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Phylogeography of the Italian wall lizard, *Podarcis sicula*, as revealed by mitochondrial DNA sequences

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Abstract

In a phylogeographical survey of the Italian wall lizard, *Podarcis sicula*, DNA sequence variation along an 887-bp segment of the cytochrome *b* gene was examined in 96 specimens from 86 localities covering the distribution range of the species. In addition, parts of the *12S rRNA* and *16S rRNA* genes from 12 selected specimens as representatives of more divergent cytochrome *b* haploclades were sequenced (together about 950 bp). Six phylogeographical main groups were found, three representing samples of the nominate subspecies *Podarcis sicula sicula* and closely related subspecies and the other three comprising *Podarcis sicula campestris* as well as all subspecies described from northern and eastern Adriatic islands. In southern Italy a population group with morphological characters of *P. s. sicula* but with the mitochondrial DNA features of *P. s. campestris* was detected indicating a probably recent hybridization zone. The present distribution patterns were interpreted as the consequence of natural events like retreats to glacial refuges and postglacial area expansions, but also as the results of multiple introductions by man.

Keywords: mitochondrial DNA, phylogeography, *Podarcis sicula*

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Introduction

The Italian wall lizard, *Podarcis sicula*, inhabits an extensive area in the central Mediterranean region. It is widespread and very abundant in Italy, on the large islands Sicily, Sardinia and Corsica and along the northern part of the east Adriatic coast as well as on many Adriatic islands. In addition, this remarkably competitive and successful species was introduced into a number of locations elsewhere in the Mediterranean region (in Portugal, Spain, France, Montenegro, Turkey, Libya, Tunisia) and the USA (Behler & King 1979; Conant & Collins 1991). Italy is thought to be the area of origin and the expansion centre of the species (Radovanović 1956; Schneider 1971; Gorman *et al*. 1975).

The east Adriatic region is inhabited by two similar *Podarcis* species, *Podarcis melisellensis*, probably autochthonous in this area, and *P. sicula*, a recent Italian invader. On the mainland and some larger islands both species are sympatric but never syntopic (Kammerer 1926; Radovanović 1966; Nevo *et al*. 1972; Gorman *et al*. 1975; Clover 1979). However, on small islands only one species always occurs: *P. melisellensis* usually becomes extinct after the arrival of the competitively superior *P. sicula* (Radovanović 1959, 1960). The distribution pattern of these two species is very complicated: on one hand, there are island groups inhabited exclusively by one of these species; on the other, there are many instances of one or two small islets inhabited by *P. sicula* which are completely surrounded by islands exclusively inhabited by *P. melisellensis*. This pattern cannot be explained only by natural immigration of *P. sicula* into the area of *P. melisellensis*. However, *P. sicula* represents a lizard species that is particularly easily transported by humans. Often it is found at beaches and in harbours which facilitate its transport through small fishing boats (Radovanović 1960). Therefore, accidental introduction by man may be an important factor in the spreading of *P. sicula* in the east Adriatic region. In this area, the species inhabits especially the northern coast and some islands. In the southern part, it is only present in narrow, isolated areas around the cities of Dubrovnik and Kotor, and on the south Dalmatian islands of Palagruža,

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Sušac, Kopište, Pod Kopište and Pod Mrčaru (Gorman *et al*. 1975). The distribution pattern of *P. sicula* in the east Adriatic region is generally explained to be the result of a recent colonization from Italy. Especially its occurrence on some south Adriatic islands and its absence from the Dalmatian mainland at this latitude was explained by immigration of a *P. s. campestris* like form from the Monte Gargano region (Apulia). Nevertheless, the mode of colonization, natural colonization or accidental introduction by man (Radovanović 1956; Witte 1965; Nevo *et al*. 1972; Gorman *et al*. 1975), was discussed controversially.

As it is the case with several other Mediterranean *Podarcis* species, a considerable number of subspecies of *P. sicula* has been described. In the latest review, Henle & Klaver (1986) recognized a total number of 52 subspecies. Twenty-four of them are restricted to Croatia, and the great majority represent local endemics of one or a few little islets. Henle & Klaver (1986) attempted to assign this abundance of taxa into five groups, where at least two of them, the *sicula* group and the *campestris* group are established on the basis of pattern similarities. The remaining three groups contain only one or a few taxa which could not be assigned with certainty to one of the two main groups. The first of them, the '*tyrrhenica* group', comprises the populations of the Tyrrhenian islands (Tuscan archipelago), from which we had, unfortunately, no samples. The second group, designated as '*cettii* group', contains only *Podarcis sicula cettii* from Sardinia, a population which has been introduced by humans according to Lanza (1982). The third group is represented only by *Podarcis sicula cattaroi*, the population of Kotor (Montenegro), which is of uncertain origin. With the exception of some isolated populations (*Podarcis sicula ragusae*, *Podarcis sicula cattaroi*, *Podarcis sicula hieroglyphica*), the mainland populations of *P. sicula* belong to only two subspecies, *P. s. sicula* and *Podarcis sicula campestris*. Schneider (1971) postulated that during the Wurm ice age the differentiation of *P. sicula* into the two main subspecies *P. s. sicula* and *P. s. campestris* may have occurred in different glacial refugia located in southern and central Italy, respectively. The present range of *P. s. sicula* comprises southern Italy and the western part of central Italy north up to Rome. Northern and eastern Italy as well as most parts of the east Adriatic coast are inhabited by the subspecies *P. s. campestris*.

So far, only a few attempts have been made to elucidate the evolution of *P. sicula* at the molecular level using allozyme electrophoresis (Gorman *et al*. 1975; Capula & Ceccarelli 2003) or sequencing of mitochondrial DNA (mtDNA) segments (Oliverio *et al*. 1998, 2001). However, all these studies were based on small samples and covered only a small part of the distribution range of the species.

We performed a detailed molecular genetic analysis of a large number of samples to reveal phylogeographical relationships of *P. sicula* and the connection between historical events and the present geographical distribution. Although the sample size may be far from ideal, it is still much bigger and covers a much greater range than any previously published molecular investigation on *P. sicula*. Therefore it seems justified to attempt a global intraspecific biogeography of *P. sicula*, although it is based on mitochondrial sequences only. Because sexually biased gene flow has been reported repeatedly on lizards (Doughty *et al*. 1994; Rassmann *et al*. 1997; Stenson *et al*. 2002), additional nuclear DNA markers should be analysed in the future to obtain a complete picture.

Materials and methods

A total of 96 samples of *Podarcis sicula* from 86 localities covering 24 of the 52 subspecies recognized to date (21 out of the 25 endemic to Croatia) were included in the analysis (Fig. 1, Table 1). Sequences of *Podarcis muralis muralis* (Austria, Baden, GenBank Accession nos AY185096, AY190305, AY190306), *Podarcis melisellensis fiumana* (Croatia, Koromačno: AY185029, AY184999, AY185010), and *Podarcis melisellensis melisellensis* (Croatia, Brusnik: AY185057, AY185006, AY185017) were used as outgroup.

Total genomic DNA was extracted by a standard phenol-chloroform protocol (Sambrook *et al*. 1989) from deep-frozen or ethanol-preserved soft tissues or tail tips, or from tongues when old, ethanol-preserved museum material was used.

In order to avoid erroneous inclusion of eventually present nuclear pseudogene sequences (numts) in the mitochondrial data set, mtDNA was purified from two samples (Sicily, Mti. Peloritani, and Krk, Ponikva) by methods described by Jones *et al*. (1988) and Beckman *et al*. (1993). The presence of a numt of the mitochondrial cytochrome *b* gene was detected (Podnar & Mayer, in preparation). Accordingly, using universal primers, the mitochondrial cytochrome *b* sequence can be obtained only from preparations of purified mtDNA, whereas the cytochrome *b* numt is preferentially amplified from total DNA extracts. Therefore, highly selective polymerase chain reaction (PCR) primers were designed (listed in Table 2), which amplify exclusively mitochondrial sequences also in presence of nuclear DNA.

Conditions for cytochrome *b*: the cytochrome *b* gene was analysed from all 95 samples. Amplification conditions involved an initial denaturation step of 2 min at 94 °C, 35 cycles of 10 s at 95 °C, 20 s at 50 °C and 90 s at 72 °C, and a final extension step of 7 min at 72 °C. Reamplification was performed directly from 1 μL of amplification mix under the same conditions as previous but in a reaction volume of 50 μL and an annealing temperature of 55 °C.

Conditions for *16S rRNA*: 12 samples were analysed (Table 1). Amplifications were performed under the same conditions as described for the cytochrome *b* gene. The

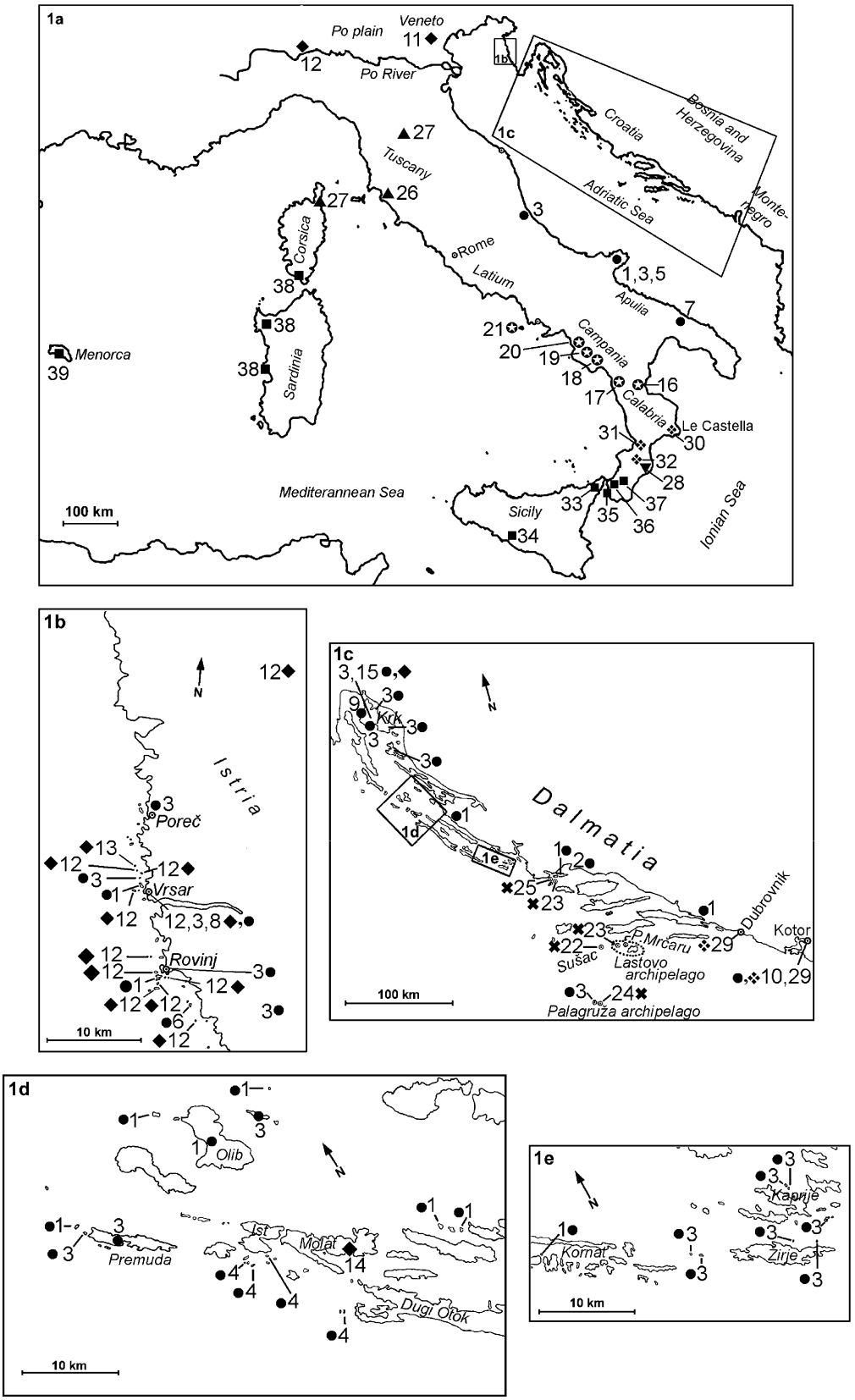

Fig. 1 Distribution of the observed haplotypes. The numbers correspond to the haplotype names presented in Table 1 and symbols to the eight haplotype groups (Fig. 2): ● = Adria, ◆ = Po plain, ✪ = Campania, ✖ = Sušac, ▲ = Tuscany, ▼ = Monasterace, ❖ = Catanzaro and ■ = Sicula.

Table 1 Cytochrome *b* haplotypes and localities of samples of *Podarcis sicula* used in this study

Cytochrome *b* haplotype	Geographic origin	Map no.
1	**I:** Pug.; Mte. Gargano (a)	1a
	HR: Istr.; Crveni Otok • (a), Istr.; Sveti Juraj • (a)	1b
	HR: Zadar (a), Čiovo • (a), **BIH**: Gradac (a)	1c
	HR: Olib • (b), Morovnik • (near Olib •) (b), Pohlib • (near Olib •) (b), Lutrošnjak • (near Premuda •) (d), Northern Sestrica • (near Sestrunj •) (a), Southern Sestrica • (near Sestrunj •) (a)	1d
	HR: Veseljuh • (near Kornat •) (e)	1e
2	**HR:** Split (a)	1c
3	**I:** Abr.; Cepagatti (a), Pug.; Mte. Gargano (a)	1a
	HR: Istr.; Poreč (a), Istr.; Galiner • (a), Istr.; Vrsar (a), Istr.; Rovinj (a), Istr.; Bale (a)	1b
	HR: Krk; Šilo • (a), Krk; Ponikva • (a), Krk; Baška • (a), Krk, Krk • (a), Rab • (a), Velika Palagruža • (c)	1c
	HR: Planik • (near Olib •) (b), Kamenjak • (near Premuda •) (a), Premuda • (f)	1d
	HR: Veli Dupinić • (near Kaprije •) (g), Mali Dupinić • (near Kaprije •) (g), Koromašna • (near Žirje •) (a), Ravan • (near Žirje •) (a), Samograd • (near Kornat •) (h), Vrtlić • (near Kornat •) (h), Gušteranski • (near Žirje •) (a)	1e
4	**HR:** Mala Sestrica • (near Ist •) (i), Dužac • (near Ist •) (i), Črnikovac • (near Ist •) (a), Veliki Laganj • (near Dugi Otok •) (j)	1d
5	**I:** Pug.; Mte. Gargano (a)	1a
6	**HR:** Istr.; Velika Sestrica • (a)	1b
7	**I:** Pug.; Ostuni (a)#	1a
8	**HR:** Istr.; Vrsar (a)	1b
9	**HR:** Krk; Poljica • (a)	1c
10	**YU**: Kotor (k)	1c
11	**I:** Ven.; Colli Euganei (a)	1a
12	**I:** Lomb.; Gropello Cairoli (near Pavia) (a)	1a
	HR: Istr.; Antonci (a), Istr.; Gusti Školj • (l), Istr.; Lakal • (m), Istr.; Lunga • (n), Istr.; Banjol • (o), Istr.; Samer • (a), Istr.; Veli Piruzi • (p), Istr.; Sveti Ivan • (n), Istr.; Sturag • (r), Istr.; Gustinja • (s), Istr.; Vrsar (a)	1b
13	**HR:** Istr.; Tovarjež • (t)	1b
14	**HR:** Molat • (a)	1d
15	**HR:** Krk; Ponikva • (a)	1c
16	**I:** Cal.; Sibari (u)	1a
17	**I:** Cal.; Scalea (u)	1a
18	**I:** Camp.; Torre Orsaia (u)	1a
19	**I:** Camp.; Vallo della Lucania (u)#	1a
20	**I:** Camp.; Paestum (u)	1a
21	**I:** Camp.; Ischia •(u)	1a
22	**HR:** Sušac • (near Lastovo •) (v/c)*	1c
23	**HR:** Pod Kopište • (near Lastovo •) (v/c)*, Pijavica • (near Trogir) (z)	1c
24	**HR:** Mala Palagruža • (x)	1c
25	**HR:** Kluda • (near Trogir) (z)	1c
26	**I:** Tusc.; Pta. Ala (a)#	1a
27	**I:** Tusc.; Florence (a), **F:** Corsica; Bastia (a)	1a
28	**I:** Cal.; Monasterace (u)	1a
29	**HR:** Dubrovnik (y), **YU**: Kotor (k)	1c
30	**I:** Cal.; Le Castella (u)	1a
31	**I:** Cal.; Lamezia Terme (u)	1a
32	**I:** Cal.; Serra San Bruno (u)	1a
33	**I:** Sic.; Mti. Peloritani (u)#	1a
34	**I:** Sic.; Agrigento (u)	1a
35	**I:** Cal.; Reggio di Calabria (u)#	1a
36	**I:** Cal.; Calanna (u)	1a
37	**I:** Cal.; Aspromonte (u)	1a
38	**I:** NW- Sardinia (w), Sard.; Oristano (w)#, **F:** Corsica; Bonifacio (w)	1a
39	**E:** Menorca • (u/w)?	1a

Localities from which *12S* and *16S rRNA* were also analysed are underlined, and those for which an additional 5′ section of the *12S rRNA* was sequenced are indicated with '#'. Letters in parentheses after the locality name indicate currently recognized subspecies: a, *campestris;* b, *pohlibensis;* c, *pelagosae;* d, *premudensis*; e, *vesseljuchi;* f, *premudana*; g, *dupinici*; h, *samogradi*; I, *mediofasciata*; j, *laganiensis;* k, *cattaroi*; l, *nikolici;* m, *zeii*; n, *insularum;* o, *bagnolensis*; p, *pirosoensis;* r, *astorgae*; s, *pretneri*; t, *bolei*; u, *sicula*; v, *cazzae*; z, *kolombatovici*; ×, *adriatica*; y, *ragusae*; w, *cettii*. *originally described as *P. s. cazzae* — put in synonymy with *P. s. pelagosae* by Henle & Klaver (1986).

Map No. refers to the maps in Fig. 1. Symbol • indicates island or islet. * originally described as *P. s. cazzae* — put in synonymy with *P. s. pelagosae* by Henle & Klaver (1986).

Abbreviations: **I**, Italy; **HR**, Croatia; **F**, France; **BIH**, Bosnia and Herzegovina; **YU**, Serbia and Montenegro; **E**, Spain; Pug, Apulia; Istr, Istria; Abr, Abruzzi; Ven, Veneto; Lomb, Lombardy; Cal, Calabria; Camp, Campania; Tusc, Tuscany; Sic, Sicily; Sard, Sardinia.

Table 2 Primer sequences for amplification (A), reamplification (R) and sequencing (S)

Primer	Sequence	Use	Reference
12S rRNA			
L-40*	5′-AAG CAT AGC ACT GAA GA-3′	A, R, S	Oliverio *et al*. (1998)
H-626*	5′-AGA ACA GGC TCC TCT AGG-3′	A, R, S	Oliverio *et al*. (1998)
L-524**	5′-AAA CTG GGA TTA GAT ACC CCA CTA T-3′	A, R	Knight & Mindell (1993)
H-981**	5′-GTA CAC TTA CCT TGT TAC GAC TT-3′	A, R	Knight & Mindell (1993)
L-775**	5′-ACG TCA GGT CAA GGT GTA GC-3′	S	Titus & Frost (1996), modified
H-909**	5′-AGG GAT GAC GGG CGG TGT GT-3′	S	Kocher *et al*. (1989), modified
16S rRNA			
L- 1625	5′-GTG GGC CTA AAA GCA GCC AC-3′	A, R	Reeder (1995)
H-2456	5′-CCG GTC TGA ACT CAG ATC ACG-3′	A, R	Heise *et al*. (1995)
L-1926	5′-CGC CTG TTT ACC AAA AAC AT-3′	S	Knight & Mindell (1993)
cytochrome *b*			
L-14253	5′-TTT GGA TCC CTG TTA GGC CTC TGC C-3′	A, R	this study
H-15425	5′-GGT TTA CAA GAC CAG TGC TTT-3′	A	this study
H-15150	5′-ATA ATA AAG GGG TGT TCT ACT GGT TGG CC-3′	R, S	this study
H-14776	5′-GGT GGA ATG GGA TTT TGT CTG-3′	S	this study

In the primer name, the number refers to the 3′ end when aligned with the *Eumeces egregius* mtDNA genome (Kumazawa & Nishida 1995), and H or L to the heavy or light strand, respectively. Marks '*' and '**' refer to the 5′- and 3′ portion of the *12S rRNA* gene, respectively.

PCR products were run on 1% agarose gels, desired fragments were cut out and purified with the QIAGEN gel extraction kit. These primary PCR products were then used as templates for reamplifications performed under the same conditions as for cytochrome *b* gene.

Conditions for *12S rRNA*: two sections of this gene were obtained separately. The 3′ section of *12S rRNA* gene was analysed from the same 12 samples as used for *16S rRNA*. Additionally, the 5′ section of *12S rRNA* was analysed from six samples (Table 1) in order to compare our data with previously published *P. sicula* sequences (Oliverio *et al*. 1998, 2001). Thermocycling for amplifications of the 5′ section of this gene consisted of an initial denaturation step of 2 min at 94 °C and 35 cycles of 10 s at 95 °C, 20 s at 50 °C and 1 min at 72 °C, and a final extension step of 7 min at 72 °C. Reamplifications were performed under the same conditions but at an annealing temperature of 52 °C. The PCR conditions for the second section were the same as described for cytochrome *b*. For both sections, amplification products were purified as described for *16S rRNA*.

All reamplification products were purified with the High Pure PCR Products Purification Kit (Roche). Sequencing was carried out by MWG-BIOTECH (Ebersberg) with primers listed in Table 2.

Sequence data and phylogenetic analyses

Sequences were aligned using CLUSTALX (Thompson *et al*. 1997) and corrected by eye. Pairwise comparison of uncorrected sequence divergences (*p*-distances), number and type of base substitutions were estimated using MEGA version 2.1 (Kumar *et al*. 2001). Calculation of *p*-distances for ribosomal sequences was performed using MATRIX 2.0 (Posada 2001) treating gaps as fifth character state.

We used three types of phylogenetic analyses: maximum parsimony (MP) and maximum likelihood (ML) as implemented in PAUP (version 4.0b10, Swofford 2002) and Bayesian inference as implemented in MRBAYES (version 3.0b4, Huelsenbeck & Ronquist 2001). All of the analyses were conducted on cytochrome *b* as well as on combined (cytochrome *b*, 3′ section of *12S* and *16S rRNA*) data set. In order to test eventual heterogeneity in phylogenetic signal among the three genes, a partition–homogeneity test with 100 replicates (PAUP version 4.0b10, Swofford 2002) was performed on a combined data set including all three partitions.

MP analyses of both data sets were conducted using the heuristic search mode with 100 repeats, randomised input orders of taxa, and tree bisection-reconnection (TBR) branch swapping with all codon positions weighted equally. Transitions and transversions were weighted differentially, according to the rates estimated by MEGA. Gaps in ribosomal sequences were treated as missing data. Nonparametric bootstrapping (1000 pseudoreplicates, 10 addition-sequence replicates) was used to assess the stability of internal branches in the trees.

For ML analyses, the optimal model of sequence evolution, was determined with MODELTEST software (version 3.06, Posada & Crandall 1998): for cytochrome *b* the HKY85 + Γ model (Hasegawa *et al*. 1985; Yang 1993) and for the combined data set the GTR + I + Γ model (Rodriguez *et al*. 1990). Analyses were performed using heuristic search mode and TBR branch swapping algorithm. The initial starting tree for cytochrome *b* data set was obtained by neighbour-joining (NJ) and for combined data set by

stepwise addition. Sequence addition option was 'AsIs'. Because of computational constraints bootstrap (BS) analysis of ML trees was performed only for the combined data set with 100 bootstrap replicates and TBR branch swapping.

Bayesian analyses were performed by running four Markov chains (one cold and three heated) for 3 000 000 generations, saving trees every 100 generations. The likelihood value stabilized already after 14 000 and 12 000 generations for cytochrome *b* and the combined data set, respectively. However, for safety reasons, we discarded the first 6000 trees ('burn in'), and estimated the Bayesian posterior probabilities from the 50% majority-rule consensus tree of the 24 000 sampled trees. The cytochrome *b* data set was analysed using the HKY85 evolutionary model ('nst = 2') with base frequencies set to the fixed values (as estimated with ModelTest), gamma distribution rate variation across sites and fixed Ti/Tv ratio (as obtained by MEGA). In the analysis of the combined data set, the GTR model ('nst = 6') with gamma distributed rates and fixed base frequencies was applied to the noncoding partition, while the settings for coding partition were the same as those for the cytochrome *b* data set.

The data set containing our *12S rRNA* 5'-sections (six samples; see Table 1) and eight sequences published by Oliverio *et al*. (1998, 2001) was analysed using the NJ method as implemented in MEGA. Oliverio's sequence set is characterized by remarkable length polymorphisms indicating some sequencing errors in his older results (Oliverio *et al*. 1998). Therefore, to minimize the effects of such errors, we used the modus 'pairwise deletion' for indel positions. To assess the stability of internal branches in the trees bootstrap analysis (2000 pseudoreplicates) was performed.

All sequences are deposited in GenBank with the following Accession nos (12S rRNA: AY184996, AY770863–AY770868, AY770906–AY770911, 16S rRNA: AY185093, AY185092, AY770912–AY770921, cytochrome *b*: AY185095, AY185094, AY770869–AY770905).

Results

Sequence data

The alignment of 95 cytochrome *b* sequences was 887 bp long. No indels were detected. From 180 variable characters 15.6% were at the first, 3.3% at the second, and 81.1% at the third codon position. Base composition was slightly A + T biased (57.6%), and the mean transition to transversion ratio was 7.5. A total of 39 different cytochrome *b* haplotypes were identified. Uncorrected (*p*) sequence divergence values among them range from 0.1% to 9.1% (Table 3).

Table 3 Observed ranges of uncorrected pairwise sequence divergence (in percentages) among clades

	Clades								
Genes	Adria	Campania	Po plain	Sušac	Tuscany	Catanzaro	Sicula	Monast	
cytochrome *b*	0.1–0.5								Adria
12S	n.d.								
16S	n.d.								
cytochrome *b*	0.7–2.5	0.8–2.3							Campania
12S	0	n.d.							
16S	0	n.d.							
cytochrome *b*	0.9–1.7	0.7–2.5	0.1–0.7						Po plain
12S	0	0	n.d.						
16S	0.4	0.4	n.d.						
cytochrome *b*	3.5–3.9	3.6–4.5	4.1–4.7	0.1–0.8					Sušac
12S	0.4	0.4	0.4	n.d.					
16S	0.6	0.6	1.0	n.d.					
cytochrome *b*	5.7–6.1	5.1–5.7	5.5–6.0	6.0–7.0	0.6				Tuscany
12S	0.9	0.9	0.9	0.9	n.d.				
16S	1.2	1.2	1.2	1.4	n.d.				
cytochrome *b*	7.4–8.6	7.3–8.6	7.9–8.9	7.6–9.0	8.6–9.1	0.2–3.7			Catanzaro
12S	2.6–3.0	2.6–3.0	2.6–3.0	2.6–3.0	2.6	0.0–0.9			
16S	2.2	2.2	2.2	2.4	2.2	0.0–0.4			
cytochrome *b*	6.0–7.0	6.2–7.3	6.5–7.8	7.3–8.6	7.4–8.6	5.9–6.9	0.1–2.4		Sicula
12S	3.6–4.3	3.6–4.3	3.6–4.3	3.6–4.3	3.2–3.9	2.1–2.6	0.4–0.9		
16S	2.6–3.0	2.6–3.0	2.6–3.0	2.8–3.2	2.6–3.0	1.2–1.8	0.2–0.4		
cytochrome *b*	7.2–7.7	7.3–7.9	7.9–8.5	8.3–8.5	7.9–8.2	7.0–7.9	5.7–6.2	n.d.	Monast.
12S	3.9	3.9	3.9	3.9	3.4	3.0	1.9–2.6	n.d.	
16S	2.6	2.6	2.6	2.8	2.6	1.2	1.0–1.4	n.d.	

Abbreviations: 'Monast.', Monasterace, 'n.d.', no data.

Translation of DNA into protein sequences revealed 21 (out of 301) variable amino acid sites. No premature stop codons were detected.

The alignments of *12S* (3′ portion) and *16S rRNA* sequences (excluding outgroups) obtained from 12 samples were 466 and 501 bp long, respectively. Base composition was also slightly A + T biased (57.2% in *12S* and 58.1% in *16S rRNA*) and the mean transitions/transversions ratio was 3.7 and 7.8, respectively. The highest observed uncorrected (*p*) sequence divergence values between the samples were 4.3% for *12S* and 3.2% for *16S rRNA* (Table 3).

Phylogenetic analyses

A partition–homogeneity test (PAUP version 4.0b10, Swofford 2002) justified the analyses of the combined data set by revealing no significant conflict between fragments (cytochrome *b* vs. *12S rRNA*: $P = 0.82$; cytochrome *b* vs. *16S rRNA*: $P = 0.20$ and *12S rRNA* vs. *16S rRNA*: $P = 0.80$).

All analyses resulted in six well-supported clades but there are several remarkable differences regarding basal tree topology depending on data set (cytochrome *b* only or combined set) and tree building algorithm.

MP trees (not shown) derived from both cytochrome *b* (two equally parsimonious trees: length = 1016.5, consistency index (CI) = 0.707, retention index (RI) = 0.859, rescaled consistency index (RC) = 0.607) and combined data set (a single most parsimonious tree: length = 1223.1, CI = 0.772, RI = 0.781, RC = 0.603) have congruent overall topologies (see BS values in Fig. 2a, 2b) with an unresolved tetratomy of the three south Italian haploclades (Sicula, Catanzaro, and Monasterace) and the northern group (Fig. 2a, 2b). Based on cytochrome *b* data, three well-supported haploclades, Campestris-Sicula, Sušac, and Tuscany, are found within the northern group. In addition, within the Campestris-Sicula haploclade two subclades, Adria and Po plain have a high BS support. The haplotypes of the samples of Campania and northernmost Calabria form a more variable assemblage, the Campania subgroup which is paraphyletic with respect to the subclades Adria and Po plain (Fig. 2a).

Both ML (single tree, $-\ln L = 5524.15262$) and Bayesian analyses of the combined data set revealed a congruent overall topology, which is, unlike the MP trees, characterized by division into two main groups, the southern one comprising the populations from southern Italy, and a northern one with the populations from the rest of the species range. However, the southern group is well supported only with Bayesian posterior probabilities. Another difference in comparison with the MP tree is the clustering of the Monasterace and Sicula haploclades, yet, it is well supported only in the Bayesian analysis (Fig. 2b). The topology of the tree obtained in the ML analysis of the cytochrome *b* data (single tree, $-\ln L = 3689$) did not reveal the northern and southern main groups found with the combined data set. Rather, the Sicula haploclade is the sister group of all the other well-supported haploclades: Catanzaro, Monasterace, Tuscany, Sušac, and finally the Campestris-Sicula haploclade. The relationships within the latter haploclade are the same as in the MP trees. The tree obtained with the Bayesian analysis of the cytochrome *b* data revealed, on the contrary, the same overall tree topology as that found in the MP trees except the clustering of the Monasterace sample with the Sicula haploclade. This node is, however, only poorly supported by Bayesian posterior probabilities (Fig. 2a).

In addition, the 5′ section of the *12S rRNA* was analysed from six samples in order to compare our data with previously published *Podarcis sicula* sequences (Oliverio *et al*. 1998, 2001). This alignment was 556 bp long (long *12S* data set). Including additional shorter sequences published by Oliverio *et al*. (2001) resulted in an alignment of 305 positions (short *12S* data set). The *p*-distances between Oliverio's Sardinia sample (Pse#4) and our Sardinia and Sicily samples (Table 1) were 0.5%, and between his (Pss#5) and our Campania samples 0.4% (Table 1, long data set). The sequences of Oliverio's (Pss#6) and our Apulia sample (short data set) were identical. The tree resulting from NJ analysis of *12S rRNA* 5′-sections data set (not shown) is characterized by two well-supported main clades, one containing our Sardinia, Sicily and Calabria samples as well as the Sardina sample of Oliverio *et al*. (1998), and another comprising two subclades (BS values 98 for both). One subclade encompasses the samples from Campania and Apulia (Oliverio *et al*. 1998, 2001), and the other one the samples from Latium and the USA (Oliverio *et al*. 2001) which cluster with our Tuscany sample.

Discussion

The basic intraspecific phylogeographical pattern of *Podarcis sicula* is characterized by the existence of six main haploclades.

The three southern haploclades correspond to the populations from Sardinia and southernmost Italy usually referred as *Podarcis sicula cettii* (Sardinia) and *Podarcis sicula sicula* (Italy and Sicily), respectively: (i) the Sicula clade from southwestern Calabria, Sicily (the terra typica of the species), as well as from Sardinia, (ii) the Monasterace group from only one locality on the Ionian coast in southeastern Calabria, and (iii) the Catanzaro group found in central Calabria.

The three remaining (northern) haploclades form a monophyletic unit in all trees which corresponds widely with the distribution area of *Podarcis sicula campestris* and related subspecies (*campestris* group according to Henle & Klaver 1986). The most basal Tuscany clade comprises the specimens from the northern areas of western Italy (Rome province northwards to Tuscany). The sequences from

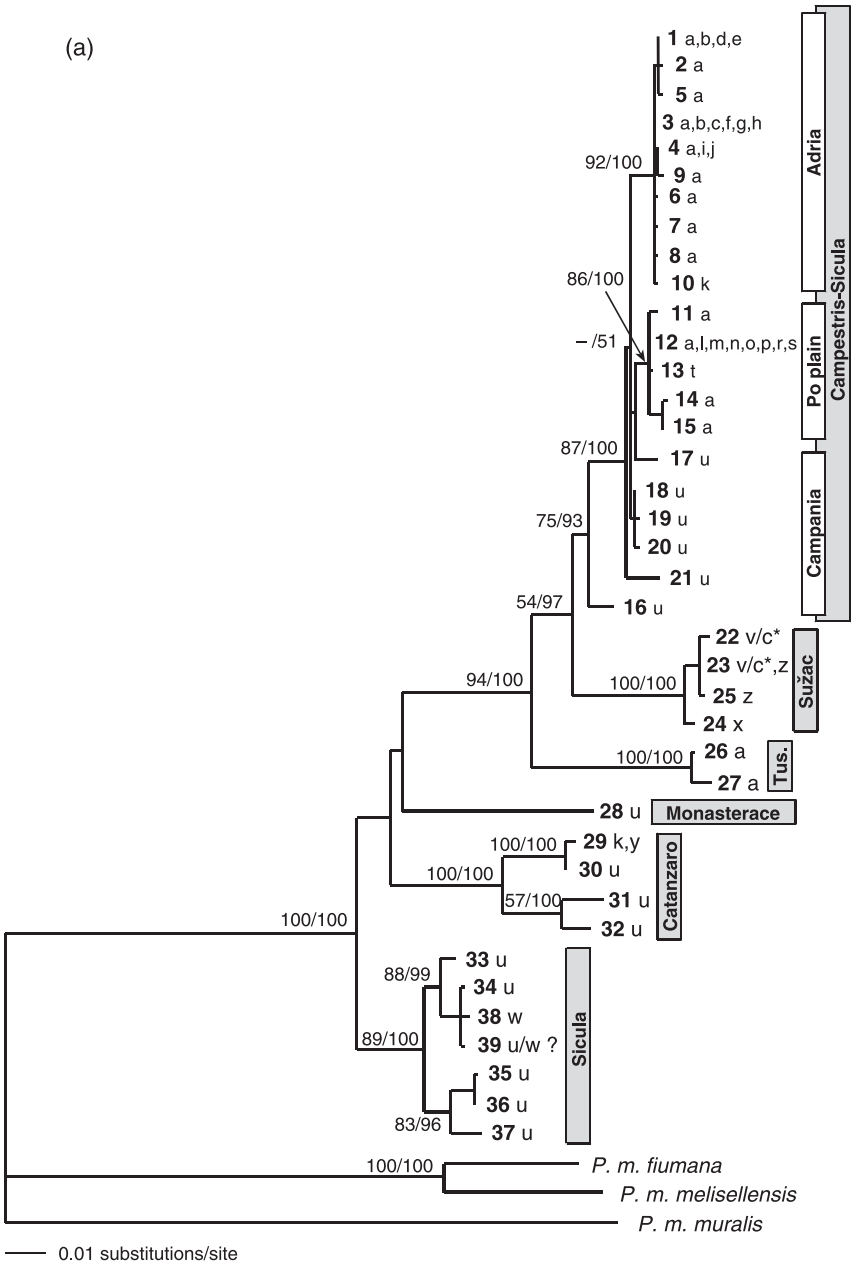

Fig. 2 Phylograms from ML analyses of (a) cytochrome *b* and (b) combined data set (for each sample the cytochrome *b* haplotype is indicated in parenthesis). Numbers on the branches indicate MP bootstrap values and Bayesian posterior probabilities (cytochrome *b*), respectively, and ML and MP bootstrap values and Bayesian posterior probabilities (combined data set), respectively. BS values smaller than 50 are indicated with dash. Abbreviation 'Tus.' = Tuscany. Letters in lower case indicate currently recognized subspecies (see Table 1).

some islands in southern and central Dalmatia form the Sušac clade which is very well separated from all other populations around the Adriatic Sea. The third clade of this group includes the populations of northern Italy and most of the Adriatic part of the species' area (*P. sicula campestris* and related subspecies) as well as populations from parts of southwestern Italy (*P. s. sicula*). Within this Campestris-Sicula clade, two well-supported subclades can be distinguished: (i) the Adria group, which has a circum-Adriatic distribution, and (ii) the Po plain group from the Po plain and the northern Adriatic region including a sample from the vicinity of the terra typica of the subspecies *campestris*. The rest of the Campestris-Sicula haploclade forms an obviously paraphyletic assemblage, the Campania group, which can be defined by its geographical area (northernmost Calabria, Campania and, most probably, southern Latium) and by the fact that it represents exclusively populations attributed to the subspecies *sicula* according to external features.

Central Mediterranean region (Italy and Corsica)

If we accept that the subspecies *P. s. sicula* has a continuous range extending from Sicily northwards as far as to the

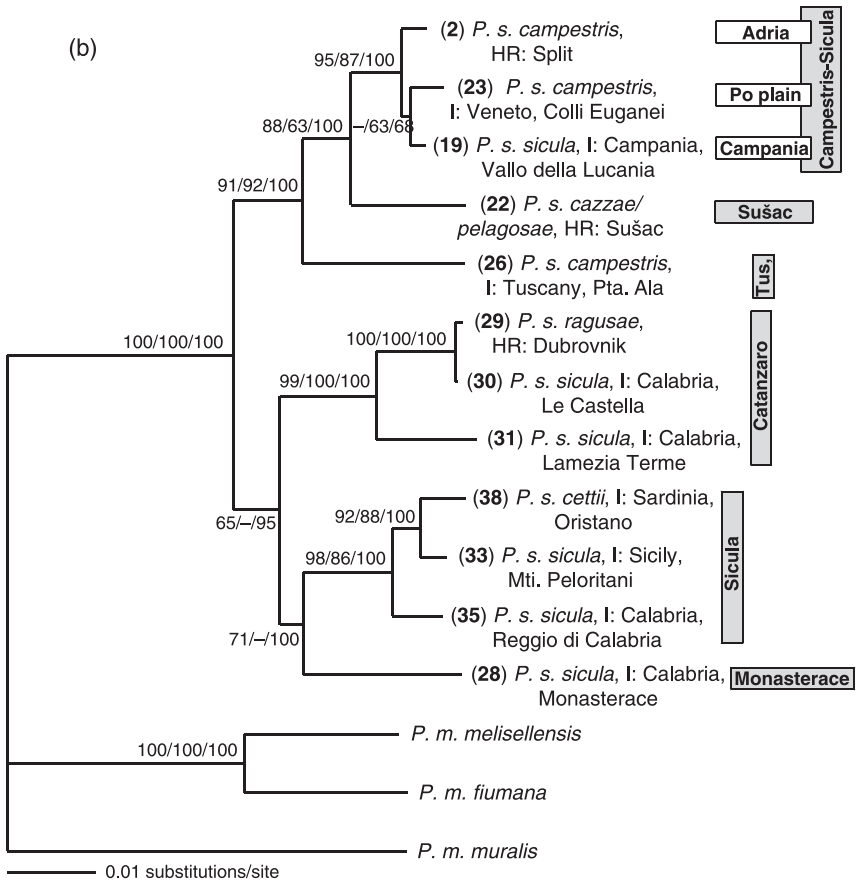

Fig. 2 *Continued*

Rome province (including all of Calabria and Campania), we can claim with certainty that representatives of this subspecies occur in four groups of mitochondrial haplotypes. It should be emphasized that the observed sequence divergences of up to 3.9% in the *12S rRNA* gene observed here within one single subspecies are in the same range or even greater than those between some *Podarcis* species. For example, in the same section of the *12S rRNA* gene, the difference between *Podarcis gaigeae* and *Podarcis milensis* is 4.1%, and 2.6% between *Podarcis lilfordi* and *Podarcis pityusensis* (Podnar & Mayer, unpublished). For the same species but in a somewhat shorter section of the *12S rRNA* gene, Harris & Arnold (1999) found 3.4% and 2.9% sequence divergence, respectively. If we accept a rough calibration of the molecular clock of 1.25% sequence difference per million year (Myr) for the *12S rRNA* gene (Lin *et al*. 2002; Podnar *et al*. 2004), those mitochondrial lineages would have diverged about 2–3 million years ago (Ma).

It is hard to believe that, over such a long period, population groups have not even slightly changed in their morphology. If we accept that the subspecies *P. s. sicula* is morphologically homogenous (although there is no any comprehensive study on that matter), the observed discrepancies cannot be explained by a survival of different but geographically well-correlated mitochondrial lineages for two or three Myr within one subspecies in a comparatively small area. The only explanation is a recent or subrecent hybridization between lineages with very different mitochondrial haplotypes. According to our hypothesis, the four haplotype groups of *P. s. sicula* (Campania, Sicula, Monasterace, and Catanzaro) correspond to formerly separated and differentiated population groups. In the past, one of those groups could have entered the ranges of the other groups and hybridized with them. Theoretically, the invader responsible for the expansion of the *sicula* morphotype, could have been any of these four hypothetical ancient forms. However, because nearly all Adriatic haplotypes of *P. s. campestris* are much closly related to the Campania haplotypes of *P. s. sicula* than to the Tuscany haplotypes of *P. s. campestris* (Fig. 2), and because it is extremely unlikely that two so morphologically similar forms as are the Tuscan and Adriatic populations of the subspecies *campestris* would have arisen independently, we can exclude the populations from Campania as the potential invaders with high probability. But all other hypothetical former morphotypes, whose mtDNA is still present in south Italy, are potential candidates for that

postulated invasion. Those hybridization events are hard to date, but at least in the Campania region they are surely pretty young, because they could not have happened before the divergence of the Po plain and Adria subclades. Although the small genetic distances between these two haplotype groups do not allow any precise dating, the splitting could be ascribed to the existence of two separate Wurm glacial refugia in southeast Italy (Apulia). Concerning the remaining groups, the previously described phenomenon could have taken place much earlier. However, we prefer the hypothesis assuming a fast postglacial expansion of one of the southern representatives. During its expansion, it entered the range of the other forms. Hybridization resulted in the final elimination of these forms whereas their mtDNA still survived. This could be explained by the preferential migration of males, as suggested by Birky *et al*. (1983), and, at least in case of the occupation of the *P. s. campestris* range by *P. s. sicula*, caused by the superior size and strength of the males of the nominate subspecies. As a consequence, reproductive success of *P. s. campestris* males would be negligible in comparison with that of the newly intruding males of *P. s. sicula*. Because we are not dealing here with a limited episode of hybridization, but rather with a continuous process, it is easy to conceive that such preferred hybridizations had repeatedly occurred with *sicula* males. Because of the dilution of the *campestris* (nuclear) alleles, such populations developed morphological traits characteristic for *P. s. sicula* while still preserving the mtDNA of *P. s. campestris* formerly autochthonous within that area.

Although the morphology of lizards is often strongly affected by local environmental constraints (Losos *et al*. 1997), it is highly unlikely that this phenomenon accounts for the appearance of the *sicula* morphotype in Campania's populations, because, under very similar environmental conditions of northern Calabria and southern Apulia, very different morphotypes corresponding to the subspecies *sicula* and *campestris*, respectively, occur. Results of Oliverio *et al*. (2001) strongly support our hypothesis. He reports on lizards found in the boundary area between the ranges of *P. s. sicula* and *P. s. campestris* (Formia, Latium, near Rome), which displayed transitional morphological characters but possessed (according to our results) solely the Tuscany mitochondrial haplotype typical for *P. s. campestri*s from Tuscany. The intermediate morphological features of these individuals suggest that hybridization in that region is very young and still an ongoing process. A similar phenomenon has been reported by Stenson *et al*. (2002) on the Dominican anole (*Anolis oculatus*) where analysis of microsatellite allele frequencies has not revealed strong phylogeographical structuring as had been observed in mtDNA haplotypes. The authors suggested male-biased gene flow consistent with highly territorial male behaviour as we propose for *P. s. sicula* males.

Recently, Capula & Ceccarelli (2003) published an allozyme-electrophoretic analysis of *P. sicula* from southwestern Italy. Their results show clearly that some alleles predominate in the areas of the 'Campania' group or the 'Catanzaro' clade, respectively. These area-characteristic electromorphs may be remnants of the ancient populations of these regions, but, unfortunately, samples from southern Calabria, Sicily and the Adriatic regions were not included in their study. Thus, comparisons with representatives of the two main subspecies from those areas are not possible.

Based on cytochrome *b* data, the southernmost haploclade, the Sicula group is divided into two subclades comprising the populations of southwestern Calabria and Sicily Sardinia (Fig. 2). The cytochrome *b* distances between these two subclades are twice as large as between the subgroups, Adria and Po plain of *P. s. campestris*, a divergence roughly corresponding to a separation since the Riss glacial period. The representatives of the Sicula group could have colonized Sicily from the Calabrian region, but colonization could also have happened in the opposite direction. According to the first scenario, *P. sicula* should have been present in northeastern Sicily for a longer time than in the south and west of the island. This is indicated by the lack of the second Sicilian *Podarcis* species, *Podarcis wagleriana*, in northeastern Sicily, which may be explained by a long-time competition with the more robust species, *P. sicula*. Therefore, we prefer the hypothesis assuming the colonization of Sicily by *P. sicula* from Calabria. As there are only three substitutions in the cytochrome *b* gene between the sample from Agrigento in southern Sicily and both Sardinian samples, we can claim with certainty that the Sardinian populations, described as *Podarcis sicula cettii*, originated from Sicily (Fig. 2). This is in agreement with the hypothesis of Lanza (1982) presuming a colonization of Sardinia from Sicily in historical time. Therefore, possible morphological differences between the subspecies *sicula* and *cettii* cannot be ascribed to a long-lasting independent evolution, but rather to founder effects and genetic drift. Müller (1905) and Eisentraut (1950) believed that the population of Menorca resulted from a recent introduction from Sardinia. However, our Menorca sample is somewhat closer to the Sicilian than to the Sardinian samples, which — in accordance with the assumption of La Greca & Sacchi (1957) — would favour an introduction from Sicily.

The cytochrome *b* sequence of our sample from Corsica is very similar to our and Fu's (2000; GenBank Accession no. AF206531) Tuscan samples and even identical to our sample from Florence confirming Schneider's (1971) assumption of a recent introduction of *P. s. campestris* from Tuscany to Corsica.

Oliverio *et al*. (1998, 2001) published some *12S rRNA* sequences of *P. sicula* from Italy and from introduced populations in the USA. However, small sample size as well as

the fact that samples from the terrae typicae have not been investigated gave rise to several taxonomic misinterpretations regarding the Italian populations of *P. sicula*. In a first study, Oliverio *et al*. (1998) investigated three *P. sicula* samples belonging to the main subspecies (*sensu* Henle & Klaver 1986): *campestris*, *sicula* and *cettii*. However, only the *cettii* sample was collected on the terra typica of this subspecies. The two other samples (*sicula* and *campestris*) originated from Campania and northern Latium, regions that are generally believed to be inhabited by the subspecies *sicula* and *campestris*, respectively, but the terrae typicae are located far away in Sicily, and Verona in the Po plain, respectively. Oliverio *et al*. (1998) found a high level of sequence divergence between the specimens of *P. s. sicula* and *P. s. cettii* and proposed (Oliverio *et al*. 2000) a long lasting isolation of the Sardinian subspecies as indicative of species status. However, as proved by our investigation, there are only very small differences between samples from Sardinia and Sicily regarding mtDNA data. The cause for Oliverio's erroneous interpretation lies simply in the fact that his reference samples of *P. s. sicula* belong to a population group originating from an area where *sicula*-like populations possess *campestris*-like mtDNA. Most of the samples studied by Oliverio *et al*. (1998, 2001) belong to the Tuscany clade distributed in Tuscany as well as (according to Oliverio's data) in northern Latium. We showed that sequence divergences between haplotypes belonging to the Tuscany group and all the other haplotypes of the northern main group are high, and therefore it is understandable that this remarkable differences were misinterpreted as characteristic of different subspecies.

East Adriatic region (Croatia and Montenegro)

In Croatia and Montenegro, we found haplotypes of *P. sicula* belonging to four haploclades: Adria, Po plain, Sušac and Catanzaro. The Adria haplotypes occupy the largest geographical area in the Adriatic region (Fig. 1). We found them in the whole region from Poreč in Istria in the north to as far as Kotor in Montenegro in the south. The most frequent haplotypes are 3 and 1 (Table 1), while almost all other haplotypes differ only by one or two substitutions from one of them. The fact that both haplotypes, 1 and 3, were found also in Apulia on the west Adriatic coast, as well as the great similarity of all Adria haplotypes, indicates a recent and rapid colonization of the Adriatic region out of Apulia.Haplotypes belonging to the Po plain group are mostly restricted to the Istrian region. The only exceptions are the haplotypes 15 and 14 that were found on the islands Krk and Molat, respectively. There are two main characteristics of the Po plain haplotypes found in the Istrian region. First, they are found mostly on the offshore islands and the hinterland of the peninsula, while along the coast, the Adria haplotypes prevail. Second, they are extremely uniform, only two different haplotypes were found (12 and 13). The widespread haplotype 12 is also found in the western Po plain and is separated by only two substitutions from the haplotype 11 found in the terra typica region of *P. s. campestris* (Veneto, Italy). The current distribution of haplotypes suggests that Istria was colonized by *P. sicula* in two waves. According to our results, the Po plain type of *P. sicula* would have colonized the Istrian peninsula and the island Krk in a first wave. During the last rise of the sea level, these populations became isolated on the newly formed Istrian islets, but were widespread on the Istrian mainland. In a second wave of colonization, lizards belonging to the Adria subgroup arrived in this region, most probably from the south, and displaced the Po plain type in the coastal area but could not reach the island already separated at this time. A similar replacement of Po plain by Adria haplotypes may currently occur on the island Krk: Haplotype 15 was found in the central part of Krk, while all samples taken from the coast showed one of the Adria haplotypes (Fig. 1c).

Within the Sušac haploclade, we found four different cytochrome *b* haplotypes (22–25) which occur on some islands on the southwestern border of the Dalmatian island world (Mala Palagruža, Sušac, Pod Kopište), but also on two small islets close to the central Dalmatian coast (Kluda and Pijavica). The channel depths surrounding Sušac and the Palagruža archipelago are deeper than 120 m, and these old islands were most probably not connected, neither with each other nor with the mainland during the last glaciation. That is why we believe that the refuge for the Sušac subgroup was located within this area.

Although the islands, Mala Palagruža and Velika Palagruža lie very close together, they are inhabited by populations with very different haplotypes. On the first island we found a haplotype of the 'Sušac' group whereas on Velika Palagruža, one of the widespread haplotypes of the Adria group (3) was found. Considerable sequence differences between the haplotypes from Sušac (22) and Mala Palagruža (24) do not support a recent introduction. Rather, it seems that the Sušac haplotype group is also the autochthonous type of both Palagruža islands. Velika Palagruža was inhabited by man as far back as Neolithic times. Because of its central position in the Adriatic it has always served as a harbour for trading ships connecting places on both sides of the Adriatic Sea. The original haplotype on Velika Palagruža had been probably replaced by the Adria haplotype 3 by introduction in historical time. Finally, because the islets, Pijavica and Kluda are surrounded either with *melisellensis* islands or *sicula* populations with Adria haplotypes, the finding of Sušac haplotypes (23 and 25) at those islets is the clear indication for a recent anthropogenic introduction from south Adriatic islands. Such obviously frequent displacements of *P. sicula* by man are the main problem to tracing natural spreading pathways of this

species. While a probable scenario could be reconstructed for the south Dalmatian islands, it is impossible to differentiate between natural colonization and introduction within the northern Adriatic region.

The population from Dubrovnik (*Podarcis sicula ragusae*) is regarded as introduced by man from the Apennine Peninsula (Radovanović 1956, 1969). Its cytochrome *b* haplotype (29) belongs to the Catanzaro group and is nearly identical to the haplotype found in Le Castella in eastern Calabria suggesting an introduction from this region, probably by merchant shipping. The population of Kotor (*Podarcis sicula cattaroi*) could not be assigned to a particular subspecies group by Henle & Klaver (1986) because of its uncertain origin. In the city of Kotor we found lizards with very different external features, some like *P. s. campestris*, some like *P. s. sicula* and some with intermediate pattern. From two haplotypes found, one is identical to that from Dubrovnik (29) and the other one (10) belongs to the Adria group. Obviously, the Kotor population originated from two independent introductions, one from Dubrovnik and the other one from any other place in the Adriatic region. Both coastal cities had a great historical importance as harbours with highly developed trade between each other, as well as with Italian harbours.

Taxonomic implications

Within *P. sicula*, 52 subspecies are currently recognized (Henle & Klaver 1986). Forty-seven of these subspecies are island endemics and most of them have been described on the basis of very small differences and / or using very small samples. Mitochondrial DNA investigations are, of course, not the key for intraspecific classifications. Primarily intraspecific hybridizations with introgression of foreign mtDNA can obscure the real evolutionary patterns. However, in some cases, our results corroborate doubts of the validity of several subspecies of *P. sicula*.

We investigated samples from eight subspecies endemic on west Istrian islets where we found, nearly exclusively, a Po plain haplotype clearly differentiated from the Adria types occurring on the mainland and on islands close to it. The authors of these subspecies (Mertens 1937; Brelih 1961) obviously realized the differences to the mainland populations but overestimated population specific features probably caused rather by founder effects and genetic drift than by long-term evolutionary processes.

From northern Dalmatia, nine endemic island subspecies are recognized. All of them show Adria haplotypes, mostly the widespread haplotypes 1 and 3 or the haplotype 4 (see Fig. 2a). The latter one was also found in the *P. s. campestris* population from Črnikovac islet south of Ist, and differs only by one substitution from haplotype 3. This is the area where natural immigration and accidental introduction by man probably overlap. Again, the assumed specific features of the taxa are more the products of founder effects and drift phenomena than of local evolutionary processes.

A similar situation is encountered with respect to the populations of Sardinia, Dubrovnik and Kotor which have obviously been introduced in historical times. We regard it as a taxonomic nuisance to accept subspecies representing just recently introduced populations, especially if no investigations on the variability within the populations of origin exist.

The phylogeographical hypotheses that we present in this study should be examined using nuclear DNA markers in further investigations. Furthermore, this investigation gave a rise to some questions concerning the ecology of the species (such are the role of sexual selection and the cause of high morphological variability) that should be addressed in further research.

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