extended to monomer variants of the same satDNA family, which were already present in a common ancestor and are currently distributed in related species in variant copy numbers (Cesari et al. 2003). As observed in *Iberolacerta*, this particular evolutionary pattern may result in species-specific profiles of satDNAs which do not reflect the phylogenetic relationships among taxa.

In conclusion, an in-depth analysis of intragenomic variability of HindIII and TaqI satDNAs in *Iberolacerta* revealed two disparate evolutionary histories which, nevertheless, showed some common traits: (i) each satDNA family is made up of a library of monomer variants or subfamilies shared by related species; (ii) species-specific profiles of satellite repeats are shaped by expansions and/or contractions of different variants from the library; (iii) different turnover rates, even among closely related species, result in great differences in overall sequence homogeneity and in concerted or non-concerted evolution patterns. Contrasting turnover rates are possibly related to genomic constraints such as karyotype architecture and the interspersed organization of diverging repeat variants in satellite arrays and maybe also to functional interactions. On the whole, these satDNA families constitute highly dynamic systems, which may have a critical role on the evolution of genome and species. Further studies aimed at investigating the genome-wide variability and organization of reptilian satDNAs may not only be useful to test current hypothesis and identify mechanisms influencing the evolution of this genomic component but also to improve its application as a molecular marker in phylogenetic studies.

Acknowledgments This work was supported by grants REN2003-02931/GLO (Ministerio de Ciencia y Tecnología, Spain), PGIDIT03RFO10301PR and PGIDIT06RFO10301PR (Xunta de Galicia, Spain) awarded to Horacio Naveira, GRC2014/050 awarded to Ana González, and by grant PRIN2009/20093HYH97 (Ministry of Education, University and Research, Italy) awarded to Vincenzo Caputo Barucchi. Verónica Rojo has been supported by a "FPU" fellowship from Ministerio de Educación, Cultura y Deporte (Spain).

Ethical standards Permissions for field work and experimental procedures were issued by the competent authorities: Xunta de Galicia (for *I. monticola* and *I. galani*), Junta de Castilla y León (for *I. cyreni* and *I. martinezricai*), Gobierno de Aragón (for *I. bonnali*), and Italian Environment Ministry (for *I. horvathi*). All institutional and national guidelines for the care and use of laboratory animals were followed.

References

Arnold EN, Arribas O, Carranza S (2007) Systematics of the palaearctic and oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa 1430:1–86

Arribas O, Odierna G (2004) Karyological and osteological data supporting the specific status of *Iberolacerta* (*cyreni*) *martinezricai* (Arribas, 1996). Amphibia-Reptilia 25: 359–367

Arribas O, Carranza S, Odierna G (2006) Description of a new endemic species of mountain lizard from Northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). Zootaxa 1240:1–55

Arribas O, Galán P, Remón N, Naveira H (2014) A new mountain lizard from Montes de León (NW Iberian Peninsula): *Iberolacerta monticola astur* ssp. nov. (Squamata: Lacertidae). Zootaxa 3796:201–236

Bachmann L, Sperlich D (1993) Gradual evolution of a specific satellite DNA family in *Drosophila ambigua*, *D. tristis*, and *D. obscura*. Mol Biol Evol 10:647–659

Bachmann L, Raab M, Sperlich D (1989) Satellite DNA and speciation: a species specific satellite DNA of *Drosophila guanchel*. J Zool Syst Evol Res 27:84–93

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Retrieved from: www.genetix.univ-montp2.fr/genetix/genetix.htm. 18/3/2015

Bulazel KV, Ferreri GC, Eldridge MDB, O'Neill RJ (2007) Species-specific shifts in centromere sequence composition are coincident with breakpoint reuse in karyotypically divergent lineages. Genome Biol 8:R170

Capriglione T (2000) Repetitive DNA as a tool to study the phylogeny of cold-blooded vertebrates. In: Olmo E, Redi CA (eds) Chromosomes today. Birkhäuser Verlag, Basel, pp 183–194

Capriglione T, Olmo E, Odierna G, Smith DI, Miller OJ (1989) Genome composition and tandemly repetitive sequence at some centromeres in the lizard *Podarcis s. sicula* Raf. Genetica 79:85–91

Capriglione T, Cardone A, Odierna G, Olmo E (1991) Evolution of a centromeric satellite DNA and phylogeny of lacertid lizards. Comp Biochem Physiol B 100:641–645

Capriglione T, Cardone A, Odierna G, Olmo E (1994) Further data on the occurrence and evolution of satellite DNA families in the lacertid genome. Chromosom Res 2:327–330

Capriglione T, de Santo MG, Odierna G, Olmo E (1998) An alphoid-like satellite DNA sequence is present in the genome of a lacertid lizard. J Mol Evol 46:240–244

Capula M, Lapini L, Capanna E (1989) The karyotype of *Lacerta horvathi* (Reptilia, Sauria, Lacertidae). Genetica 79:11–16

Cesari M, Luchetti A, Passamonti M, Scali V, Mantovani B (2003) Polymerase chain reaction amplification of the Bag320 satellite family reveals the ancestral library and past gene conversion events in *Bacillus rossius* (Insecta Phasmatodea). Gene 312:289–295

Chaiprasertsri N, Uno Y, Peyachokinagui S et al (2013) Highly species-specific centromeric repetitive DNA sequences in lizards: molecular cytogenetic characterization of a novel family of satellite DNA sequences isolated from the water monitor lizard (*Varanus salvator macromaculatus*, Platynota). J Hered 104:798–806

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371: 215–220

Ciobanu D, Grechko VV, Darevsky IS (2003) Molecular evolution of satellite DNA CLsat in lizards from the Genus *Darevskia* (Sauria: Lacertidae): correlation with species diversity. Russ J Genet 39:1527–1541

Ciobanu D, Grechko VV, Darevsky IS, Kramerov DA (2004) New satellite DNA in *Lacerta* s. str. lizards (Sauria: Lacertidae): evolutionary pathways and phylogenetic impact. J Exp Zool B Mol Dev Evol 302:505–516

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657–1659

de la Herrán R, Fontana F, Lanfredi M et al (2001) Slow rates of evolution and sequence homogenization in an ancient satellite DNA family of sturgeons. Mol Biol Evol 18:432–436

Dover GA (1982) Molecular drive: a cohesive mode of species evolution. Nature 299:111–117

Dover GA (2002) Molecular drive. Trends Genet 18:587–589

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792–1797

Enukashvily NI, Ponomartsev NV (2013) Mammalian satellite DNA: a speaking dumb. Adv Protein Chem Struct Biol 90: 31–65

Feliciello I, Akrap I, Brajković J, Zlatar I, Ugarković Đ (2015) Satellite DNA as a driver of population divergence in the red flour beetle *Tribolium castaneum*. Genome Biol Evol 7:228–239

Ferree PM, Barbash DA (2009) Species-specific heterochromatin prevents mitotic chromosome segregation to cause hybrid lethality in *Drosophila*. PLoS Biol 7, e1000234

Fry K, Salser W (1977) Nucleotide sequences of HS-A satellite DNA from kangaroo rat *Dipodomys ordii* and characterization of similar sequences in other rodents. Cell 12:1069–1084

Giovannotti M, Nisi Cerioni P, Caputo V, Olmo E (2009) Characterisation of a GC-rich telomeric satellite DNA in *Eumeces schneideri* Daudin (Reptilia, Scincidae). Cytogenet Genome Res 125:272–278

Giovannotti M, Nisi Cerioni P, Splendiani A, Ruggeri P, Olmo E, Caputo Barucchi V (2013) Slow evolving satellite DNAs: the case of a centromeric satellite in *Chalcides ocellatus* (Forskål, 1775) (Reptilia, Scincidae). Amphibia-Reptilia 34: 401–411

Giovannotti M, Rojo V, Nisi Cerioni P et al (2014) Isolation and characterization of two satellite DNAs in some Iberian rock lizards (Squamata, Lacertidae). J Exp Zool B Mol Dev Evol 322:13–26

Grechko VV, Ciobanu DG, Darevsky IS, Kramerov DA (2005) Satellite DNA of lizards of the genus *Lacerta* s. str. (the group *L. agilis*), the family Lacertidae. Dokl Biochem Biophys 400: 44–47

Grechko VV, Ciobanu DG, Darevsky IS, Kosushkin SA, Kramerov DA (2006) Molecular evolution of satellite DNA repeats and speciation of lizards of the genus *Darevskia* (Sauria: Lacertidae). Genome 49:1297–1307

Henikoff S, Ahmad K, Malik HS (2001) The centromere paradox: stable inheritance with rapidly evolving DNA. Science 293: 1098–1102

Kearse M, Moir R, Wilson A et al (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

Kuhn GCS, Sene FM, Moreira-Filho O, Schwarzacher T, Heslop-Harrison JS (2008) Sequence analysis, chromosomal distribution and long-range organization show that rapid turnover of new and old pBuM satellite DNA repeats leads to different patterns of variation in seven species of the *Drosophila buzzatii* cluster. Chromosom Res 16:307–324

Kuhn GCS, Schwarzacher T, Heslop-Harrison JS (2010) The non-regular orbit: three satellite DNAs in *Drosophila martensis* (*buzzatii* complex, *repleta* group) followed three different evolutionary pathways. Mol Genet Genomics 284:251–262

Kuhn GCS, Küttler H, Moreira-Filho O, Heslop-Harrison JS (2011) The 1.688 repetitive DNA of *Drosophila*: concerted evolution at different genomic scales and association with genes. Mol Biol Evol msr173

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452

Luchetti A, Cesari M, Carrara G et al (2003) Unisexuality and molecular drive: Bag320 sequence diversity in *Bacillus* taxa (Insecta Phasmatodea). J Mol Evol 56:587–589

Macas J, Navrátilová A, Koblížková A (2006) Sequence homogenization and chromosomal localization of VicTR-B satellites differ between closely related *Vicia* species. Chromosoma 115:437–447

Martinsen L, Venanzetti F, Johnsen A, Sbordoni V, Bachman L (2009) Molecular evolution of the *pDo500* satellite DNA family in *Dolichopoda* cave crickets (Rhaphidophoridae). BMC Evol Biol 9:1–14

Meštrović N, Plohl M, Mravinac B, Ugarković D (1998) Evolution of satellite DNAs from the genus palorus—

experimental evidence for the 'library' hypothesis. Mol Biol Evol 15:1062–1068

Meštrović N, Castagnone-Sereno P, Plohl M (2006) Interplay of selective pressure and stochastic events directs evolution of the MEL172 satellite DNA library in root-knot nematodes. Mol Biol Evol 23:2316–2325

Navajas-Pérez R, Quesada del Bosque ME, Garrido-Ramos MA (2009) Effect of location, organization, and repeat-copy number in satellite-DNA evolution. Mol Genet Genomics 282: 395–406

Nijman IJ, Lenstra JA (2001) Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite repeats. J Mol Evol 52:361–371

Odierna G, Aprea G, Arribas O, Capriglione T, Caputo V, Olmo E (1996) The karyology of the Iberian rock lizards. Herpetologica 52:542–550

Palomeque T, Lorite P (2008) Satellite DNA in insects: a review. Heredity 100:564–573

Pezer Ž, Brajković J, Feliciello I, Ugarković D (2012) Satellite DNA-mediated effects on genome regulation. In: Garrido-Ramos MA (ed) Repetitive DNA. Genome dynamics vol 7. Karger, Basel, pp 153–169

Plohl M, Luchetti A, Meštrović N, Mantovani B (2008) Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. Gene 409:72–82

Plohl M, Petrovic V, Luchetti A et al (2010) Long-term conservation vs high sequence divergence: the case of an extraordinarily old satellite DNA in bivalve mollusks. Heredity 104: 543–551

Plohl M, Meštrović N, Mravinac B (2012) Satellite DNA evolution. In: Garrido-Ramos MA (ed) Repetitive DNA, vol 7, Genome dynamics vol. Karger, Basel, pp 126–152

Robles F, de la Herrán R, Ludwig A, Rejón CR, Rejón MR, Garrido-Ramos MA (2004) Evolution of ancient satellite DNAs in sturgeon genomes. Gene 338:133–142

Rojo V, Giovannotti M, Naveira H et al (2014) Karyological characterization of the endemic Iberian rock lizard, *Iberolacerta monticola* (Squamata, Lacertidae): insights into sex chromosome evolution. Cytogenet Genome Res 142:28–39

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Schwarzacher T, Heslop-Harrison P (2000) Practical in situ hybridization. BIOS Scientific Publishers Ltd., Oxford

Sindaco R, Jeremčenko VK (2008) The reptiles of the western paleartic, volume 1: annotated checklist and distributional atlas of the turtles, crocodiles, amphisbaenians and lizards of Europe, North Africa, Middle East and Central Asia. Latina: Edizioni Belvedere

Smith GP (1976) Evolution of repeated DNA sequences by unequal crossover. Science 191:528–535

Stephan W (1986) Recombination and the evolution of satellite DNA. Genet Res 47:167–174

Strachan T, Webb D, Dover G (1985) Transition stages of molecular drive in multiple-copy DNA families in *Drosophila*. EMBO J 4:1701–1708

Talbert PB, Henikoff S (2010) Centromeres convert but don't cross. PLoS Biol 8, e1000326

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 101:11030–11035

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619–633

Ugarković D (2009) Centromere-competent DNA: structure and evolution. Prog Mol Subcell Biol 48:53–76

Ugarković D, Plohl M (2002) Variation in satellite DNA profiles—causes and effects. EMBO J 21:5955–5959

Waters PD, Marshall Graves JA, Thompson K, Sankovic N, Ezaz T (2008) Identification of cryptic sex chromosomes and isolation of X- and Y-borne genes. Methods Mol Biol 422: 239–251

Wei KH-C, Grenier JK, Barbash DA, Clark AG (2014) Correlated variation and population differentiation in satellite DNA abundance among lines of *Drosophila melanogaster*. PNAS 111:18793–18798