Aspects of the evolutionary history of *Podarcis taurica* (Pallas 1814), *P. gaigeae* (Werner 1930) and *P. milensis* (Bedriaga 1882) in Greece

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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 *fall into four main groups that have substantial geographic coherence (Western Island group, Southwestern group, Italian group and Balkan group). The Balkan species, are divided in two subgroups, the subgroup of* P. taurica, P. milensis, P. gaigeae, P. melisellensis *and the subgroup of* P. erhardii *and* P. peloponnesiaca, *which are highly diversified and present great morphological and ecological plasticity, inhabiting many different ecotypes. We address 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) and nuclear (c-mos) sequences. Our data suggest that* P. gaigeae *is closely related to* P. milensis *and both* P. gaigeae *and* P. milensis *to* P. taurica. *However the specimens of P. taurica are subdivided in two different groups. The first includes the specimens from Crimea (Ukraine) and Northeastern Greece and the other the specimens from the rest of continental Greece and Ionian Islands. This result suggests that the evolutionary history of* P. taurica *in Greece is more complex than a single evolutionary invasion. The data analyzed stress the need for a reconsideration of the evolutionary history of Greek* Podarcis *species and help to overcome difficulties that classical taxonomy has encountered at both the specific but mostly the subspecific level of this genus.*

Keywords: *Podarcis taurica, Podarcis gaigeae, Podarcis milensis,* molecular phylogeny, mtDNA and nuclear markers, evolution, Greece.

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Introduction

The reconstruction of phylogenies is of primary importance in the understating of the dynamic patterns of evolution (the biogeography of a group, the bases of its biological diversity at any level). Although the phylogeny of the genus *Podarcis* has been the subject of much discussion, the relationship among the species are still unclear.

Wall lizards of the genus *Podarcis* (Sauria, Lacertidae) comprises 17 [or 18 if we consider that *P. carbonelli* is a distinct species (Sá-Souza & Harris 2002)] currently recognized species in southern Europe, where they are the predominant reptile group, but their taxonomy is complex and continuously revised, largely because the species are morphologically very similar but exhibit substantial levels of intraspecific variation (Arnold 2002). *Podarcis* is a complex of species, diffused in central Europe and in the circum-Mediterranean regions, with the exception of the xeric south-eastern areas and of Anatolia, and includes several endemic insular species (Oliverio *et al.* 1998, 2000).

There is substantial morphological evidence that *Podarcis* is a clade (Arnold 1973, 1989). Morphology also suggests that the closest relatives are the Moroccan *Lacerta andreasnkyi* which in turn are sister to *L. persicillata* and *L. dugesii* (Arnold 1973). The same (the monophyly of *Podarcis*) is extrapolated by several molecular studies that have been done in the last 5-6 years (Oliverio *et al.* 1998, 2000; Harris & Arnold 1999; Fu 2000; Poulakakis *et al.* 2003).

However, within *Podarcis* relationships are poorly understood. Because morphology is so uniform, it provides few characters for phylogenetic analysis, and these tend to conflict (Arnold 1973, 1989). Various karyological, immunological and protein electrophoretic studies have been made, but these usually involve only a minority of species, and results from different species combinations often conflict (Lanza & Cei 1977; Tiedemann & Mayer 1980; Mayer & Tiedemann 1980, 1981; Olmo *et al.* 1986, 1987; Capula 1994, 1996, 1997, Chondropoulos *et al.* 2000).

Recent studies on the lacertid genus *Gallotia* (Thorpe *et al.* 1993, 1994) and on the iguanid genus *Anolis* (Losos *et al.* 1997, Jackmann *et al.* 1999), among others, confirmed the general power of DNA sequencing as a means to reconstruct phylogenies and zoogeography. Based on DNA sequence data (cytochrome b, cyt-*b* and 12S rRNA genes) the species of *Podarcis* fall into four main groups with substantial geographic coherence: (1) Western Island group (*P. filfolensis, P. pityusensis, P. tiliguerta,* and *P. lilfordi*), (2) Southwestern group (*P. atrata, P. bocagei, P. hispanica*, and perhaps *P. carbonelli*), (3) Italian group (*P. muralis, P. raffonei,* and *P. sicula*) and (4) Balkan group (*P. taurica, P. gaigeae, P. milensis, P. melisellensis* and perhaps *P. peloponnesiaca, P. erhardii,* and *P. wagleriana* (Harris & Arnold 1999). 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 both these studies support the monophyly of a Balkan group of *Podarcis*, which includes *P. gaigeae, P. milensis, P. melisellensis, P. taurica*, and perhaps *P. wagleriana, P. erhardii*, and *P. peloponnesiaca*. However, Oliverio *et al.* (2000) do not agree with Harris & Arnold (1999) that *P. wagleriana* is part of the "Balkan" clade. The *Podarcis* species of Iberian Peninsula have been studied by Harris & Sá-Souza (2001, 2002), using partial 12S rRNA and cyt-*b* mitochondrial DNA sequences. Oliverio *et al.* (1998, 2000), Pinho *et al.* (2004), and Pretus *et al.* (2004) used partial mtDNA sequences (cyt-*b*,12S rRNA) in order to explore the phylogenetic relationships of Italian and western islands species. So, the least studied group is the last one, the Balkan group, for which the only thing we know is that *P. erhardii*, which is closely related to *P. peloponnesiaca*, is probably a species complex (Poulakakis *et al.* 2003).

The Balkan group of species is probably divided, on the basis of the preliminary results of the molecular study of Poulakakis *et al.* (2003), in two subgroups, the subgroup of *P. taurica, P. milensis,* and *P. gaigeae* and the subgroup of *P. erhardii* and *P. 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 closely related to *P. milensis* and both *P. gaigeae* and *P. milensis* to *P. taurica* (Tiedemann & Mayer 1980; Mayer & Tiedemann 1980, 1981), and that *P. taurica* has closer relation to *P. milensis* than to *P. peloponnesiaca* (Chondropoulos *et al.* 2000).

The Balkan wall lizard, *P. taurica*, is distributed in a large area of the Balkans, as well as in Hungary, Crimean Peninsula and NW Anatolia (Gasc *et al.* 1997). It exhibits a notable geographic variation in colour, pattern size. So far three subspecies have been described on the basis of coloration, patterning, and relative leg length: *P. t. taurica* (the largest part of species' range), *P. t. thasopoulae* (Thasopoula isl.), *P. t. ionica* (west part of the Greek mainland and Ionian islands) (Chondropoulos *et al.* 1993).

The complex geological history of Hellenic area during the late Tertiary has influenced the distribution of all *Podarcis* species in the region (i.e., *P. erhardii, P. peloponnesiaca, P. muralis, P. taurica, P. milensis*, and *P. gaigeae*) and contributed to the diversification of each species. This diversity is thought to reflect the submergence and re-emergence of landmasses, due to tectonic, volcanic and eustatic events.

In the present study we examine the phylogenetic relationships of several populations of *P. taurica, P. gaigeae*, and *P. milensis* across Greece, using partial mtDNA (cyt *b* and 16S rRNA) and nuclear (c-mos) sequences. We combine this information with previously published sequences and use the results to produce a historical interpretation of the species' distribution and morphological diversification.

Materials and Methods

Total genomic DNA was extracted from 40 specimens of *Podarcis* (Table 1, Fig. 1). Three target genes were selected for molecular phylogenetic analysis: (1) a partial sequence (425 bp) of the mitochondrial protein encoding cytochrome b gene (cyt b), (2) a partial sequence of the non-protein coding mitochondrial 16S rRNA (16S), and (3) a partial sequence (397bp) of the nuclear proto-oncogene that encodes a kinase expressed in germ cells. Sequencing was done on a PE-ABI377 automated sequencer.

Individuals from two closely related species of the same family (Lacertidae) were used as outgroup *taxa*: *L. andreasnkyi* (cyt *b* – AF206537: Fu 2000, 16S rRNA – AF206603: Fu 2000, c-mos – AF211203: Brehm *et al.* unpub. data) and *Gallotia stehlini* (cyt *b* – AF439949: Rando *et al.* unpub. data, 16S rRNA – AF149936: Beyerlein & Mayer 1999, c-mos – AF435108: Maca-Mayer *et al.* unpub. data).

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 in MEGA computer package (v.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.

Table 1.

List of the examined specimens of *Podarcis*, with *taxon* name, geographic origins, population map codes (see Fig. 1), and number of samples. Individuals from two closely related species were used as outgroup *taxa*: *Lacerta andreanskyi* and *Gallotia stehlini*. Note: Asterisk (*) indicates gene regions previously published. For the specimen 37 there is not information about the c-mos gene.

Map Code	Species	Samples	Locality
1-9	*P. gaigeae gaigeae*	9	Skyros island and islets around it
10-11	*P. gaigeae weigandi*	2	Piperi island
12-16	*P. milensis milensis*	5	Milos island
17-20	*P. taurica ionica*	4	Ionian islands
21-27	*P. taurica taurica*	7	Peloponnesus
28-30	*P. taurica taurica*	3	Central Greece
31-34	*P. taurica taurica*	4	North Greece
35-36	*P. taurica thasopoulae*	2	Thasopoula island
37	*P. taurica taurica*	1	Out of Greece*
38-40	*P. muralis*	3	Continental Greece
41-42	*P. erhardii*	2	Crete and Cyclades
43	*P. peloponnesiaca*	1	Peloponnesus
44-45	Outgroup	2	Out of Greece*

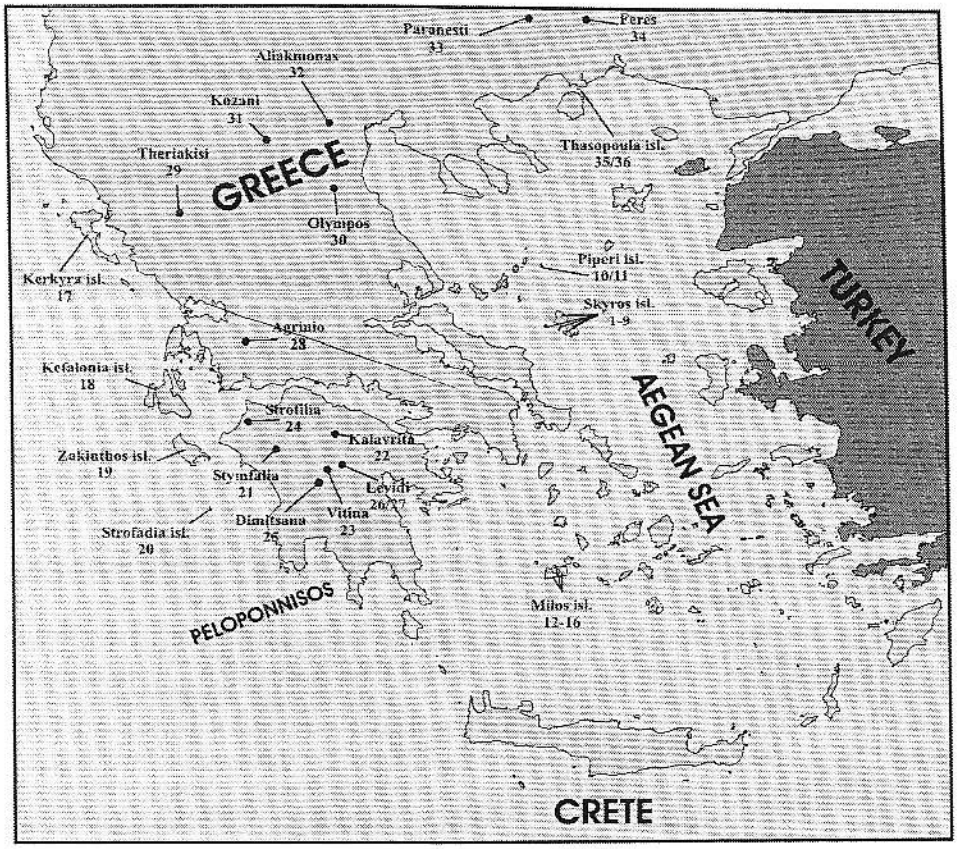

Fig. 1. Map showing the sampling localities of the 42 specimens used for the DNA analysis. 21 specimens of *P. taurica*, 11 specimens of *P. gaigeae*, 5 specimens of *P. milensis*, 3 specimens of *P. muralis*, 2 specimens of *P. erhardii*, and 1 specimen of *P. peloponnesiaca*. Individuals from two closely related species were used as outgroup *taxa*: *Lacerta andreanskyi* and *Gallotia stehlini*.

Analyses for phylogenetic inference were conducted using three methods: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). Nucleotides were used as discrete, unordered characters. To examine whether the sequences from the three 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 (v.4.0b10, Swofford 2002), and significance was estimated by 1000 repartitions. Maximum parsimony analysis was performed with 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 maximum likelihood (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 (v.3.06, Posada & Crandal 1998). Likelihood-ratio tests and Akaike Information Criterion (AIC; Akaike 1973) indicated that the Tamura-Nei (TrN) model+I+G 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. ML bootstraps employed only 100 iterations.

We performed Bayesian analysis with the program MrBayes (v3.0B, Huelsenbeck & Ronquist 2001) using the TrN model of substitution with rate heterogeneity set to a gamma distribution, hence applying the fewest possible number of constraints to the dataset. 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 $-lnL$ 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 (4 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.

Results

Of the 1330 sites examined, there were 295 variable sites, 221 of which were parsimony informative (403 and 251 respectively when the outgroups were included in the analysis). Pairwise sequence divergence in *Podarcis* species (Tamura & Nei 1993) ranged from 0 to 13.2% (Table 2).

A partition homogeneity test indicated no conflicting phylogenetic signals between the datasets ($P = 0.971$) and the mtDNA and nuclear genes were analyzed together.

Tree length distribution, determined from random sampling of 106 unweighted trees, was significantly skewed to the left ($g1 = -0.49$), suggesting a strong phylogenetic signal in the data ($P < 0:01$; Hillis & Huelsenbeck 1992).

The heuristic parsimony analysis produced 3 equally parsimonious trees of 705 steps (CI = 0.583, RI = 0.809). Maximum likelihood analysis under the TrN+I+G model resulted in a topology with $lnL = -5046.9971$. For the Bayesian inference method, identical topologies were recovered for each of the 4 runs with the full dataset (Fig. 2).

Table 2.

(A) Sequence divergences (%) among the main clades/lineages of *Podarcis*. Values in diagonal are within clade sequence divergences. (B) Sequence divergences (%) among the major clades of *P. taurica* subgroup (3 clades of *P. taurica*, 1 clade of *P. gaigeae* and 1 clade of *P. milensis*).

A. Species	**1**	**2**	**3**	**4**	**5**	**6**
1. *P. taurica* (A)	(3.6)					
2. *P. milensis* (B)	6.4	(0.5)				
3. *P. gaigeae* (C)	6.5	6.2	(0.6)			
4. *P. erhard ii* (D)	8.6	8.8	8.9	(3.7)		
5. *P. peloponnesiaca* (D)	8.9	9.1	9.2	3.4	(1.1)	
6. *P. muralis* (E)	8.3	8.6	8.9	9.7	10.3	(6.2)

B. Species	**1**	**2**	**3**	**4**
1. *P. taurica* (A1)				
2. *P. taurica* (A2)	2.8			
3. *P. taurica* (A3)	4.8	5.7		
4. *P. milensis* (B)	6.4	6.4	6.6	
5. *P. gaigeae* (C)	6.4	6.3	6.9	6.2

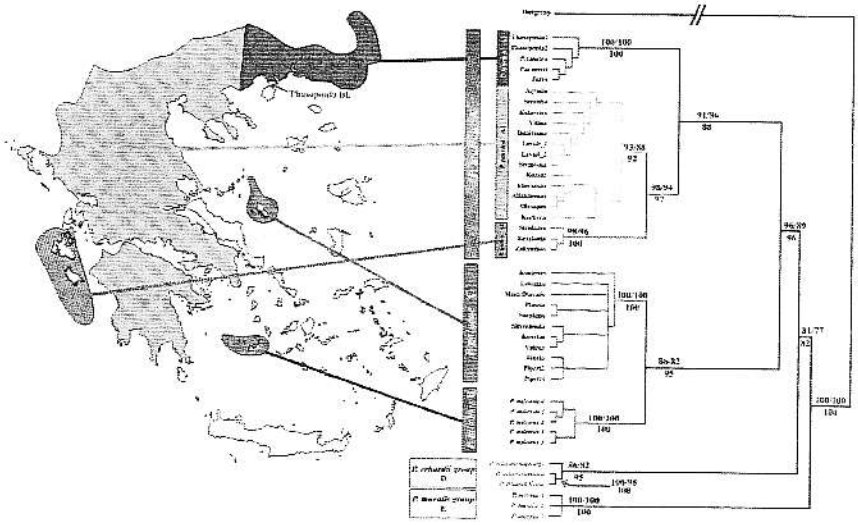

Fig. 2. Phylogenetic relationships among the 45 specimens of the *Podarcis* spp. and outgroups. Phylogenetic analyses (all the methods used: MP, ML, and BI) produced trees with the same topology. Only the Maximum Parsimony tree is presented. Numbers above branches indicate nonparametric bootstrap values (1000 pseudoreplicates) on MP and ML analysis respectively (MP/ML). Numbers below branches indicate posterior probabilities of Bayesian analysis (BI).

Discussion

The results of this study reveal a well-resolved phylogeny and identify a number of haplotype clades which, on the basis of 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 the genus is a monophyletic group (bootstrap value 100%), fact that comes in agreement with previous mtDNA studies (Harris & Arnold 1999, Oliverio *et al.* 2000). Concerning the *taxa* of the area of Greece (6 species in total), three phylogenetic lineages can be distinguished. The first includes *P. taurica, P. gaigeae,* and *P. milensis* (and perhaps *P. melisellensis*, but this species is not included in this study). The second includes *P. erhardii, P. peloponnesiaca* and the third includes *P. muralis*.

Within the first clade (the subgroup of *P. taurica*), our results recognize the three species (*P. taurica, P. gaigeae,* and *P. milensis*) as separate phylogenetic clades. In addition, *P. taurica* is the first species that diverges from the other two species, while *P. gaigeae* and *P. milensis* form a closely related pair, an observation that agrees with previously published results (Mayer & Tiedemann 1980, 1981; Tiedemann & Mayer 1980; Harris & Arnold 1999; Oliverio *et al.* 2000).

Within the clade of *P. taurica* (A), there are three groups of haplotypes. A1 includes populations from continental Greece and Peloponnisos. A2 corresponds to populations confined in the Ionian Islands, and A3 represents the haplotypes from Thasopoula Isl. and NE Greece. Harris & Sá-Souza (2002) and Poulakakis *et al.* (2003) have already referred to the inconsistencies between the molecular and morphological groupings of the various species within the genus *Podarcis*. With regard to morphological subspecies of *P. taurica*, it is clear that they do not represent monophyletic units and should be reevaluated in the light of new evidences. An interesting example supporting this claim is the assignment of the population from northern Greece (Paranesti, Feres) to *P. t. taurica*. In our analysis these individuals were assigned to the subclade A3 (Fig. 2), together with specimens from Thasopoula Island, which belong to a different subspecies (*P. t. thassopoulae*), whereas the other *P. t. taurica* individuals (from the rest continental Greece) were assigned to subclades A1. Constraining *P. taurica* subspecies to be monophyletic, performing a heuristic search to find the shortest tree with this constraint, and comparing this to the optimal tree using the Shimodaira & Hasegawa (SH) (1999) test shows that the difference is significant (SH test using 1000 RELL bootstraps, $P > 0.001$). This brings into question the practice of subspecies recognition and subsequent assignment of local populations into these subspecies on the basis of an exclusive or limited collection of characters, be that morphological, behavioural or molecular. It is already known that the current taxonomy of *Podarcis* species in the Balkan Peninsula needs revision (Poulakakis *et al.* 2003). Given that the borders between the ranges of the currently recognized subspecies of *P. taurica* are not evident, the above subclades could correspond to the described subspecies of *P. taurica*.

In addition to providing a means for evaluating the validity of morphological taxonomy, the molecular data may provide insights concerning the biogeography of the

genus. The topology of the producing phylogenetic trees and the genetic distances among the clades of *P. taurica* subgroup may discriminate the biogeographic hypothesis of these species in the area of Greece. Given that the molecular clock hypothesis cannot be rejected (the likelihood-ratio test did not reject the null hypothesis of a homogeneous clocklike rate, LRT = 33.34, *d.f.* = 34, *P*-value = 0.49), we consider that there is a homogeneous clocklike rate for the tree produced by the *Podarcis* sequences from Greece. Based on the Table 2A, the genetic distances among the species of the subgroup of *P. taurica* are similar. In particular, the genetic distance between *P. taurica* and *P. gaigeae* is 6.5, the distance between *P. taurica* and *P. milensis* is 6.4 and between *P. gaigeae* and *P. milensis* is 6.2. However, the intraspecific variation differs from species to species. For example in *P. gaigeae* and *P. milensis* is 0.6% and 0.5% respectively, whereas in *P. taurica* is 3.6%. This intriguing result led us to seek the genetic distances among the major clades of *P. taurica* species (namely A1, A2, and A3). As shown in Table 2B, the genetic distances between clade A3, which includes specimens from north-eastern Greece and Thassopoula Island, and the other clades (A1 and A2) of the same species (*P. taurica*), are 4.8 and 5.7 respectively. They are close to the distances between clade A3 and the species *P. gaigeae* and *P. milensis* (6.6 and 6.9 respectively), whereas the corresponding distance between clade A1 (specimens from continental Greece) and A2 (specimens from Ionian islands, except Kerkyra) is only 2.8%. Furthermore, the sequence divergence between *P. gaigeae* and *P. milensis* is 6%, which is almost equal to the intraspecific variation of *P. taurica* that we mentioned before (A3 vs A2 is 5.7%).

If we consider that *P. taurica* is a single species then the above genetic distances suggest that the evolutionary history of *P. taurica* in the area of Greece is not a simple case of one invasion during the past. The distribution of *P. taurica* subgroup (*P. taurica, P. gaigeae, P. milensis,* and perhaps *P. melisellensis*) mainly in Balkan Peninsula and its absence in the rest of Europe, suggests that the ancestral species of this group originated somewhere in the Balkan peninsula and expanded to this area. A historical fact, probably the arrival of the ancestral form of *P. erhardii* (Poulakakis *et al.* 2003) from northwest, following the eastward path of Dinaric Alps and Hellenides, led to the restriction of the distribution of the ancestral form of *P. taurica* subgroup in few small populations. Two of them (one south-east and the other central-east of Greece) produced the *taxa* we recognize today as *P. milensis* and *P. gaigeae*, which are distributed on the corresponding archipelagos of Milos and Skyros respectively.

Concerning the third species of this subgroup (*P. taurica*), there are two possible scenarios to explain its distribution. The first, the most parsimonious one, suggests the remaining of one population in northeast Greece or Balkan peninsula (clade A3), and, when the situation calmed down after the arrival of the ancestral form of *P. erhardii*, its recolonization of the area of Greece, producing clade A1 (continental Greece)

and A2 [Ionian islands – except Kerkyra – that according to the paleogeographic maps was a paleogeographic unit Dermitzakis (1990)]. The second scenario suggests the remaining of two populations, one in the northeast which produced clade A3 and the other in southwest Greece (clade A1), which produced by colonization clade A2 in the Ionian Islands. Considering the observed genetic distances mentioned above, if we accept the second scenario as the true one, since there is a homogeneous clock-like 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* and *P. milensis*. However, this distance (A1 *vs.* A3) is much smaller (4.8%) than the distances between the clade A1 and the species *P. gaigeae* and *P. milensis* (6.4% and 6.5% respectively).

From this evidence, we favor the first scenario, by which the colonization of Greece derived from the ancestral population of *P. taurica* of northeast area of Greece, following the dispersal route we mentioned above.

The results presented in this study 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 specific and mostly at the subspecific level of this genus.

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