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Phylogeography of Balkan wall lizard (*Podarcis taurica*) and its relatives inferred from mitochondrial DNA sequences

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Abstract

Wall lizards of the genus *Podarcis* (Sauria, Lacertidae) comprise 17 currently recognized species in southern Europe, where they are the predominant reptile group. The taxonomy of *Podarcis* is complex and unstable. Based on DNA sequence data the species of *Podarcis* falls into four main groups that have substantial geographical conherence (western island group, southwestern group, Italian group and Balkan group). The Balkan species are divided in two subgroups: the subgroup of *Podarcis taurica* (*P. taurica, P. milensis, P. gaigeae* and perhaps *P. melisellensis*), and the subgroup of *Podarcis erhardii* (*P. erhardii* and *P. peloponnesiaca*). We addressed the question of phylogenetic relations among the species of the *P. taurica* subgroup encountered in Greece, as they can be inferred from partial mtDNA (cyt *b* and 16S) sequences. Our data support the monophyly of *P. taurica* subgroup and suggest that *P. gaigeae, P. milensis* and *P. melisellensis* form a clade, which thereinafter connects to *P. taurica*. Within the previous clade, *P. gaigeae* is more closely related to *P. milensis* than to *P. melisellensis*. However, the specimens of *P. taurica* were subdivided in two different groups. The first one includes the specimens from northeastern Greece, and the other group includes the specimens from the rest of continental Greece and Ionian islands. Because the molecular clock of the cyt *b* and 16 rRNA genes was not rejected in our model test, it is possible to estimate times of speciation events. Based on the splitting of the island of Crete from Peloponnisos [*c.* 5 million years ago (Ma)], the evolutionary rate for the cyt *b* is 1.55% per million years (Myr) and for the 16S rRNA is 0.46% per Myr. These results suggest that the evolutionary history of *P. taurica* in Greece is more complex than a single evolutionary invasion. The data analysed, stress the need for a reconsideration of the evolutionary history of Greek *Podarcis* species and help overcome difficulties that classical taxonomy has encountered at both the species level.

Keywords: Balkan Peninsula, molecular clock, molecular phylogeny, mtDNA markers, *Podarcis*

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Introduction

Studying patterns of genetic variation in a geographical context via gene trees (i.e. phylogeography) has contributed considerably to our understanding of what factors have influenced population structure and species divergence (Avise 1994). Phylogeography is seen as the bridge between population genetics and phylogenetic systematics (Avise *et al*. 1987; Avise 2000). Phylogenetic methods can be used to infer haplotype trees and estimate patterns of relatedness among haplotypes focusing on the historical relationships of gene lineages. By comparing the phylogenetic tree to the geographical structure of the data, one can infer historical patterns of population subdivision, understand the current distribution of the studied species, and estimate the divergence time among the species under study.

The complex geological history of the Balkan Peninsula and especially of the Hellenic area (multiple geological events of land connections) during the late Tertiary have

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likely contributed to the diversification and distribution of many terrestrial animals (Beerli *et al*. 1996; Douris *et al*. 1998; Parmakelis 2003; Poulakakis *et al*. 2003, 2005). Located at the margin of the Eurasian and African plates, this area has experienced tremendous geological alterations since the late Tertiary (Creutzburg 1963; Steininger & Roegl 1984; Meulenkamp 1985; Dermitzakis 1990). Connections offered the opportunities for biological dispersal, while submergence of land bridges brought about distributive isolation among related taxa. If this was a major element influencing the formation of the rich fauna of the Hellenic area, then the phylogenetic relationships of terrestrial taxa should reflect these palaeogeographical events. Compared with what is known for the geological history of this region, the impact of those geological events on the historical biogeography of terrestrial animals is less well understood.

Phylogenetic relationships among *Podarcis* species have attracted attention from many researchers (Castilla *et al*. 1998a, b; Oliverio *et al*. 1998, 2000; Harris & Arnold 1999; Sá-Sousa *et al*. 2000; Harris & Sá-Sousa 2001, 2002; Harris *et al*. 2002). *Podarcis* is a complex of species, diffused in Europe and northwest Africa. Although 17 to 18 species of *Podarcis* have been recognized (Harris & Sá-Sousa 2002; Sá-Sousa & Harris 2002) and subdivided into four groups [the western island, the southwestern, the Italian, and the Balkan group (Harris & Arnold 1999)], there is considerable controversy and gaps in knowledge about the taxonomic status and biogeographical history of these groups.

So far, only a few attempts have been made to elucidate the evolution of *Podarcis taurica* and its relatives (*Podarcis gaigeae* and *Podarcis milensis*) at the molecular level using allozyme electrophoresis or sequencing of mitochondrial DNA (mtDNA) segments. Using partial mitochondrial DNA (mtDNA) sequences, Harris (1999), Harris & Arnold (1999) and Oliverio *et al*. (2000) concluded that the relationships among *Podarcis* species cannot be definitively resolved with the data sets they used. Nevertheless all these studies support the monophyly of a Balkan group of *Podarcis*. Based on the preliminary results of the molecular study of Poulakakis *et al*. (2003), the Balkan group of species is divided in two subgroups, the subgroup of *taurica* (*P. taurica*, *P. milensis*, *P. gaigeae*, and perhaps *P. melisellensis*, a species which was not included in the previous study) and the subgroup of *erhardii* (*Podarcis erhardii* and *Podarcis peloponnesiaca*, which are highly diversified and present great morphological and ecological plasticity, inhabiting many different ecotypes). Within the former subgroup, protein electrophoresis indicates that *P. gaigeae* is more closely related to *P. milensis*, whereas both *P. gaigeae* and *P. milensis* are more similar to *P. taurica* (Mayer & Tiedemann 1980, 1981; Tiedemann & Mayer 1980), and that *P. taurica* has closer relation to *P. milensis* than *P. peloponnesiaca* (Chondropoulos *et al*. 2000). However, all these studies were based on only one sample per species and covered a very small part of their distribution range.

Podarcis taurica is distributed in a large area of the Balkans, as well as Hungary, the Crimean Peninsula and northwestern Anatolia (Gasc *et al*. 1997). It exhibits a notable geographical variation in colour and pattern size. So far, three subspecies have been described on the basis of colouration, patterning and relative leg length: *Podarcis taurica taurica* is found in the largest part of species' range, *Podarcis taurica thasopoulae* is found in the island of Thasopoula in Greece and *Podarcis taurica ionica* is found in the western part of the Greek mainland and the Ionian islands (Chondropoulos *et al*. 1993). On the other hand, *P. gaigeae* and *P. milensis* are endemic insular species, which are distributed only on the island group of Skyros and Milos, respectively and *P. melisellensis* is distributed on the east coast of Adriatic Sea (see Fig. 1).

In the work presented here, *taurica*'s subgroup specimens from several localities of the Balkan Peninsula were collected, and the partial mitochondrial cytochrome *b* (cyt *b*) and 16S rRNA genes were sequenced. This information was combined with previously published sequences in order to construct phylogenetic relationships of *P. taurica* and its relative species (*P. milensis*, *P. gaigeae*, *P. melisellensis*). Specifically, we tried to evaluate alternative models of the biogeographical history of *P. taurica* and its relatives on the Balkan area, discussing general implications on the historical biogeography of this region and to estimate the chronology of diversification events among those species, using a calibrated molecular clock.

Materials and methods

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from 36 specimens of *Podarcis* (Table 1, Fig. 1). Tissue samples were homogenized in a digest buffer and total genomic DNA was extracted using proteinase K dissolution, purified by two extractions with phenol–chloroform–isoamyl alcohol (25:24:1), one with chloroform–isoamyl alcohol (24:1), and precipitated using isopropanol. Two target genes were selected for molecular phylogenetic analysis: (i) a partial sequence (425 bp) of the mitochondrial protein-encoding cytochrome *b* gene (cyt *b*), and (ii) a partial sequence of the mitochondrial gene encoding 16S rRNA (16S). The universal L14724 and H15149 primers (Kocher *et al*. 1989) were used to amplify the cyt *b* region of the mtDNA. The polymerase chain reaction (PCR) was performed as follows in the presence of 3 mM $MgCl_2$: 35 cycles of denaturation at 94 °C for 45 s, annealing at 47 °C for 45 s and extension at 72 °C for 60 s. Primers 16Sar-L and 16Sbr-H (Palumbi *et al*. 1991) were used to amplify a segment of approximately 530 bp from the 16S rRNA region of the mtDNA, according to the

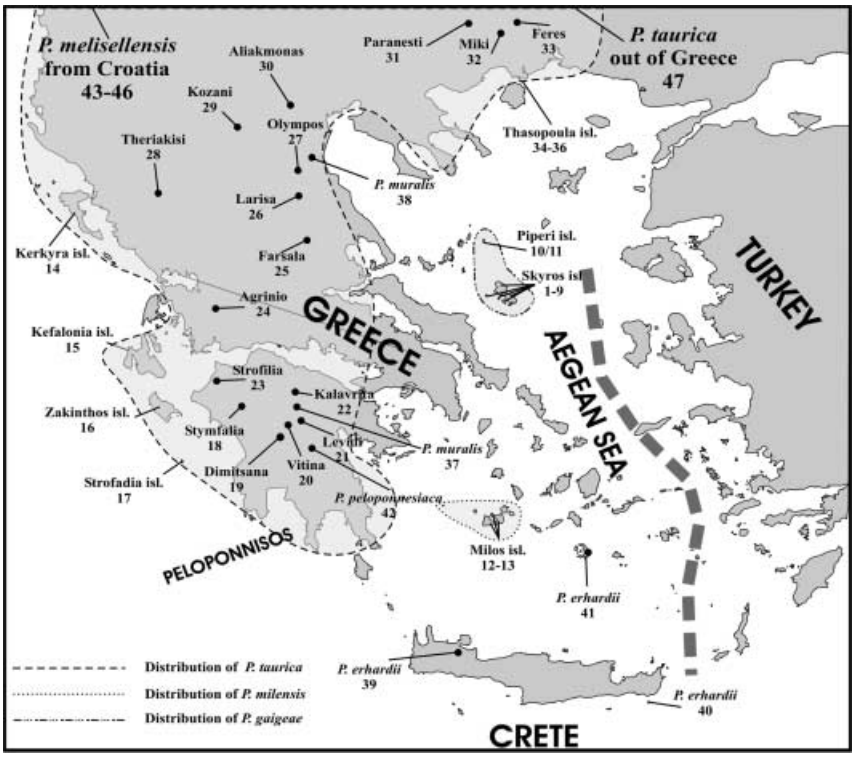

Fig. 1 Map showing the sampling localities of the 47 specimens used for the DNA analysis. Twenty-four specimens of *Podarcis taurica*, 11 specimens of *Podarcis gaigeae*, 2 specimens of *Podarcis milensis*, 2 specimens of *Podarcis muralis*, 3 specimens of *Podarcis erhardii*, 1 specimen of *Podarcis peloponnesiaca*, and 4 specimens of *Podarcis melisellensis*. The thick dashed line presents the Mid-Aegean trench.

Table 1 List of the specimens of *Podarcis* examined, with population map codes (see Fig. 1), taxon name, number of samples, geographical origins, and the accession numbers. Individuals from three closely related species were used as outgroup taxa: *Lacerta andreanskyi*, *Gallotia stehlini*, and *Gallotia galloti*

				Accession no.	
Map code	Species	Samples	Locality	cyt *b*	16S rRNA
1–9	*P. gaigeae gaigeae*	9	Skyros I.	AY768706–AY768741	AY768742–AY768777
10–11	*P. g. weigandi*	2	Piperi I.	AY768706–AY768741	AY768742–AY768777
12–13	*P. milensis milensis*	2	Milos I.	AY768706–AY768741	AY768742–AY768777
14–17	*P. taurica ionica*	4	Ionian Is.	AY768706–AY768741	AY768742–AY768777
18–23	*P. t. taurica*	6	Peloponnisos	AY768706–AY768741	AY768742–AY768777
24–28	*P. t. taurica*	5	Central Greece	AY768706–AY768741	AY768742–AY768777
29–33	*P. t. taurica*	5	North Greece	AY768706–AY768741	AY768742–AY768777
34–36	*P. t. thasopoulae*	3	Thasopoula I.	AY768706–AY768741	AY768742–AY768777
37–38	*P. muralis*	2	Cont. Greece	AF4862–32, 33	AY8961–81, 80
39–41	*P. erhardii*	3	Crete/Cyclades	AF4862–13, 16, 25	AY896–147, 153, 225
42	*P. peloponnesiaca*	1	Peloponnisos	AF486231	AY896172
43–46	*P. melisellensis*	4	Croatia	AY1850–20, 23, 29, 52	AY185009–012
47	*P. taurica*	1	Out of Greece	AF080280	AF019653

following PCR profile: 35 cycles of denaturation at 94 °C for 60 s, annealing at 47 °C for 60 s and extension at 72 °C for 60 s. The light strands were sequenced for all 36 individuals using a PE-ABI377 automated sequencer. Two specimens of *Podarcis muralis*, three specimens of *Podarcis erhardii*, one specimen of *Podarcis peloponnesiaca* (Poulakakis *et al*. 2003, unpublished), four specimens of *Podarcis melisellensis* (Pondar *et al*., unpublished), and one specimen of *Podarcis taurica* (Harris *et al*. 1998) retrieved from GenBank were included in the phylogenetic analysis (Table 1).

Individuals from three closely related species of the same family were used as outgroup taxa: *Lacerta andreasnkyi* (cyt *b*: AF206537 and 16S: AF206603; Fu 2000), *Gallotia stehlini* (cyt *b*: AF439949; Rando *et al*., unpublished; and 16S: AF149936; Beyerlein & Mayer 1999), and *Gallotia galloti* (cyt *b*: AF439946 and 16S: AF019651; Rando *et al*., unpublished).

Alignment and genetic divergence

The alignment of the concatenated cyt *b* and 16S rRNA sequences was performed with CLUSTAL_X (Thompson *et al*. 1997) and corrected by eye. Sequence divergences were estimated using the MEGA computer package (version 2, Kumar *et al*. 2001) using the Tamura–Nei model of evolution (Tamura & Nei 1993) to adjust for differences in nucleotide frequencies and substitution-rate heterogeneity. The alignment used is available on request from the authors. GenBank Accession numbers for the sequences obtained are AY768706–AY768741 for cyt *b* and AY768742–AY768777 for 16S rRNA.

Phylogenetic analyses

Analyses for phylogenetic inference were conducted using three methods: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). To examine whether the sequences from the two genes should be combined in a single analysis, a partition homogeneity test which was described as the incongruence-length difference test by Farris *et al*. (1995), was run in PAUP (version 4.0b10, Swofford 2002) and significance was estimated by 1000 repartitions.

MP analysis was performed using PAUP 4.0b10, with heuristic searches using stepwise addition and performing tree-bisection–reconnection (TBR) branch swapping (Swofford *et al*. 1996). Confidence in the nodes was assessed by 1000 bootstrap replicates (Felsenstein 1985) with random addition of taxa.

For ML analysis (Felsenstein 1981), the best-fit model of DNA substitution and the parameter estimates used for tree construction were chosen by performing hierarchical likelihood-ratio tests (Huelsenbeck & Crandall 1997) in MODELTEST (version 3.06, Posada & Crandall 1998). Likelihood-ratio tests and the Akaike information criterion (AIC; Akaike 1973) indicated that the GTR model + I + G (Rodriguez *et al*. 1990) had the lowest likelihood score and showed a significantly better fit than the other, less complicated models. Heuristic ML searches were performed with 10 replicates of random sequence addition and TBR branch swapping. Since an ML tree search with such a complex model would be computationally excessive, we employed only 100 iterations.

The BI method was performed with the software MRBAYES (version 3.0B, Huelsenbeck & Ronquist 2001) using the GTR model of substitution with rate heterogeneity set to a gamma distribution, hence applying the fewest possible number of constraints to the data set. The analysis was run with four chains for 10^7 generations and the current tree was saved to file every 100 generations. This generated an output of 10^5 trees. The –ln *L* stabilized after approximately 10^5 generations and the first 10^4 trees (10% 'burn-in' in Bayesian terms, chain had not become stationary) were discarded as a conservative measure to avoid the possibility of including random, suboptimal trees. The percentage of samples recovering any particular clade in a Bayesian analysis represents that clade's posterior probability (Huelsenbeck & Ronquist 2001). We used one of the methods of Leaché & Reeder (2002) to assure that our analyses were not trapped on local optima. In particular, the posterior probabilities for individual clades obtained from separate analyses (four runs) were compared for congruence (Huelsenbeck & Imennov 2002), given the possibility that two analyses could appear to converge on the same ln-likelihood value while actually supporting incongruent phylogenetic trees.

Divergence time and molecular-clock

To estimate divergence times, the net nucleotide divergence (*Da*) between geographical groups was calculated from Tamura & Nei (1993) pairwise distances using the software MEGA (version 2, Kumar *et al*. 2001). This metric corresponds to the between-group variation corrected for the within-group variation in haplotypes and can be used to calculate the splitting time of groups (Nei 1987). A molecular-clock likelihood-ratio test (LRT), $\lambda = -2(\ln L_{\text{nonclock}} - \ln L_{\text{clock}})$, which is distributed as χ^2 with $n - 2$ degrees of freedom, where n is the number of sequences, was performed to determine whether there was a statistical difference in evolutionary rates among clades (Muse & Weir 1992; Huelsenbeck & Crandall 1997).

Results

Of the 925 sites examined, there were 135 variable cyt *b* sites, 119 of which were parsimony informative (154 and 133 respectively when the outgroups were included in the analysis), and 76 variable 16S rRNA sites, 67 of which were parsimony informative (148 and 99 respectively including outgroups). For cyt *b*, ingroup sequence divergence (Tamura & Nei model) ranged from 0% to 20.8%, while for 16S rRNA sequence divergence ranged between 0% and 7.9% (Table 2).

A partition homogeneity test indicated no conflicting phylogenetic signals between the data sets ($P = 0.971$), and the mtDNA genes were analysed together. Tree length distribution, determined from random sampling of 10^6 unweighted trees, was significantly skewed to the left ($g1 = -0.48$), suggesting a strong phylogenetic signal in the data ($P < 0.01$; Hillis & Huelsenbeck 1992).

Equally weighted parsimony analysis of the 232 parsimony-informative characters (186 for the ingroup) produced more than 10 000 most-parsimonious trees with a length of 688 steps (consistency index, CI = 0.697 and retention index, RI = 0.840). The 50% majority rule tree is presented

Table 2 Pairwise comparison (Tamura–Nei model) of cyt *b* (below diagonal) and 16S rRNA (above diagonal) sequences among the major mtDNA clades/lineages of *Podarcis* included in this study. Values in diagonal are within clade sequence divergences, cyt *b*/16S rRNA. No values were calculated (nc) where only one individual was sampled

	1	2	3	4	5	6	7	8	9	10
1. *P. taurica* (A1)	**2.5/0.8**	2.5	2.9	4.5	4.4	4.7	5.5	4.4	5.0	6.6
2. *P. taurica* (A2)	7.0	**1.0/0.1**	3.4	5.0	4.8	5.2	6.2	4.9	6.4	7.7
3. *P. taurica* (A3)	9.2	11.0	**1.6/1.4**	4.5	4.5	5.0	5.7	4.8	5.2	6.6
4. *P. gaigeae* (B)	12.2	13.7	12.9	**0.8/0.5**	3.1	5.5	5.7	4.4	5.2	6.6
5. *P. milensis* (C)	12.3	12.9	12.7	11.4	**0.7/0.4**	5.3	5.7	4.7	6.2	6.8
6. *P. melisellensis* (D)	13.0	15.0	13.8	12.4	12.2	**3.5/1.4**	4.3	4.4	5.7	6.6
7. *P. muralis* (F)	15.3	15.6	15.4	18.6	18.7	17.5	**3.7/1.0**	5.8	5.5	6.4
8. *P. erhardii* — Cyclades (E)	17.1	15.9	17.4	20.8	16.9	20.0	15.7	**nc**	4.4	6.2
9. *P. peloponnesiaca* (E)	17.4	16.8	17.5	19.4	17.8	19.6	16.1	13.9	**nc**	3.0
10. *P. erhardii* — Crete (E)	15.6	16.3	16.4	17.6	16.8	18.4	15.5	17.0	8.5	**3.5/1.0**

in Fig. 2. The number of equally parsimonious solutions was largely due to terminal branch swapping, particularly among specimens belonging to the same species and coming from the same populations such as the specimens of *Podarcis gaigeae* from Skyros and *Podarcis melisellensis* from Croatia. It is characteristic that when we retained a single sequence from each of the branches mentioned, the MP analysis produced a single most parsimonious tree, the topology of which, concerning the species under study, was identical to that of MP with the large data set.

ML analysis under the GTR + I + G model resulted in a topology with ln L = –4513.42, which is consistent with the parsimony one (final parameters estimates: base frequencies A = 0.32, C = 0.27, G = 0.13, T = 0.28, a = 0.6451, Pinv = 0.4059, and A/G = 3.87 and C/T = 9.11). Two ML trees were identified, differing in a single node [the relationship of the specimen of *Podarcis taurica* from Kerkyra Island with the other specimens of the same species from continental Greece (Agrinio, Olympos, Farsala, Larisa)].

For the BI method, identical topologies were recovered for each of the runs with the full data set, although posterior probabilities for some of the nodes differed slightly between each of the Bayesian runs.

The likelihood-ratio test did not reject the null hypothesis of a homogeneous clocklike rate for the tree produced by the *Podarcis* sequences from Greece (LRT = 52.68, d.f. = 47, $\chi^2_{0.05} = 64$). This result suggests that we can use the genetic distances between populations inhabiting different geographical regions, in conjunction with the geological information about the age of the tectonic events that are responsible for the separation of these regions, in order to estimate a local rate of evolution for the *Podarcis* species.

Discussion

The results of this study revealed a well-resolved phylogeny and identified a number of haplotype clades which, based on the observed levels of sequence divergence (Table 2), represent long-separated lineages and diverse evolutionary histories within *Podarcis*. All outgroups used in this analysis indicate that *Podarcis* is a monophyletic group (bootstrap value 100%), a fact that comes in agreement with previous mtDNA studies (Harris & Arnold 1999; Oliverio *et al*. 2000) and the phylogenetic affiliations of *taurica*'s subgroup agree with the species groupings predicted from the morphological classification of the populations sampled.

The basic intraspecific phylogeographical pattern of *taurica*'s subgroup is characterized by the existence of six main lineages. Three lineages (A1, A2, and A3), which form a monophyletic unit, correspond to the populations of *Podarcis taurica* from the whole studied area. The lineage A1 includes populations from continental Greece and Peloponnisos, the lineage A2 corresponds to populations confined in the Ionian islands (except Kerkyra Island), and the lineage A3 represents the haplotypes from Thasopoula Island and northwest Greece (Fig. 2). Within *P. taurica* (A), the first that diverge from the others is lineage A3, while A1 and A2 form a monophyletic group, fact that is in agreement with geographical origin and palaeogeographical (see below) situation of the above populations.

The three remaining lineages form another monophyletic unit and correspond to the three species of *taurica*'s subgroup (lineage B: *Podarcis gaigeae*, lineage C: *Podarcis milensis*, and lineage D: *Podarcis melisellensis*), in which *P. gaigeae* and *P. milensis* are more closely related to each other than to *P. melisellensis*. This observation is consistent with previously published results (Mayer & Tiedemann 1980, 1981; Tiedemann & Mayer 1980; Harris & Arnold 1999; Oliverio *et al*. 2000).

The topology of the producing phylogenetic tree and the knowledge of the geological history of Hellenic area may help to describe a biogeographical hypothesis for *P. taurica* and its relative species (*P. gaigeae*, *P. milensis*, and *P. melisellensis*) in the Balkan area.

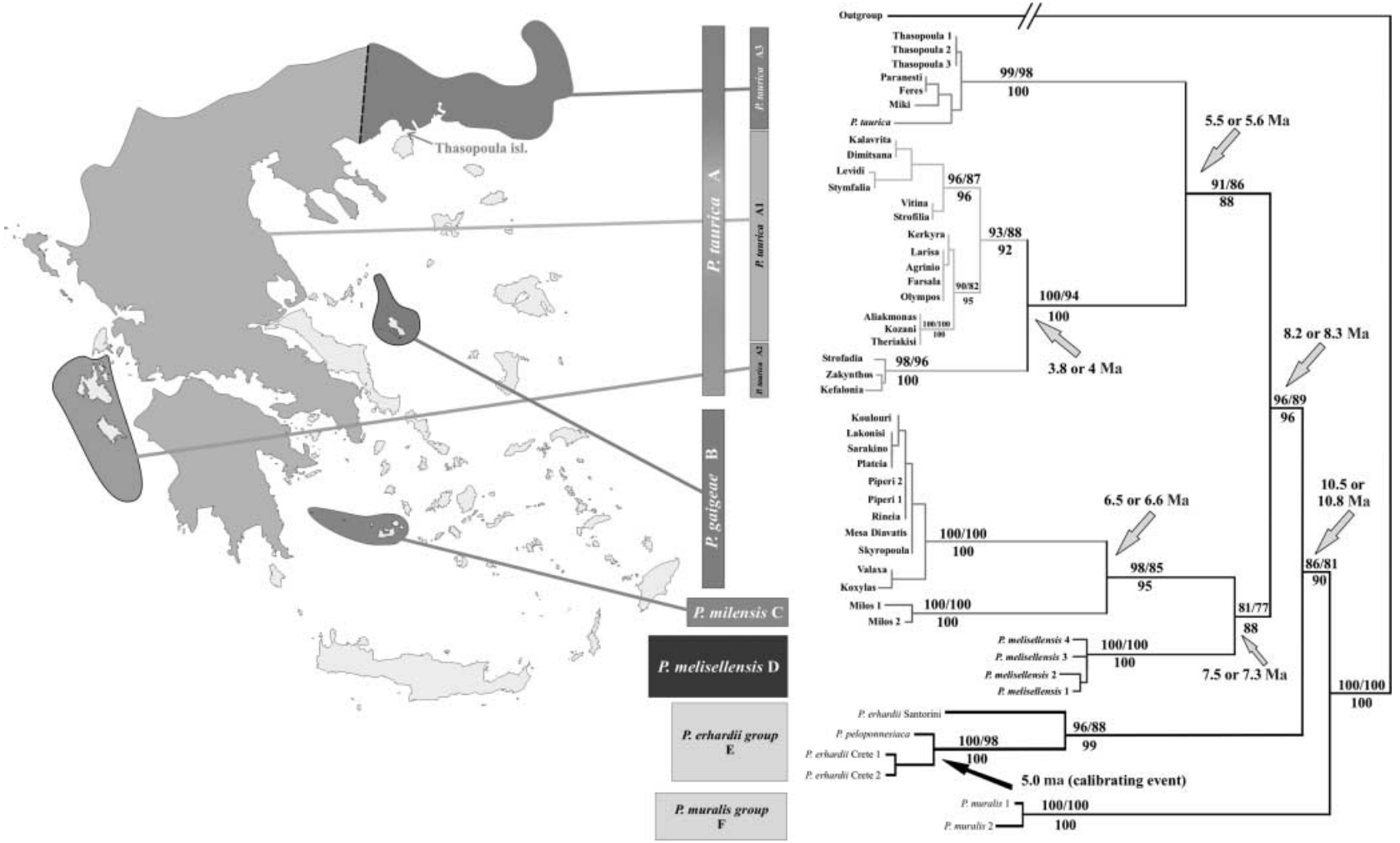

Fig. 2 Phylogenetic relationships among the seven specimens of the *Podarcis* spp. Individuals from three closely related species were used as outgroup taxa: *Lacerta andreanskyi*, *Gallotia stehlini* and *Gallotia galloti*. Phylogenetic analyses (all the methods used: MP, ML, and BI) produced trees with the same topology. Only the ML tree is presented. Numbers above branches indicate nonparametric bootstrap values on MP and ML analysis respectively (MP/ML). Numbers below branches indicate posterior probabilities of Bayesian analysis (BI). The thick grey arrows indicate the separation events based on a calibrated molecular clock, whereas the thick black arrow indicates the geological event based on which we calibrate the molecular clock.

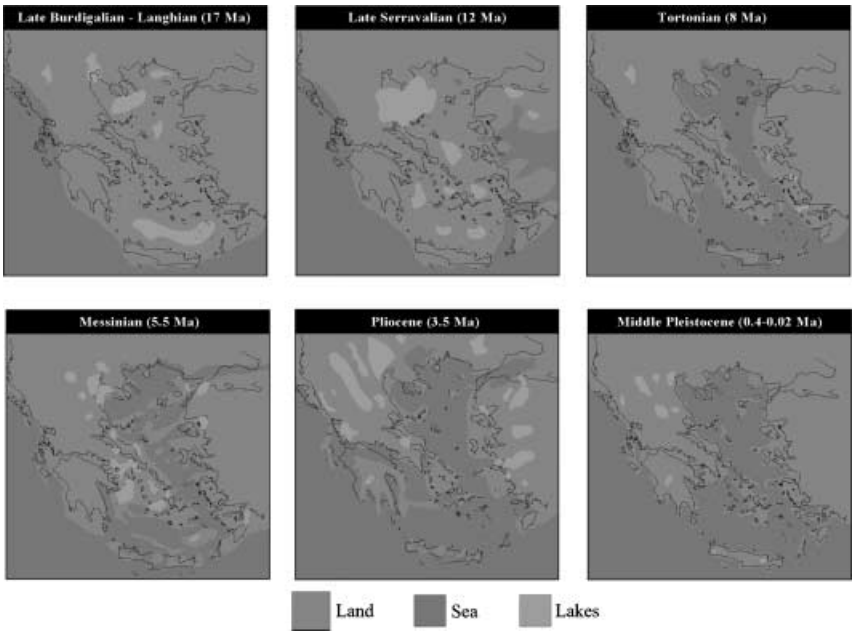

Fig. 3 Greece from Miocene to present (redrawn after Creutzburg 1963; Dermitzakis & Papanikolaou 1981; Dermitzakis 1990). The maps are drawn based on present geography. The actual positions of landmasses have been constantly changing from the Upper Serravalian, due to the fanlike, southwards, expansion of the southern Aegean region.

The palaeogeographical evolution of the Hellenic region during the Tertiary is described in Fig. 3 by six palaeogeographical sketches, which indicate the relative positions of various palaeogeographical domains during some critical periods (from 17 Ma to 0.02 Ma). The Aegean region was part of a united landmass (known as Agäis) during the Upper and Middle Miocene (23–12 Ma) (Dermitzakis & Papanikolaou 1981). Geomorphological change in the Mediterranean region is driven by the collision of the African and Arabian plates with the Eurasian plate. The late Seravallian to early Tortonian (12–8 Ma) tectonic movements probably initiated the modern history of the Aegean region and the surrounding areas. These movements caused the break-up of a southern Aegean landmass. At the end of the Middle Miocene (12 Ma), the forming of the Mid-Aegean trench (east of Crete and west of Kasos-Karpathos) (Figs 1 and 3) began and was fully completed during the early late Miocene (10.6 Ma) (Creutzburg 1963; Dermitzakis & Papanikolaou 1981). This phenomenon caused the separation of west Aegean from east Aegean islands. In the Messinian (latest Miocene, 6.5 Ma) the entire Mediterranean basin dried up, as a result of the closing of the Strait of Gibraltar (Hsü 1972). The Mediterranean islands then became mountains in a steppe or desert, so that overland migration between islands and from the mainland was possible. However, Crete was isolated from Cyclades and Peloponnisos by deep canyons during the Messinian salinity crisis (Schüle 1993). Some 5.2 Ma, the Strait of Gibraltar reopened and the basin was refilled from the Atlantic Ocean in about 1000 years (Beerli *et al*. 1996; Krijgsman *et al*. 1999). Crete became permanently isolated (5.0 Ma), both from Anatolia to the east and Peloponnisos to the west, as well as from the islands of the southern Aegean arc (Meulenkamp 1985; Schüle 1993). Kithira was submerged in the early Pliocene, and did not re-emerge until the late Pliocene (Meulenkamp 1985). In the Pleistocene, all of today's islands (i.e. Crete) were in the same position as present and remained completely isolated.

Given that the molecular clock hypothesis cannot be rejected, we suppose that there is a homogeneous clocklike rate for the tree produced by the *Podarcis* sequences included in this study. At least one independently timed event has to be used for calibrating any clock (Busack 1986). Based on the geological events mentioned above, the splitting of the island of Crete from Peloponnisos is dated some 5 Ma (Meulenkamp 1985; Schüle 1993). Given that the corrected pairwise divergence (*Da*) between *Podarcis peloponnesiaca* of Peloponnisos and *Podarcis erhardii* of Crete is 7.75% for cyt *b* and 2.3% for the 16S rRNA, the evolutionary rate for the cyt *b* is 1.55% per Myr and for the 16S rRNA is 0.46% per Myr. On the basis of these evolutionary rates we infer the divergence events of the major clades of the phylogenetic tree of Fig. 2 (Table 3).

The above evolutionary rate for cyt *b* is similar to the corresponding rate of *P. erhardii* (1.45–1.59% per Myr for cyt *b*) (Poulakakis *et al*. 2003), but is lower than the rate of evolution of Iberian *Podarcis* species (Harris *et al*. 2002). In the latter study, the authors used an evolutionary rate of 2% per Myr in order to estimate some separation events among the Iberian *Podarcis* lineages. Moreover, if we consider that the evolutionary rate of Iberian species is based on two gene fragments (cyt *b* and 12S rRNA) and that the cyt *b* gene is faster evolving than 12S rRNA gene then we can conclude that the difference is much larger. Although

Table 3 Date estimated for divergence events, using the net nucleotide divergences, within and between major groups of *Podarcis* included in this study

Separation event	Evolutionary rate	Separation time (Myr)
taurica subgroup (*P. taurica*, *P. gaigeae*, *P. milensis*, and *P. melisellensis*)	Cyt *b*: 1.55%	10.5
	16S: 0.46%	10.8
P. taurica (*P. melisellensis*, *P. gaigeae*, *P. milensis*)	Cyt *b*: 1.55%	8.2
	16S: 0.46%	8.3
P. melisellensis (*P. gaigeae*, *P. milensis*)	Cyt *b*: 1.55%	7.5
	16S: 0.46%	7.3
P. gaigeae–*P. milensis*	Cyt *b*: 1.55%	6.5
	16S: 0.46%	6.6
P. taurica (A3)–*P. taurica* (A1 and A2)	Cyt *b*: 1.55%	4.9
	16S: 0.46%	5.2
P. taurica (A1)–*P. taurica* (A2)	Cyt *b*: 1.55%	3.8
	16S: 0.46%	4.0

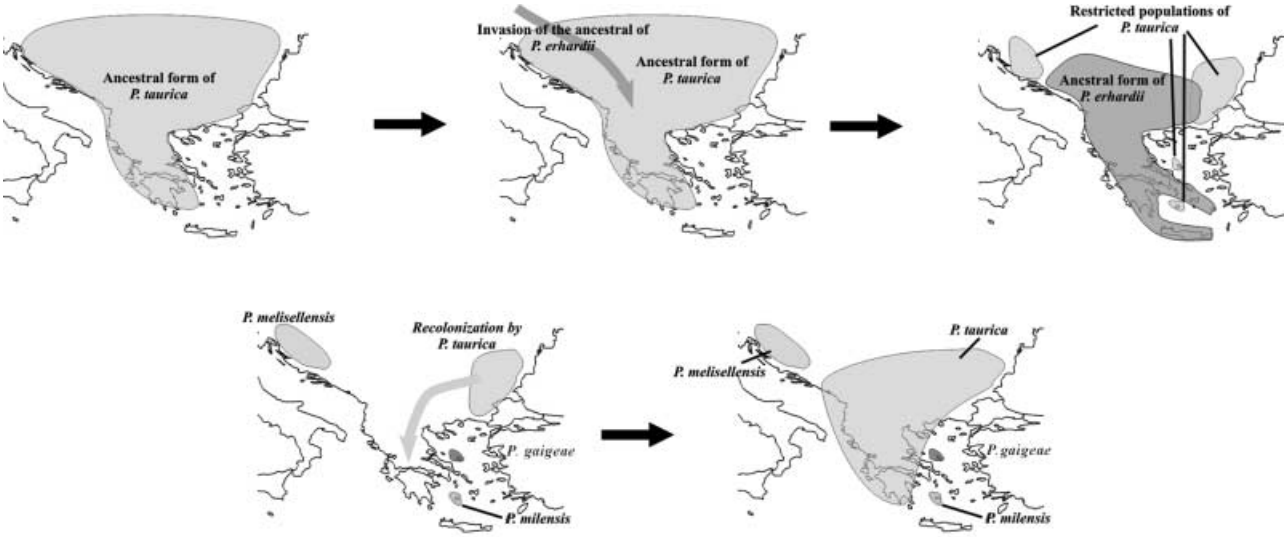

Fig. 4 The hypothetical biogeographical scenario of *Podarcis taurica* and its relatives (*Podarcis gaigeae*, *Podarcis milensis*, and *Podarcis melisellensis*) in Balkan Peninsula.

some genes appear to provide reliable clocks (Pesole *et al*. 1991; Beerli *et al*. 1996), other studies have demonstrated that there is no standardized clock for all genes and species (Gillespie 1991; Beerli *et al*. 1996). A molecular clock with known relationships among the taxa under study and with well-dated isolation times will give the most accurate estimates, although extrapolation to other groups and beyond the time interval requires caution (Beerli *et al*. 1996). The significant difference between the evolutionary rates of Iberian and Balkan *Podarcis* species is due to the fact that the rate that Harris *et al*. (2002) used in their study was based on a molecular clock which was calibrated for other lizard species (*Tarentola* sp., Carranza *et al*. 2000).

If we consider that *P. taurica* is a single species, then the genetic distances and the molecular clock hypothesis suggest that the evolutionary history of *P. taurica* in the Balkan Peninsula does not consist of a simple invasion occurring during the past (Fig. 4). The distribution of the *P. taurica* subgroup (*P. taurica*, *P. gaigeae*, *P. milensis*, and *P. melisellensis*) mainly in the Balkan Peninsula and its absence from the rest of Europe, suggest that the ancestral species of this group originated somewhere in the Balkan Peninsula and expanded to this area. This probably happened after the formation of the Mid-Aegean trench (Figs 1 and 3), which was fully completed about 10.6 Ma (Poulakakis *et al*. 2003). This hypothesis is based on the fact that species of the genus *Podarcis* are not found presently in any Aegean island to the east of the trench. An exception is the small islet Pachia near the island of Nisyros (Valakos *et al*. 1999), which could be a case of recent colonization from the Cyclades.

This information fits well with the divergence time estimated in this study for the beginning of the diversification of *P. taurica* subgroup (10.5–10.8 Ma from cyt *b* and 16S rRNA, respectively). A historical fact, probably the arrival (approximately 9 Ma) of the ancestral form of *P. erhardii* (Poulakakis *et al*. 2003) from the northwest, following the eastward path of the Dinaric Alps and the Hellenides, led to the restriction of the distribution of the ancestral form of *P. taurica* subgroup in few small populations.

One of the above-mentioned population restrictions occurred, perhaps, near the Dalmatian coast, and produced the species recognized today as *P. melisellensis*. Two other populations, the first in the southeast and the second in the central-east part of Greece — corresponding to the area of Milos and Skyros island groups — produced the taxa recognized today as *P. milensis* and *P. gaigeae*. Concerning the fourth species of *taurica*'s subgroup (*P. taurica*), there are two possible scenarios to explain its distribution. The first, which is the most parsimonious one, suggests the remaining of one population in northeast Greece or the northeast part of Balkan peninsula (clade A3), which, when the situation from the arrival of the ancestral form of *P. erhardii* was stabilized, recolonized the area of Greece, producing the clade A1 (continental Greece) and A2 [Ionian islands — except Kerkyra — that based on the palaeogeographical maps was a palaeogeographical unit (Dermitzakis 1990)]. The second scenario suggests the remaining of two populations, northeastern one producing clade A3 and a southwestern Greece one (clade A1), which produced the clade A2 in the Ionian islands by colonization. Based on the observed genetic distances, if we accept the second scenario as the true one, then, given that there is a homogeneous clocklike rate in the producing tree, we would expect the genetic distance between clade A1 (continental Greece) and clade A3 to be equal to the genetic distances of clade A1 and that of the species *P. gaigeae*, *P. milensis*, and *P. melisellensis*, since the time of divergence was the same. However, this distance (A1 vs. A3) is much smaller (9.2% for cyt *b* and 2.9% for 16S rRNA) than the distances between the clade A1 and the species *P. gaigeae*, *P. milensis*, and *P. melisellensis* (12.2%, 12.3%, and 13.0% for cyt *b*, respectively and 4.5%, 4.4%, and 4.7% for 16S rRNA, respectively). In addition, if we accept the presence of two ancestral populations, then these populations diverged before 7.5 or 7.3 Ma, as in the case of *P. gaigeae*, *P. milensis* and *P. melisellesnsis*, and the most probable result would be the evolution of the second population (southwest Greece, clade A1) in a separate species, as in the cases of *P. gaigeae*, *P. milensis*, and *P. melisellensis*. From this evidence, and given that the morphological and molecular data suggest that all *P. taurica* specimens consist of a clade, it appears that colonization of Greece came from the hypothetical ancestral population of *P. taurica* of northeast Greece or Balkan Peninsula (before 4.9 or 5.2 Ma), following the dispersal route mentioned, in which the latter population gave the haplotypes group of Ionian islands (A2) (before 3.8 or 4.0 Ma).

Conclusively, the most possible scenario of the biogeographical history of *P. taurica* and its relatives in the Balkan Peninsula is the vicarianistic one, in which an old, widely distributed species (ancestral form of *P. taurica*) became subdivided during the Upper Miocene into populations that were isolated on several Greek islands (island group of Milos and Skyros for *P. milensis* and *P. gaigeae*, respectively) and Dalmatian coast (*P. melisellensis*). Vicariance is probably the major cause of the differentiation of the Aegean animal species with poor overseas abilities, such as *Rana* spp. (Beerli *et al*. 1994, 1996), *P. erhardii* (Poulakakis *et al*. 2003), *Mastus* spp. (Parmakelis 2003). Especially in the studies of Beerli *et al*. the phylogenetic analyses of electrophoretic data showed that the geological isolation of Crete and Karpathos, an analogue case of the isolation of Skyros and Milos island groups, led to the differentiation of Cretan and Karpathian *Rana* populations at the species level.

The phylogeographical hypotheses that we present in this study should be examined using nuclear markers in further investigations. Nevertheless these results underline the need for a reconsideration of the evolutionary history of Greek *Podarcis* species and contribute to the amelioration of difficulties encountered by classical taxonomy at the species level.

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