Accepted on 2 February 2009
J Zool Syst Evol Res doi: 10.1111/j.1439-0469.2009.00532.x

[1]*CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Vairão, Portugal;* [2]*Departament de Biologia Animal (Vertebrats), Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain;* [3]*Departamento de Zoologia e Antropologia, Faculdade de Ciências da Universidade do Porto, Porto, Portugal*

Genetic admixture between the Iberian endemic lizards *Podarcis bocagei* and *Podarcis carbonelli*: evidence for limited natural hybridization and a bimodal hybrid zone

C. Pinho[1], A. Kaliontzopoulou[1,2], M. A. Carretero[1], D. J. Harris[1,3] and N. Ferrand[1,3]

Abstract

When recently diverged taxa come into contact, the extent of introgression between them is related to the degree of differentiation that they have achieved. Studying contact zones is therefore essential to understand if differentiated taxa are reproductively isolated and, ultimately, if they are likely to remain distinct. Recent work on Iberian and North African wall lizards (*Podarcis*) has documented the existence of multiple evolutionary units, diagnosable both by genetic markers and morphology, but suggests that gene flow between distinct forms has occurred. Therefore, we were interested in evaluating how species boundaries are maintained in the areas where they meet. In this work, we study the contact zone between *Podarcis bocagei* and *Podarcis carbonelli*. We sampled a transect including the only locality where these two lizards are known to occur in syntopy and analysed a battery of 15 unlinked nuclear genetic markers and mitochondrial DNA. We also conducted a preliminary analysis of morphology and fertility. Using model-based clustering approaches, we show that the two species hybridize in the population where they have direct contact, but evidences of introgression are low for nearby populations. Although a significant number of individuals show evidence of admixture, this hybrid zone is clearly bimodal, suggesting strong barriers to gene flow, of which the putative nature are discussed. Interestingly, morphological analyses do not support the existence of intermediate forms among individuals that are admixed genetically. Taken together, these results constitute further evidence validating *P. bocagei* and *P. carbonelli* as distinct species.

Key words: bimodality – fertility – hybridization – Lacertidae – multilocus genotype – morphology – speciation

Introduction

Speciation is usually thought of as the development of reproductive isolation between diverging taxa. Early views on this subject (Dobzhansky 1937; Muller 1940, 1942) and recent empirical work (e.g. Presgraves et al. 2003) suggest that the genetic basis of reproductive isolation essentially consists in the acquisition of epistatic incompatibilities in genes responsible for ecological, physiological or behavioural differentiation. If only a few loci have developed such incompatibilities between diverging populations, then gene flow between these populations may be extensive, except for these few loci. The more genes that are involved in functional divergence between populations, the more difficult it becomes for them to exchange genes, because different loci become co-adapted within these populations (e.g. Orr 1995), and the less likely it is that populations will fuse in the future. At some point in this process (Wu (2001) calls it 'the point of no return'), the populations will have diverged sufficiently so that they will not merge even if the barrier that led to their separation is removed. The process of divergence continues thereon even in the presence of some degree of gene flow, until full reproductive isolation is achieved. Contact zones provide us with the unique opportunity of assessing whether species are reproductively isolated and allow us to understand the stage that they have reached in the process of differentiation. When differentiated genetic entities come into contact, different situations may occur: in one extreme of the divergence process, they do not interbreed or, if they do, are unable to produce viable or fertile offspring and are thus considered to be fully reproductively isolated; in the other extreme, they may interbreed freely and eventually merge into a single unit. In between these two situations, they may often interbreed in a narrow hybrid zone, the width of which is dependent upon a balance between dispersal and selection (a tension hybrid zone, e.g. Barton 1983; Barton and Hewitt 1985), and maintain their genetic integrity over most of their distribution area. By analysing the dynamics of contact zones between recently diverged taxa, we may therefore predict how the future of the two populations will be in terms of fusion or divergence.

Contact zones between forms of wall lizards in the Iberian Peninsula and North Africa constitute one such opportunity. The taxonomy of these animals has been a long-standing matter of debate and is in the process of re-examination, mainly drawing from recent studies on morphology and genetic variation. Analyses of mitochondrial DNA (mtDNA) sequences (Pinho et al. 2006) suggest the existence of as many as 11 differentiated forms, which were suggested to deserve species status based on the high genetic distances observed between them. Four species are presently recognized: *Podarcis bocagei* (Seoane, 1884), *Podarcis carbonelli* Pérez-Mellado, 1981, *Podarcis vaucheri* (Boulenger, 1905) and *Podarcis hispanica* (Steindachner, 1870). Although these studies are ongoing, *P. hispanica* is currently recognized as a paraphyletic assemblage of divergent monophyletic mtDNA lineages distinguished as 'types' by Harris and Sá-Sousa (2001) and other subsequent investigations. Although distribution maps are still incomplete and most contact zones have not yet been described, the distribution of most

Corresponding author: Catarina Pinho (catarina@mail.icav.up.pt)
Contributing authors: Antigoni Kaliontzopoulou (antigoni@mail.icav.up.pt), Miguel A. Carretero (carretero@mail.icav.up.pt), D. James Harris (james@mail.icav.up.pt), Nuno Ferrand (nferrand@mail.icav.up.pt)

lineages is likely parapatric, except for the pairs *P. bocagei*/*P. hispanica* type 1 and *P. carbonelli*/*P. hispanica* type 2, which are essentially sympatric and even syntopic. Other cases of sympatry have been reported, such as *P. carbonelli*/*P. hispanica* type 1 (Iberian 'Sistema Central') and the former with *P. vaucheri* (Doñana National Park). In general, these different partitions observed in mtDNA are also identifiable on the basis of multiple nuclear markers (Pinho et al. 2007a), but nuclear intron genealogies reveal that both fully recognized species and groups within *P. hispanica* are not reciprocally monophyletic, due to incomplete lineage sorting of ancestral polymorphism coupled with the existence of limited gene flow between forms (Pinho et al. 2008). It has therefore been suggested that forms of *Podarcis* are not completely reproductively isolated but the extent and dynamics of gene exchange between forms remains to be completely understood. As a result, the characterization of contact zones between the various forms is of utmost importance to evaluate if and how species boundaries are maintained (Pinho et al. 2007a).

One of the first contact zones to be identified was that between *P. bocagei* and *P. carbonelli* (Carretero et al. 2002). Both species are mostly ground-dwelling, exhibit similar ecological requirements, and reproduce at the same time of the year (Carretero et al. 2006; MA Carretero et al. unpublished data). Formerly regarded as conspecific because of their morphological and ecological similarities, these taxa are now considered to be separate species (Sá-Sousa and Harris 2002) and indeed are not sister taxa with respect to mtDNA (Pinho et al. 2006). Their distributions are parapatric, overlapping in a narrow zone, only a few kilometres wide. Based on inferred biogeographic and phylogeographic patterns (Sá-Sousa 2001a; Pinho et al. 2007b), it seems likely that *P. bocagei* and *P. carbonelli* have come into contact after a post-glacial expansion of their distributions, which implies a relatively recent origin for this contact. *P. bocagei* and *P. carbonelli* have been shown to hybridize in captivity (Galán 2002), but there is no further information on the sterility or fitness of their hybrids. Thus far, there are no field observations available suggesting that the two species could interbreed in natural conditions. Instead, an apparent lack of morphological intermediate individuals between both species has been reported (Carretero et al. 2002; Kaliontzopoulou 2004; Kaliontzopoulou et al. 2007). It has also been demonstrated that males from both species are able to recognize conspecific or heterospecific females based on chemical cues (Barbosa et al. 2005), suggesting assortative mating.

In this work, we studied a battery of mitochondrial and nuclear markers in individuals sampled from populations situated along a North-South transect that crosses the contact zone. We also investigated morphological and fertility aspects of individuals collected in the area. Our main aims were (1) to determine if these species are able to produce hybrids; in the case that they are hybridizing, (2) to determine if this hybridization leads to gene flow; (3) to investigate if there are barriers to gene flow or if hybrids are formed freely; (4) to investigate the morphological characteristics of hybrids; (5) to assess which factors might influence the dynamics of this contact zone; and, ultimately, (6) to clarify whether or not *P. bocagei* and *P. carbonelli* are good species.

Materials and Methods

Genetic analyses

Sampling

Samples were obtained along a North-South transect of the Portuguese coast, centred in the only locality – Espinho (Carretero et al. 2002) – where the two species are found in strict syntopy. We analysed a total of 146 individuals from five localities (Fig. 1): Vairão (n = 30), Madalena (n = 22), Espinho (n = 59), Esmoriz (n = 18) and Aveiro (n = 17). Individuals from Vairão and Madalena were morphologically identified as *P. bocagei*, whereas individuals from Esmoriz and Aveiro were assigned to *P. carbonelli*. The sample from Espinho included only adult individuals that were examined by the authors immediately after capture and could be assigned to one of the two species on the basis of various empirical diagnostic characters that principally include size, colour pattern, and head dimensions and shape (Sá-Sousa 2001b). This classification was used throughout the study to *a priori* classify individuals into species. The sample included 26 individuals assigned to *P. bocagei* and 28 to *P. carbonelli*. Both sexes (18 females and 36 males) were represented. Five samples of unknown sex and specific origin, collected in the area by non-experts and consisting only of tail tissue, were also included. Samples were divided into two portions, one of which was stored frozen at −80°C (for allozyme analyses) and the other kept in 96% ethanol (for DNA analyses).

Data collection

Allozyme data from the populations of Vairão and Aveiro have been obtained previously (see Pinho et al. 2003, 2004a, 2007a). Tissue extraction, protein separation by starch gel electrophoresis and isoelectric focusing and enzymatic detection of all loci in the remaining populations followed the procedures described in these papers. The set of 10 markers was previously shown to distinguish between *P. bocagei* and *P. carbonelli* individuals in a Bayesian assignment framework (Pinho et al. 2007a).

For microsatellite analyses, DNA was extracted following standard procedures (Sambrook et al. 1989). We used three from a set of nine microsatellites developed for *P. bocagei* by Pinho et al. (2004b) (*Pb11*,

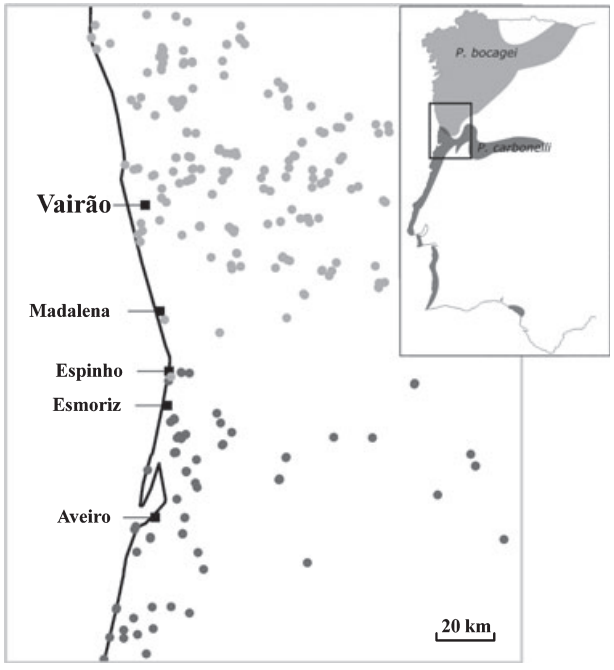

Fig. 1. Distribution of *Podarcis bocagei* and *Podarcis carbonelli*. (a) General pattern in W Iberia. (b) Detailed records in the study area (based on the most recent distribution data, Loureiro et al. 2008) and sampling sites. The two species are only found in syntopy in Espinho

Pb50 and *Pb66)*, which were amplified according to the described conditions with the exception of the annealing temperature, that was lowered to 53°C in all cases. The electrophoretic separation of the amplified fragments was carried out in 6% denaturing polyacrylamide gels and silver stained as described in Pinho et al. (2004b).

We also analysed genetic variation at a nuclear intron using Single Strand Conformation Polymorphism (SSCP). Pinho et al. (2008) described a novel nuclear intron [intron 7 of the 6-phosphogluconate dehydrogenase (*6-Pgdint7*) locus] that was found to be diagnostic between *P. bocagei* and *P. carbonelli*. We used specific primers *PgdA* (5′-GGAATTCCCCATCTCTGACTTAG-3′) and *PgdB* (5′-GCATG CAAAGACAGGTTCTGGTG-3′) to target a 160-bp fragment containing three diagnostic single nucleotide polymorphisms (SNPs) according to data from Pinho et al. (2008). Polymerase chain reaction (PCR) was carried out in 10 μl volumes, containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 0.5 U of *Ecotaq* DNA polymerase (Ecogen, Barcelona, Spain) and approximately 25 ng of genomic DNA. Amplification conditions were as follows: an initial denaturation step for 3 min at 94°C, 40 cycles consisting on 30 s at 94°C, 20 s at 53°C and 20 s at 72°C, and a final extension of 72°C for 2 min. Before loading PCR products into SSCP gels, they were diluted 1 : 5 in denaturing loading buffer (95% deionized formamide, 10 mM NaOH, 0.01% bromophenol blue), denaturated for 5 min at 94°C and kept on ice until loading. Preliminary SSCP tests were performed with samples known to carry different sequences in order to optimize separation conditions. For the small fragment of 160 bp, all *P. bocagei* sequenced by Pinho et al. (2008) exhibit one single sequence, whereas *P. carbonelli* shows four distinct alleles, two of which (differing in one SNP and corresponding to alleles PR3 and PR4 in Pinho et al. (2008)) were impossible to separate under all tested conditions. Optimal resolution for the separation of *P. bocagei* versus *P. carbonelli* alleles was obtained using 12% polyacrylamide gels (29 : 1 acrylamide : methylbisacrylamide) with 1× Tris/Borate/EDTA (TBE) on a vertical electrophoresis system, run at a constant voltage of 170 V at 15°C for 16 h. Previously genotyped samples were always added to gels in order to ensure correct genotype scoring. Previously undetected alleles were PCR-amplified for the whole intron and sequenced following Pinho et al. (2008). It should be noted that we were *a priori* expecting strong linkage disequilibrium (LD) between *6-Pgdint7* and the allozyme *PGD*, because this enzyme is encoded by the *6-PGD* gene. However, the two loci behave as unlinked (see Results), probably because of recombination outside the intron, leading to a lack of association between the allozyme and the intron genotypes. Because of this, we were able to treat both loci as independent in subsequent analyses.

As an addition to the study of nuclear markers, we also developed a protocol for discriminating the mtDNA lineage carried by each individual, consisting of a Restriction Fragment Length Polymorphism (RFLP) analysis. Based on available sequences of the 12S rRNA mtDNA gene, we used BioEdit v. 7.0.5.2 (Hall 1999) to produce restriction maps and selected restriction enzymes that allow species distinction. Although a single enzyme would have sufficed, we selected two, *Mse* I and *Taq* I, to reduce the possibility of misclassification due to homoplasy. Each of these enzymes produces a single restriction profile for each species. PCR amplification followed the conditions described in Pinho et al. (2006). PCR products were digested with each of the enzymes separately and run on 3% high resolution agarose gels side by side with previously typed samples.

Analytical methods

Allelic frequencies were calculated using GENETIX 4.05.2 (Belkhir et al. 1996–2004). We used ARLEQUIN v 2.000 (Schneider et al. 2000) to perform Analyses of Molecular Variance (AMOVA, Excoffier et al. 1992) to evaluate and compare the usefulness of the markers in discriminating between both species. For this purpose, we used data from the four populations outside the contact zone and used a F_{ST}-like calculation (i.e. based on allele frequencies and disregarding allele size, in the case of microsatellite markers) to generate distance matrices for all types of markers. The GENEPOP software (v. 3.1b; Raymond and Rousset 1995) probability test was used to determine whether populations were in Hardy–Weinberg and in linkage equilibrium.

We further analysed individual assignment based on multilocus genotypes. In these analyses, individuals with more than 20% of missing data were discarded. We followed three main methodologies: first, we used GENETIX to perform a Factorial Correspondence Analysis. Second, we used STRUCTURE 2.0 (Pritchard et al. 2000) to estimate the proportion of each individual genome originating in each parental species. This software implements a Bayesian model-based clustering algorithm that creates population structure and attempts to find clusters of individuals that minimize Hardy–Weinberg and linkage disequilibria. The parameter settings included the assumption of admixture and of independent allele frequencies. We forced the number of populations (K) to 2 in order to evaluate if individuals from the two species could be discriminated. In exploratory runs, we did not provide the software with prior population information regarding the origin of the individuals, but in final runs individuals from the two populations located in the extremes of the transect (Vairão and Aveiro), assumed to represent pure *P. bocagei* and *P. carbonelli*, were used as a proxy for determining the degree of admixture of all the other individuals. STRUCTURE was run for 550 000 steps, of which the first 50 000 were discarded as burn-in and we conducted five independent replicates of the Markov Chain Monte Carlo. We also performed analyses using the correlated allele frequencies model to test for robustness of our conclusions to the violation of prior assumptions. Third, we used NEWHYBRIDS (Anderson and Thompson 2002), which calculates the posterior probability (PP), under a model-based Bayesian approach, of multilocus individual genotypes belonging to one of six different categories (pure of each parental type, F1 or F2 hybrids and F1-parental backcrosses in both directions). This software was run three times, each with 250 000 steps along the Markov Chain. To test the validity and limitations of this approach, we additionally simulated 100 individuals from each class using HYBRIDLAB 1.0 (Nielsen et al. 2006), assuming Vairão and Aveiro as pure representatives of *P. bocagei* and *P. carbonelli*, respectively. NEWHYBRIDS was run on this simulated data set under the same conditions as the previous analyses. With this, we expected to evaluate if the markers contained enough information for the assignment of individuals into hybrid classes.

In addition, we simulated a scenario of random admixture between the two species for comparison with the observed situation. For this we generated 1000 putative individuals from 5, 10 and 50 generations of random admixture using a program written for this effect (M. Carneiro, unpublished data). To allow comparisons with our sample, instead of a 1 : 1 *P. bocagei*/*P. carbonelli* ratio, in these analyses, the initial population included 57.1% of pure *P. bocagei* individuals and 42.9% of pure *P. carbonelli*, which corresponded to the overall genetic composition of our contact zone sample estimated using STRUCTURE. Three independent simulations of each generation were run using STRUCTURE, under the same conditions described above. To ensure the analyses were not affected by the comparatively lower number of non hybrid individuals sampled, we simulated individuals from Vairão, Madalena, Esmoriz and Aveiro using HYBRIDLAB and included them in STRUCTURE analyses in the same proportions as those found in our original sample.

Morphological analyses

In order to examine the possible effect of hybridization on the morphology of the studied species, we typified morphologically the individuals from the area of syntopy (Espinho) that were also used for genetic analyses. To explore the morphological properties of hybrids, we investigated the posterior probabilities of belonging to each of the two species, ascribed to each individual of the hybrid zone by a morphology-based discriminant analysis. For this purpose, we first analysed a large dataset of allopatric populations of both species, that were previously genetically investigated and were reliably found to present no intermixing with other Iberian Podarcis species (Pinho et al. 2007a,b) and covering the whole range of the species' distribution (Table S1). This dataset consisted of a total of 134 male *P. bocagei* and 113 male *P. carbonelli*, representing eight different populations of each species. All of these individuals were morphologically typified by quantifying 13 linear biometric traits (see Kaliontzopoulou et al. 2007, 2008 for details) and four pholidotic characters (i.e. number of femoral

pores, collar, gular and ventral scales). Half of these specimens were used to build a discriminant function to distinguish between both species based on morphological characters. Then, the other half of allopatric specimens, as well as 34 specimens from the contact zone (18 *P. bocagei* and 16 *P. carbonelli*), were subjected to this discriminant function in order to examine the morphological position of hybrids. The PP assigned to each of the hybrid zone individuals was examined for correlation with the PP calculated from genetic data using STRUCTURE. Since a marked sexual dimorphism exists in both species (Kaliontzopoulou et al. 2007, 2008) and low sample size did not permit the analysis of female specimens, morphological analyses were restricted to males only.

Analysis of fertility

Adult specimens from Vairão, Espinho and Torreira (a coastal locality around 10 km south of Esmoriz) analysed morphologically were sacrificed humanely and dissected for visual inspection of the reproductive organs. For those collected during the reproductive season (February–July), gonads were measured and weighed according to standard protocols (Carretero 2006). Furthermore, the density of spermatozoa in testes was estimated following Carretero et al. (2006) and vitellogenic follicles and oviductal eggs were also counted. Individuals were classified as 'hybrids' when they showed posterior probabilities of assignment to a species (using STRUCTURE) lower than the lowest value observed in reference ('pure') populations (94.2%). Comparisons of gonad size and number of spermatozoa, follicles and eggs between 'pure' specimens and putative hybrids were performed through ANCOVA. Since such variables were dependent on body size we used snout-vent length (SVL) as a covariate. Because the slopes between SVL and those variables were not homogeneous between species (Carretero et al. 2006), we used a model for separate slopes. All variables were log-transformed prior to the analyses to ensure normality and homoscedasticity. All the data sets (genetic, morphology and fertility) are available from the authors upon request.

Results

Genetic polymorphism outside the contact zone and distinction between species

Allele frequencies for all loci are given in Table S2. The following paragraphs describe the results obtained in the various markers separately.

Allozymes

A total of 37 enzyme alleles were detected amongst the four populations outside the contact zone. AMOVA analyses showed that using this data set differentiation between the two species accounts for 43.97% of the total genetic variation (Table 1; F_{CT} from the locus by locus average is highly significant). The different loci vary on their ability to separate the two species. The most informative loci in discriminating between the two species were *GOT* and *PGD*. Although existing data suggest that allele *GOT*B* is fixed in northern *P. carbonelli* populations (C. Pinho, D. J. Harris, N. Ferrand, unpublished data), the population of Esmoriz shows a frequency of 22% of allele *GOT*A*, the most frequent in *P. bocagei* and fixed in Madalena. In *PGD*, the most frequent allele in *P. carbonelli* is *PGD*A*, which is absent from all *P. bocagei* populations studied to date but is present at a very low frequency (2%) in Madalena. Excluding these situations, both populations (Esmoriz and Madalena) are typical for the allozyme genotypes observed in each species.

Microsatellites

Cross-amplification of the microsatellites developed for *P. bocagei* was successful in *P. carbonelli*. All three microsatellites were highly polymorphic and showed mostly overlapping sequence sizes between both species. Sixteen alleles were found in *Pb11*, 24 in *Pb50* and 19 in *Pb66*. Microsatellite loci appear not to be as good discriminators between *P. bocagei* and *P. carbonelli* as the other markers, since only 12.40% of the variation is explained by between species differentiation (F_{CT} from the locus by locus average is significant, $p < 0.05$), which is most likely due to the huge amount of within-population variability (Hedrick 1999), probably coupled with some degree of homoplasy. Nevertheless, the combination of the three microsatellite markers performs well in discriminating between the two species (using STRUCTURE, results not shown). In particular, *Pb66* showed high frequencies of diagnostic alleles, which is reflected in a higher than average 24.35% of variation found between species.

Table 1. Utility of the nuclear markers analysed in the distinction between *Podarcis bocagei* and *Podarcis carbonelli*, as shown by Hierarchical Analyses of Molecular Variance (AMOVA) using populations outside the contact zone

	Percentage of variation		
	Among species	Within species, among populations	Within populations
Allozymes	43.97	4.58	51.44
GOT1	87.49	2.01	10.50
GPI	1.50	−2.31	100.81
IDH1	4.91	1.75	93.33
LDH2	−4.77	10.62	94.15
MPI	10.23	−1.07	90.84
PEPA	47.37	5.35	47.28
PEPB	−0.49	11.21	89.27
PEPD	30.45	1.50	68.06
PGD	66.37	6.34	27.29
PGM1	0.39	3.23	96.38
Microsatellites	12.40	3.56	84.03
Pb11	9.05	3.02	87.92
Pb50	3.21	5.82	90.97
Pb66	24.35	1.92	73.72
6-Pgdint7	93.43	0.22	6.36
Total	38.77	3.70	57.52

6-Pgdint7 SSCP genotyping

As expected from sequence data, this marker was fully diagnostic between *P. bocagei* and *P. carbonelli*. Only one allele was found in *P. bocagei* populations outside the contact zone, whereas two of the previously detected *P. carbonelli* alleles, corresponding to alleles PR3/PR4 and PR7 defined by Pinho et al. (2008), were found in *P. carbonelli* populations. Allele PR3/PR4 was the most abundant, found at an average frequency of 92.9%. Over 93% of the variation at this locus is explained by differences between the two species.

Mitochondrial DNA RFLP analyses

Only the *P. bocagei* mtDNA type was found in populations situated north of the contact zone, whereas the *P. carbonelli* haplotype was fixed in the southern populations.

Genetic composition of the contact zone

In the locality where the two morphotypes contact, Espinho, both mtDNA types were detected. In the allozyme loci, six alleles not detected in the other surveyed populations were

observed, one of which was in locus *LDH1*, in which polymorphism had not been previously observed (Pinho et al. 2003). Genetic variants not found in the remaining populations of the transect were also detected in microsatellites (six alleles) and the nuclear intron (one allele; after sequencing, it was shown to be allele PR8 from Pinho et al. (2008), which had previously not been found in *P. bocagei* or *P. carbonelli*). In the nuclear markers, in loci exhibiting contrasting allele frequencies between both species, intermediate frequencies were observed (results not shown). Individuals simultaneously carrying alleles that were diagnostic for both species were detected, suggesting hybridization.

Hardy–Weinberg and linkage equilibrium

Most loci did not show significant ($p < 0.05$) deviations from Hardy–Weinberg expectations outside the contact zone. There were a few exceptions: e.g. *PEPD* in Vairão (already reported in previous studies; Pinho et al. 2003, 2004a, 2007a) and microsatellite *Pb50* in the same locality. However, in the population of Espinho, five significant cases of disequilibria were detected (*GOT*, *PEPD*, *Pb50*, *Pb66* and *6-Pgdint7*), mostly resulting from a deficit of heterozygotes. LD follows the same pattern: outside the contact zone there are no significant cases of association between different loci. However, in the contact zone LD was detected between several pairs of loci ($p < 0.05$): *PEPA* and *PEPD*, *PEPA* and *GOT*, *PEPD* and *GOT*, *IDH* and *GOT*, *PEPA* and *PGD*, *PEPD* and *PGD*, *GOT* and *PGD*, *PEPA* and *LDH1*, *GOT* and *LDH1*, *GOT* and *LDH2*, *PEPA* and *Pb50*, *PEPD* and *Pb50*, *GOT* and *Pb50*, *PGD* and *Pb50*, *GOT* and *Pb66*, *PEPA* and *6-Pgdint7*, *PEPD* and *6-Pgdint7*, *IDH* and *6-Pgdint7*, *GOT* and *6-Pgdint7*, *PGD* and *6-Pgdint7*, and *Pb50* and *6-Pgdint7*.

Individual multilocus genotype analyses

Factorial Correspondence Analyses show that the populations from outside the contact zone are clearly separable (Fig. 2). However, some individuals from Espinho are placed in between these two, suggesting an admixed origin. This was confirmed in the analyses using STRUCTURE and NEWHYBRIDS, shown in Fig. 3; the mtDNA type carried by each individual is also presented. Initial runs without using prior information clearly identified two sets of individuals corresponding to the two species, suggesting that the set of markers performs well in their separation. Using prior population information by flagging individuals from Aveiro and Vairão as pure of each species, results are highly similar to these initial results: a high proportion of the genome of the individuals from Madalena and Esmoriz is assigned to *P. bocagei* and *P. carbonelli*, respectively, suggesting that in general these represent 'pure' individuals of each species. However, one individual from Esmoriz shows a low but non-neglectable portion of its genome assigned to *P. bocagei*. The same individual fails to be classified as 'pure' with high PP using NEWHYBRIDS. This is probably due to the presence of alleles *GOT*A* and *Pb66*132* in this individual, which seem to be characteristic of *P. bocagei*. Other individuals from Esmoriz also present these alleles, but not simultaneously.

Unlike the general trend outside the contact zone, in the contact population of Espinho many individuals show signs of admixture. Hybrid individuals identified using STRUCTURE are rarely straightforwardly assigned to a category by NEWHYBRIDS. Five individuals show over 75% PP of representing a backcross of a F1 with a pure *P. bocagei* and two with *P. carbonelli*. Clear first generation hybrids were not detected in the sample, although three individuals show over 50% PP of being one. The fact that the PP of assignment is scattered through the four hybrid classes in most hybrid individuals could be either an outcome of multiple generations of backcrossing or of insufficient resolution of the markers. We therefore tested the degree of confidence in our hybrid assignment by running NEWHYBRIDS on individuals of known hybrid classes simulated using HYBRIDLAB. These results are summarized in Table S3. Ninety-one per cent of simulated F1 individuals are detected as such by NEWHYBRIDS with over 75% PP (62% with higher than 90%). This high assignment coincidence observed for F1 individuals was not matched by the other hybrid classes, because only 63%, 63% and 56% of F2s, backcrosses to *P. bocagei* and backcrosses to *P. carbonelli*, respectively, were classified as such with over 75% PP (54%, 32% and 23% when considering a threshold of 90% PP). This suggests that the markers used would be efficient in identifying F1 individuals in the case they existed in our sample (hence suggesting that the three individuals mentioned above are probably not F1s), but may not provide similar resolution to accurately discriminate the exact class of second generation hybrids. Nevertheless, because a small fraction of hybrids are assigned to the wrong class, we may be relatively confident in the backcrosses pinpointed by NEWHYBRIDS. Importantly, only 1.5% of all simulated hybrid were assigned as pure representatives of a species, whereas none of pure individuals was assigned as hybrid, highlighting that the markers used are powerful detectors of hybridization.

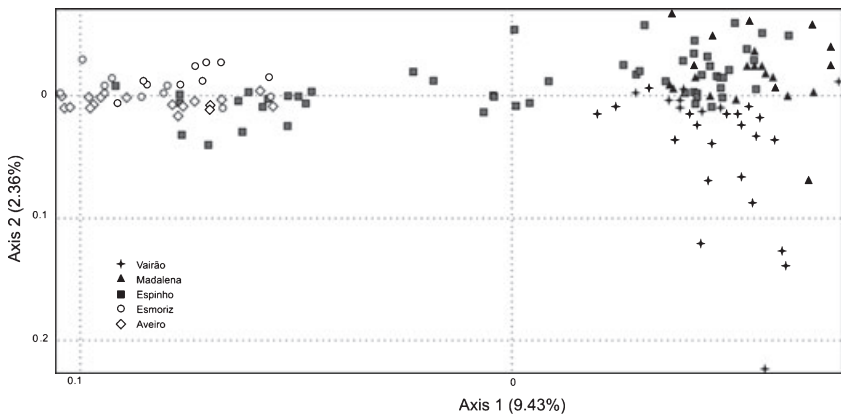

Fig. 2. Results of the factorial correspondence analyses performed using GENETIX. Each square represents an individual

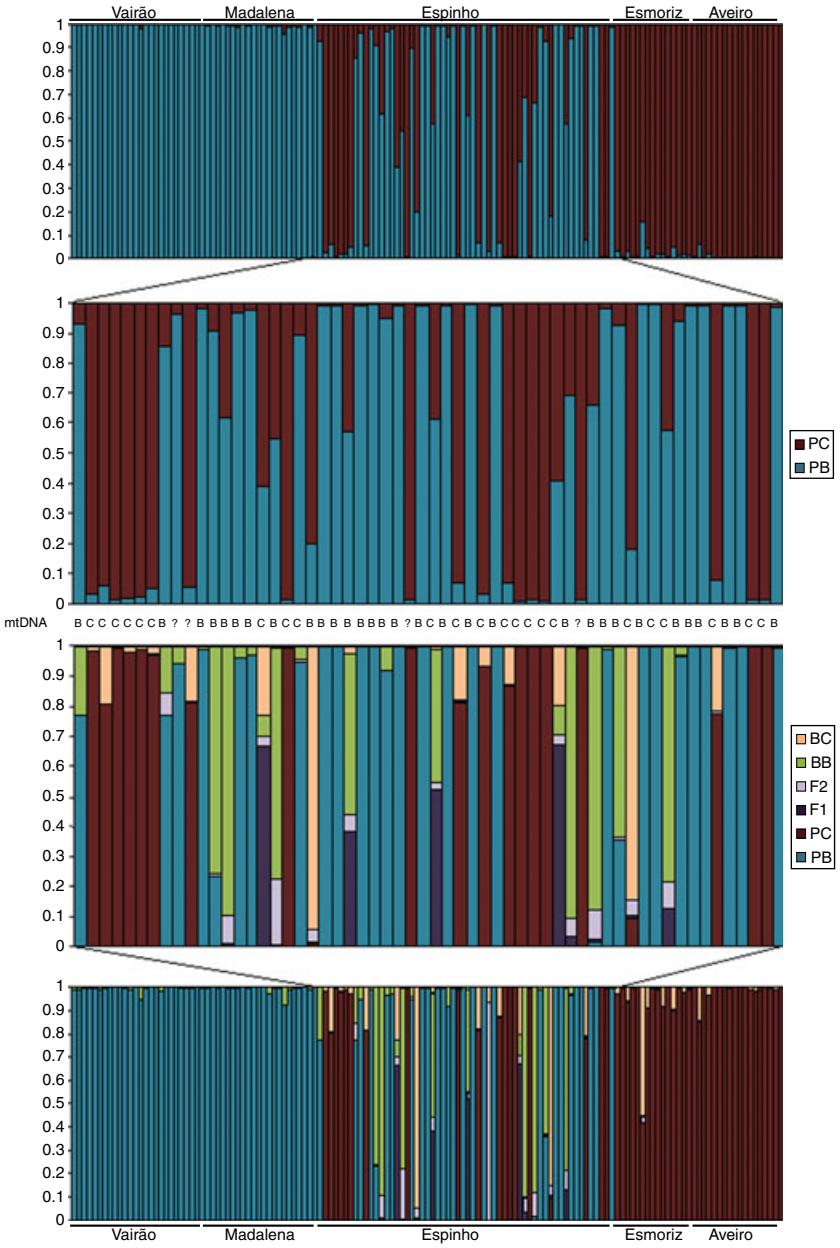

Fig. 3. Model-based multilocus genotype analyses and mitochondrial DNA results. Upper graphs correspond to the proportion of the genome of each individual originating in each of the two species, estimated using STRUCTURE. Each individual is represented by a vertical bar divided in two segments, the length of which is proportional to the estimated percentage of the genome of *Podarcis bocagei* (PB) or *Podarcis carbonelli* (PC), respectively. On the bottom, results obtained using NEWHYBRIDS are shown. Again, each individual corresponds to a bar divided in six portions, each proportional to the estimated posterior probabilities of assignment to pure *P. bocagei* (PB), pure *P. carbonelli* (PC), F1, F2, and backcross of a F1 hybrid with a pure *P. bocagei* (BB) or with a pure *P. carbonelli* (BC). The graphs shown in the centre of the figure demonstrate the results obtained in the contact zone. Letters B or C presented between these graphs correspond to the mitotype (*P. bocagei* and *P. carbonelli*, respectively) detected in the individuals. ?, no information is available for the mtDNA

Although it is clear that hybrid individuals exist, few of them, however, have similar proportions of their genome coming from either species; the most common admixed individuals, although being obviously 'not pure', show a high proportion of the genome assigned to one of the species. This clearly contrasts with the expected trends for a panmictic population, since simulations reveal that a unimodal curve peaking close to 50% should be obtained after only a few generations of free admixture (Fig. 4).

Comparison with the morphology

The discriminant analysis of the reference sample gave highly significant results, assigning correct specific classification to 92.44% of the individuals examined (95.52% for *P. bocagei* and 88.46% for *P. carbonelli*). In contrast, the level of correct classification of the total cross-validation sample (both allopatric and hybrid-zone individuals) was lower for both species (81.18% for *P. bocagei* and 82.09% for *P. carbonelli*). Within the cross-validation sample, individuals from the hybrid zone showed lower levels of correct classification (61.11% for *P. bocagei* and 81.25 for *P. carbonelli*) than those from allopatric populations (86.57% for *P. bocagei* and 82.35% for *P. carbonelli*) suggesting a higher morphological proximity of the two species in Espinho. However, there was no correlation between the genetically and morphologically assigned posterior probabilities of belonging to each species, nor for specimens of *P. bocagei* (Spearman $R = 0.099$, $p = 0.695$) neither for those of *P. carbonelli* (Spearman $R = 0.289$, $p = 0.278$) (Fig. 5).

Fertility

Data were available for 49 lizards from the contact zone (six males and nine females 'pure' *P. carbonelli*; 13 males and three female 'pure' *P. bocagei* and 14 male and four females putative hybrids) as well as for 86 *P. bocagei* (51 males and 35 females) from Vairão and 71 *P. carbonelli* (37 males and 34 females) from Torreira. None of them displayed any

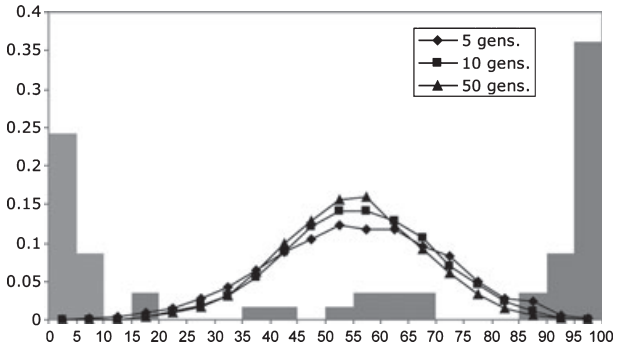

Fig. 4. Genetic bimodality within the hybrid zone. The *X* axis of the histogram represents the proportion of the genome attributed to *Podarcis bocagei* estimated using STRUCTURE and is divided in classes of 5%. The *Y* axis corresponds to the proportion of individuals falling in each class. Observed data are represented by grey bars. For comparison, results obtained from simulating 5, 10 and 50 generations of free admixture among the two species are also shown

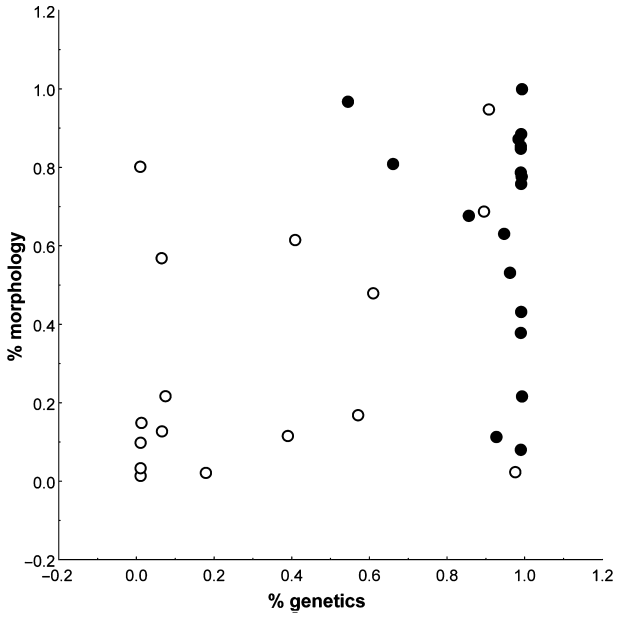

Fig. 5. Scatter-plot of the genetically versus the morphologically assigned posterior probabilities of belonging to *Podarcis bocagei* for the individuals analysed from the hybrid zone. Closed circles represent individuals empirically assigned to *P. bocagei* and open circles those assigned to *Podarcis carbonelli*

apparent abnormality in the sexual organs. In the contact zone, male hybrids had similar testis relative to their body size to 'pure' *P. carbonelli* but bigger than *P. bocagei* (volume ANCOVA, $F_{3,27} = 21.17$, $p < 10^{-6}$, Scheffé's *post-hoc* test $p < 0.02$; weight ANCOVA, $F_{3,27} = 21.14$, $p < 10^{-6}$, Scheffé's *post-hoc* test $p < 0.03$). The number of spermatozoa also displayed variation but it could not be attributed to any specific pairwise comparison (ANCOVA, $F_{3,27} = 9.69$, $p = 0.0002$, Scheffé's *post-hoc* test $p > 0.32$). However, both *P. carbonelli* and *P. bocagei* carried more spermatozoa in the contact zone than in allopatric populations (Carretero et al. 2006). Regarding females, no differences between hybrids and 'pure' specimens (allopatric or sympatric) were recorded (ovary weight, number of follicles and eggs, ANCOVAs $p > 0.15$).

Discussion

Our results are unambiguous in showing that *P. bocagei* and *P. carbonelli* hybridize in the area where they contact, since several individuals from the contact zone show a multilocus genotype presenting signs of admixture between both species. By a simple observation of multilocus genotypes, we are also able to answer our second question, since hybrids are not only viable but also show at least some degree of fertility, because successive generations of hybrid individuals were found in the contact zone (implying some degree of introgression).

Genetic bimodality within the hybrid zone

Although a large number of individuals seem to have a hybrid origin (Fig. 3), more individuals than expected under a scenario of free admixture were found to be genetically pure. In addition, the majority of hybrid individuals are not intermediate in composition; no F1s were detected, and most individuals bearing signs of admixture present a large fraction of their genome assigned to one of the species, contrasting to the expectations assuming panmixia (Fig. 4). This produces a strong deviation from Hardy–Weinberg expectation and strong LD. This contact zone therefore entirely fits the description of a bimodal hybrid zone (Jiggins and Mallet 2000). Although this pattern could in theory result from recurrent dispersal of pure individuals from neighbouring populations, we detected no evidence for dispersal in the opposite direction. It is therefore highly likely that this pattern results from barriers to gene flow.

Possible prezygotic isolating mechanisms

It has been suggested that prezygotic isolating mechanisms generally evolve faster than postzygotic barriers (see example from *Drosophila* in Coyne and Orr 1997), although this remains controversial (see review in Coyne and Orr 2004). Many bimodal hybrid zones show strong prezygotic isolation as a result of assortative mating (see Jiggins and Mallet (2000) and references therein). In the contact zone, the two species occupy similar habitats in close contact and breed during the same period of the year (Carretero et al. 2002, 2006); in other words, there are no obvious ecological or temporal barriers preventing gene flow. It is therefore likely that the inferred segregation between *P. bocagei* and *P. carbonelli* results from ethological barriers. The dynamics of mate recognition between these two species have been studied from a chemosensory point of view (Barbosa et al. 2005). It has been shown that both *P. bocagei* and *P. carbonelli* males tend to show a higher rate of tongue-flicks when presented with chemical stimuli from conspecific females when compared to heterospecific females, which is usually a sign of a larger interest in pursuing those stimuli. It seems therefore likely that at least female chemical cues and male chemosensory responses have coevolved in allopatry and interactions between these traits may be acting as partial barriers to gene flow.

These and other behavioural interactions may reduce initially heterospecific matings and could be sufficient to maintain bimodality within the hybrid zone. However, the first hybridizations are often rather rare but once this first barrier is overcome it is likely that backcrosses and F2s may hybridize more freely since behavioural incompatibilities tend to be attenuated (Coyne and Orr 2004; Mallet 2005). It is therefore reasonable to assume that the bimodal nature of this hybrid zone is also maintained by postzygotic isolating mechanisms.

Putative postzygotic isolating mechanisms

Possible selective forces operating against hybrids have not been studied in these species through controlled cross-species matings. In this context, we were interested in making a preliminary evaluation of patterns indicating decreased hybrid survival or reproductive success. First, we investigated whether Haldane's (1922) rule is verified on this hybrid zone. This empirical rule states that, when there is absence, rarity or sterility of first generation hybrids of one sex in a population of hybridizing taxa, that sex is usually the heterogametic one. As in most lacertids, females of *Podarcis* are the heterogametic sex (Olmo 2005). If Haldane's rule were applicable, we would expect to see (1) no females as F1 (in the case of female inviability) and/or (2) backcrosses to a parental species presenting mtDNA strictly from that species (in the case of complete F1 female sterility, resulting from the mating of a male hybrid with a pure female and not the opposite). Because we did not detect F1s in our sample, we were unable to test the first prediction. However, one out of the five individuals with over 0.75 PP of being a backcross with *P. bocagei* had *P. carbonelli* mtDNA, whereas one out of two backcrosses with *P. carbonelli* showed the other species' mitotype. Assuming that these individuals are real backcrosses, we may therefore rule out the possibilities of complete female inviability or sterility, but the possibility that the survival and reproductive success of F1 females might be lower than those of F1 males remains to be tested.

Second, we compared fertility indices in hybrids to those in pure individuals. Although sample size is a limiting factor, our study failed to detect a decrease in the fertility of the hybrids. Hybrid males have apparently normal gonads and their numbers of spermatozoa fall within the usual values of the species they were morphologically assigned to (Carretero et al. 2006). Although less represented in the sample, hybrid females also do not appear to show reduced ovary weights, number of follicles or eggs. Therefore, if selection is acting against hybrid fertility, it is probably doing so at later stages of the life cycle, such as embryo development.

We further explored the nature of possible postzygotic isolating mechanisms by looking at the morphological characteristics of hybrids. With this respect, no evident pattern was observed. However, it is evident that some morphological overlap between the two species exists. These lizards are known to present a very high intra-specific morphological variability (Arnold 1989) and the two species examined have been described as very similar (Sá-Sousa 2001b; Sá-Sousa and Harris 2002), justifying the morphological overlap observed between them. Although levels of correct classification of individuals into species were lower in the contact zone (70.59% as compared with 92.57% in the rest of the range of the two species), the analysis of correlation with the genetically assigned posterior probabilities revealed no relation between morphological and genetic identities. This suggests that some other mechanism, other than hybridization, may be responsible for the higher resemblance of both species in the contact zone, such as short-term adaptation to the same ecological conditions.

The validity of present assessments is certainly restricted by low sample sizes in comparisons between genetic and morphological and fertility data. Also, to correctly study and identify postzygotic mechanisms impeding gene flow it is crucial to produce hybrid offspring in captivity and analyse their morphological and reproductive characteristics.

Populations near the contact zone appear to be genetically and morphologically typical for their species, but we did find some individuals in these populations bearing foreign alleles. This suggests that the transition between species is geographically steep and that the hybrid zone is narrow, but introgression reaches areas outside the present contact zone. This could result either from neutral diffusion, positive selection for some alleles (Piálek and Barton 1997) or from the back and forth movement of the hybrid zone over time (e.g. as a consequence of climatic oscillations). A detailed geographic analysis of the extent of hybridization is beyond the scope of this article; nevertheless, it is clear that a formal cline analysis (Barton 1983) would certainly improve our knowledge on the dynamics of this hybrid zone.

Even considering the above mentioned limitations, it is clear from the data presented here that there are mechanisms preventing gene flow between both species. According to Jiggins and Mallet (2000), '(…) bimodality within a local population indicates that speciation of the parental forms is nearly complete.' Therefore, although it is clear that *P. bocagei* and *P. carbonelli* exchange genes, they probably represent 'good' species. Further examination of other Iberian *Podarcis* lineages is necessary to detect contact zones and to investigate levels of gene flow across them and ultimately to determine how many of the described mtDNA lineages may also deserve recognition as full species.

Acknowledgements

This work was partially financed by FCT research projects POCTI/40987/BSE/2001, POCTI/BSE/45664/2002 and POCI/BIA-BDE/55865/2004. CP, MAC and DJH are funded by postdoctoral grants (SFRH/BPD/28869/2006, SFRH/BPD/27025/2006 and SFRH/BPD/26738/2006, respectively) and AK was supported by a PhD grant (SFRH/BD/28565/2006), all from FCT. Collecting permits were provided by Instituto de Conservação da Natureza (ICN, Portugal). The authors would like to thank all the people that participated in sampling campaigns (Raquel Ribeiro, Bruno Ribeiro, Diana Barbosa, Ricardo Pereira, Sara Rocha, Gonçalo Themudo, Raquel Godinho and Elsa Froufe). The authors would also like to thank Armando Loureiro for the distribution data, Ettore Olmo for information on sexual chromosomes in *Podarcis*, and Rui Faria and Hélder Freitas for helpful advice regarding data analyses and discussion. The authors are also grateful to Miguel Carneiro for developing the program for hybrid genotype simulations.

Resumen

Mezcla genética entre los saurios endémicos ibéricos Podarcis bocagei y P. carbonelli*: evidencia de hibridación natural limitada y de una zona híbrida bimodal*

Cuando taxones que han divergido recientemente entran de nuevo en contacto, la extensión de la introgresión entre los mismos está relacionada con el grado de diferenciación alcanzado. Estudiar las zonas de contacto resulta, pues, esencial para interpretar si estos taxones se hallan aislados reproductivamente y, en última instancia, si van permanecer diferenciados en el futuro. Estudios recientes sobre las lagartijas ibéricas y norteafricanas del género *Podarcis* han demostrado la existencia de múltiples unidades evolutivas diagnosticables mediante marcadores genéticos y a través de la morfología si bien sugieren que ha tenido lugar flujo entre las distintas formas. Por consiguiente, estamos interesados en evaluar cómo se mantienen los límites entre las especies en las áreas donde contactan. En este trabajo, estudiamos la zona de contacto entre *P. bocagei* y *P. carbonelli*. Muestreamos en un transecto que incluía la única localidad conocida donde ambas se hallan en sintopía y analizamos una batería de 15

marcadores nucleares no ligados y de ADN mitocondrial. También llevamos a cabo análisis preliminares de morfología y fertilidad. Mediante el empleo de enfoques de agregación basados en modelos, mostramos que ambas especies hibridan en las poblaciones que contactan pero la evidencia de introgresión es reducida para las poblaciones adyacentes. Aunque un número significativo de individuos muestra evidencia de mezcla, esta zona híbrida es claramente bimodal, lo cual sugiere fuertes barreras al flujo genético cuya posible naturaleza se discute. Curiosamente, los análisis morfológicos no apoyan la existencia de formas intermedias entre los individuos genéticamente mixtos. En su conjunto, estos resultados constituyen una prueba más de la validez de *P. bocagei* y *P. carbonelli* como especies distintas.

References

Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. Genetics **160:**1217–1229.

Arnold EN (1989) Towards a phylogeny and biogeography of the Lacertidae, relationships within an Old-World family of lizards derived from morphology. Bull Br Mus **55:**209–257.

Barbosa D, Desfils E, Carretero MA, Font E (2005) Chemical stimuli mediate species recognition in *Podarcis* wall lizards. Amphibia-Reptilia **26:**257–263.

Barton NH (1983) Multilocus clines. Evolution **37:**454–471.

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. Annu Rev Ecol Syst **16:**13–148.

Belkhir K, Borsa P, Goudet J, Chikhi L, Bonhomme F (1996–2004) GENETIX, logiciel sous WindowsTM pour la génétique des populations.

Carretero MA (2006) Reproductive cycles in Mediterranean lacertids: plasticity and constraints. In: Corti C, Lo Cascio P, Biaggini M (eds), Mainland and Insular Lizards. A Mediterranean Perspective. Firenze University Press, Florence, pp 33–54.

Carretero MA, Sá-Sousa P, Barbosa D, Harris DJ, Pinho C (2002) Sintopía estricta entre *Podarcis bocagei* y *P. carbonelli*. Bol Asoc Herp Esp **13:**20–24.

Carretero MA, Ribeiro R, Barbosa D, Sá-Sousa P, Harris DJ (2006) Spermatogenesis in two Iberian *Podarcis* lizards: relationships with male traits. Anim Biol **56:**1–12.

Coyne JA, Orr HA (1997) Patterns of speciation in *Drosophila* revisited. Evolution **51:**295–303.

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA.

Dobzhansky T (1937) Genetics and the Origin of Species. Columbia University Press, New York, NY.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics **131:**479–491.

Galán P (2002) Hibridación en laboratorio de *Podarcis bocagei* y *Podarcis carbonelli*. Bol Asoc Herp Esp **13:**28–31.

Haldane JBS (1922) Sex-ratio and unisexual sterility in hybrid animals. J Genet **12:**101–109.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser **41:**95–98.

Harris DJ, Sá-Sousa P (2001) Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetol J **11:**129–136.

Hedrick PW (1999) Highly variable loci and their interpretation in evolution and conservation. Evolution **53:**313–318.

Jiggins C, Mallet J (2000) Bimodal hybrid zones and speciation. Trends Ecol Evol **15:**250–255.

Kaliontzopoulou A (2004) Efecto de la simpatría en la morfología de dos especies del género Podarcis en Portugal, DEA Thesis. University of Barcelona, Spain.

Kaliontzopoulou A, Carretero MA, Llorente G (2007) Multivariate and geometric morphometrics in the analysis of sexual dimorphism variation in *Podarcis* lizards. J Morphol **268:**152–165.

Kaliontzopoulou A, Carretero MA, Llorente G (2008) Head shape allometry and proximate causes of head sexual dimorphism in *Podarcis* lizards: joining linear and geometric morphometrics. Biol J Linn Soc **93:**111–124.

Loureiro A, Ferrand N, Carretero MA, Paulo OS (eds.) (2008) Atlas dos Anfíbios e Répteis de Portugal. Instituto da Conservação da Natureza e da Biodiversidade, Lisboa.

Mallet J (2005) Hybridisation as an invasion of the genome. Trends Ecol Evol **20:**229–237.

Muller HJ (1940) Bearing of the *Drosophila* work on systematics. In: Huxley J (ed.), The New Systematics. Clarendon press, Oxford, pp 185–268.

Muller HJ (1942) Isolating mechanisms, evolution and temperature. Biol Symp **6:**71–125.

Nielsen EE, Bach LA, Kotlicki P (2006) HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. Mol Ecol Notes **6:**971–973.

Olmo E (2005) Chromorep: a reptile chromosomes database. Available at: http://ginux.univpm.it/scienze/chromorep/.

Orr HA (1995) The population genetics of speciation: the evolution of hybrid incompatibilities. Genetics **139:**1805–1813.

Piálek J, Barton NH (1997) The spread of an advantageous allele across a barrier: the effects of random drift and selection against heterozygotes. Genetics **145:**493–504.

Pinho C, Harris DJ, Ferrand N (2003) Genetic polymorphism of 11 allozyme loci in populations of wall lizards (*Podarcis* spp.) from the Iberian Peninsula and North Africa. Biochem Genet **41:**343–359.

Pinho C, Ferrand N, Harris DJ (2004a) Genetic variation within the *Podarcis hispanica* species complex: new evidence from protein electrophoretic data. In: Pérez-Mellado V, Riera N, Perera A (eds), The Biology of Lacertid Lizards: Evolutionary and Ecological Perspectives. Institut Menorquí d'Estudis, Recerca 8, pp 269–277.

Pinho C, Sequeira F, Godinho R, Harris DJ, Ferrand N (2004b) Isolation and characterisation of 9 microsatellite loci in *Podarcis bocagei* (Squamata: Lacertidae). Mol Ecol Notes **4:**286–288.

Pinho C, Ferrand N, Harris DJ (2006) Reexamination of the Iberian and North African *Podarcis* (Squamata: Lacertidae) phylogeny based on increased mitochondrial DNA sequencing. Mol Phylogenet Evol **38:**266–273.

Pinho C, Harris DJ, Ferrand N. (2007a) Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol J Linn Soc **91:**121–133.

Pinho C, Harris DJ, Ferrand N. (2007b) Contrasting patterns of population structure and historical demography in three western Mediterranean lizard species inferred from mtDNA variation. Mol Ecol **16:**1191–1205.

Pinho C, Harris DJ, Ferrand N (2008) Non-equilibrium estimates of gene flow inferred from nuclear genealogies suggest that Iberian and North African wall lizards are an assemblage of incipient species. BMC Evol Biol **8:**63.

Presgraves DC, Bagalavan L, Abmayr SM, Orr HA (2003) Adaptive evolution drives divergence in a hybrid inviability gene between two species of *Drosophila*. Nature **423:**715–719.

Pritchard JK, Stephens M, Donnelly P. (2000) Inference of population structure using multilocus genotype data. Genetics **155:**945–959.

Raymond M, Rousset F (1995) GENEPOP (version 3.1): population genetics software for exact tests and ecumenicism. J Heredity **86:**248–249.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor Press, New York, NY.

Sá-Sousa P (2001a) Comparative chorology between *Podarcis bocagei* and *P. carbonelli* (Sauria: Lacertidae) in Portugal. Rev Esp Herpetol, **15:**85–97.

Sá-Sousa P (2001b) A controversa sistemática das lagartixas do género Podarcis Wagler, 1830 (Sauria, Lacertidae) em Portugal, PhD Thesis. University of Lisbon, Portugal.

Sá-Sousa P, Harris DJ (2002) *Podarcis carbonelli* is a distinct species. Amphibia-Reptilia, **23:**459–468.

Schneider S, Roessli D, Excoffier L (2000) Arlequin Ver. 2.000: A Software for Population Genetics Analyses. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
Wu C-I (2001) The genic view of the process of speciation. J Evol Biol **14:**851–861.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of localities of the specimens of *Podarcis bocagei* and *Podarcis carbonelli* examined for morphological analyses.

Table S2. Allele frequencies for nuclear loci in the five populations analysed.

Table S3. Validation of hybrid class assignment using HYBRIDLAB and NEWHYBRIDS.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.