Molecular phylogeny of the Greek legless skink *Ophiomorus punctatissimus* (Squamata: Scincidae): The impact of the Mid-Aegean trench in its phylogeography

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Abstract

Sequence data derived from three mitochondrial markers (cytochrome *b*, 16S rRNA and 12S rRNA genes) were used to infer the evolutionary history of several insular and mainland populations of the Greek legless skink (*Ophiomorus punctatissimus*), covering most of its distributional range. All phylogenetic analyses produced topologically identical trees that revealed a well-resolved phylogeny. These trees support two *O. punctatissimus* clades, which are geographically separated (west and east of the mid-Aegean trench). The assumption of a clock-like evolution could not be rejected, and thus a local clock was calibrated for the *O. punctatissimus* lineages. The non-overlapping geographic distributions of the major clades suggest a spatial and temporal sequence of diversification that coincides with paleogeographic separations during the geological history of the Aegean region. It seems that *O. punctatissimus* is an old eastern Mediterranean species that has been differentiating in this region at least from middle Miocene. It is possible that the ancestral form of *O. punctatissimus* invaded the Aegean region from Anatolia before the complete formation of the mid-Aegean trench, when the Aegean was still a uniform landmass, while other vicariant events have led to its present distribution.
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1. Introduction

The scincid lizards of the genus *Ophiomorus*—a group of elongated skinks with greatly reduced appendages—comprise ten currently recognized species (Anderson, 1999; Anderson and Leviton, 1966; Nilson and Andren, 1978), ranging from southeastern Europe (Greece) to extreme northwestern India (Fig. 1). Peculiarly, no species of *Ophiomorus* are found in eastern Turkey, northern Syria, Iraq or northern Iran (Anderson, 1999; Anderson and Leviton,

1966; Nilson and Andren, 1978). The species are largely cryptozoic and characterized by two major adaptive trends, based on which two groups were identified (Anderson and Leviton, 1966). The western group - characterized by extremely elongated cylindrical bodies, blunt conical snouts, and limbs that have either been entirely lost (*Ophiomorus punctatissimus*, *Ophiomorus latastii*) or greatly reduced (*Ophiomorus persicus*)—is adapted to live either under rocks or burrowed in loose soil.

Ophiomorus punctatissimus is the only extant representative of its genus in Europe, occurring in southeast mainland Greece (Peloponnesos) and on the Greek islands of Kythira (southeast of Peloponnesos) and Kastelorizo (southwest of Turkey). The remaining part of its disjunct distribution covers a very small area in southwestern Turkey (Fig. 1).

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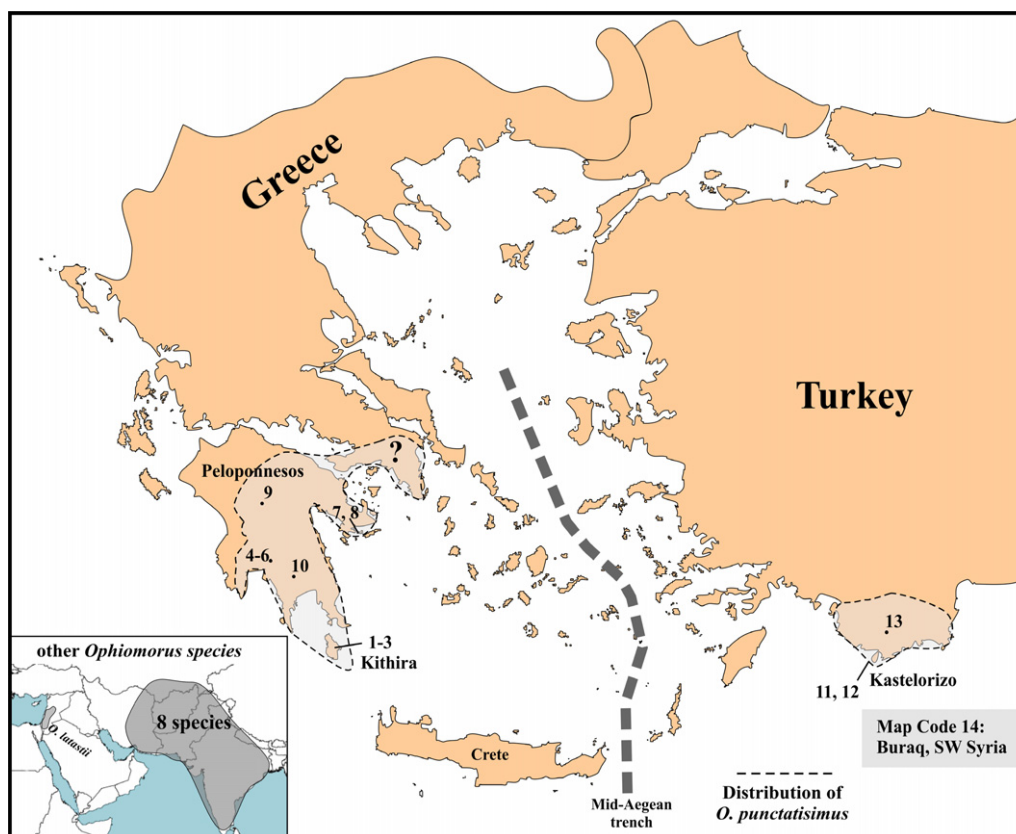*E-mail address:* poulakakis@nhmc.uoc.gr (N. Poulakakis).

Fig. 1. Map showing the sampling localities of the 14 specimens (13 of *O. punctatissimus* and 1 of *O. latastii*) used for the phylogenetic analysis. Codes correspond to those in Table 1. Thin dashed line represents the current distribution of *O. punctatissimus* (its presence in Attiki is questionable). The thick dashed line denotes the mid-Aegean trench.

The taxonomic status and the population abundance of this species are unclear, and many aspects of its evolutionary history remain uncertain. How did its present disjunct distribution arise? The patchy and poorly known distribution of *O. punctatissimus,* along with the absence of any taxonomic study on this species, led Lymberakis et al. (2005) to characterize it as Least Concern (IUCN, 2006), thus emphasizing the need for more taxonomic and phylogenetic studies for both the Greek and Turkish populations, with a view to unraveling the evolutionary history of this species.

During the last decade, several studies have used molecular data to explore the biogeographic affinities that occur in various parts of the northeastern Mediterranean region. Most of these studies have focused on the Aegean region, since it is characterized by high levels of diversity and endemism as well as a complex palaeogeographic history (Parmakelis et al., 2006a). Located at the margin of the Eurasian and African plates, this area has experienced tremendous geological alterations since the late Tertiary (Creutzburg, 1963; Dermitzakis, 1990; Dermitzakis and Papanikolaou, 1981; Meulenkamp, 1985; Meulenkamp and Sissingh, 2003; Steininger and Rögl, 1984). Connections created opportunities for dispersal, while submergence of land bridges geographically fragmented populations. If these were major elements influencing the formation of the rich fauna of the Hellenic area, then the phylogenetic relationships of terrestrial taxa should reflect these paleogeographic events. The above-mentioned biogeographic studies involve both invertebrate (Douris et al., 1995; Gantenbein et al., 2000; Gantenbein and Largiader, 2002; Parmakelis et al., 2005, 2006a,b) and vertebrate (Beerli et al., 1996; Kasapidis et al., 2005; Lymberakis et al., 2007; Poulakakis et al., 2003, 2005a,b,c; Weisrock et al., 2001) terrestrial taxa, as well as plants (Bittkau and Comes, 2005). Most of these studies conclude that a principally vicariant pattern of differentiation, directly related to the geotectonic evolution of the Aegean area, has shaped the present-day distribution of the taxa studied.

Until now, no attempts have been made to elucidate the molecular phylogeny and phylogeography of *O. punctatissimus*. In the present study, *O. punctatissimus* specimens were collected from several localities throughout its distribution, and DNA sequences were obtained for three mitochondrial genes (cytochrome *b*, 16S rRNA, and 12S rRNA). The aims of this study are to identify the most influential evolutionary processes (vicariance *versus* dispersal) shaping the geographic distributions of *O. punctatissimus* lineages in the Aegean region and geological explanations for these patterns.

2. Material and methods

2.1. DNA extraction, amplification and sequencing

The number and geographic locations of specimens used in this study are given in Table 1 and Fig. 1. All voucher specimens (12 specimens of *O. punctatissimus* from Greece and one of *O. latastii* from SW Syria) were deposited in the Natural History Museum of Crete (NHMC), Greece. Total genomic DNA was extracted from small pieces of the tail (preserved in ethanol) using standard methods (Sambrook et al., 1989).

A partial sequence (∼310 bp) of the mitochondrial protein-coding cytochrome *b* (*cyt b*) gene was amplified with the primers L14841 and H15149 (Palumbi, 1996). An approximately 500 bp fragment of the non-protein coding mitochondrial 16S rRNA gene was amplified using the universal primers 16aR2 and 16d (Reeder, 1995; Schmitz et al., 2005). Furthermore, a 500 bp segment of the non-protein coding mitochondrial 12S rRNA gene was amplified using the universal primers tPhe and 12 g (Leaché and Reeder, 2002; Wiens and Reeder, 1997).

Amplification of gene regions involved an initial cycle of denaturation at 94 °C for 5 min, and 35 subsequent cycles of 94 °C for 30 s, 47 °C (*cyt b*) or 59 °C (*16S*) or 60 °C (*12S*) for 30 s and 72 °C for 1 min, using single Taq DNA polymerase (Minotech, IMBB, Crete). PCR products were purified with the QIAquick PCR purification kit (Qiagen). Single-stranded sequencing of the PCR product was performed using the Big-Dye Terminator (v3.1) Cycle Sequencing kit on an ABI 377 automated sequencer following the manufacturer's protocol. Primers used in cycle sequencing were those used in the PCR amplification. The mtDNA genes were sequenced in both directions for all taxa (for accession numbers see Table 1).

In addition, two sequences (*12S*: AY649127 and *16S*: AY649168) of *O. punctatissimus* (Brandley et al., 2005) were retrieved from GenBank and included in the analysis. Individuals from two skink species were used as outgroup taxa: *Eumeces egregius* [*cyt b*, *16S*, *12S*: NC000808 (Kumazawa and Nishida, 1999)] and *Chalcides ocellatus* [*cyt b*: Z98040 (Cabrera et al. unpublished data), *16S*: AY308184 (Schmitz, 2003), *12S*: AY649106 (Brandley et al., 2005)].

2.2. Alignment and genetic divergence

The alignment of the concatenated *cyt b*, *16S*, and *12S* sequences was performed with ClustalX (Thompson et al., 1997) and manually improved. Alignment gaps were inserted to resolve length differences between sequences. Cytochrome *b* sequences were translated into amino acids prior to analysis and did not show any stop codons. Sequence divergences were estimated in PAUP* v.4.0b10 (Swofford, 2002) using the corresponding model of evolution for each gene across the entire dataset (see below). In addition, a saturation analysis was performed in DAMBE (Xia and Xie, 2001).

2.3. Phylogenetic analyses

Phylogenetic inference analyses were conducted using Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian inference (BI) methods. Maximum Parsimony analysis was performed with PAUP*, with branch and bound searches. Confidence in the nodes was assessed by 1000 bootstrap replicates (Felsenstein, 1985) with random addition of taxa. The model used for the ML analyses [General Time Reversible, GTR; (Rodriguez et al., 1990) + gamma (G)] was selected using Modeltest v.3.7 (Posada and Crandall, 1998) under the Akaike Information

Table 1
List of the specimens of *Ophiomorus* included in the study, with population map codes (see Fig. 1), taxon names, geographic origin, and accession numbers

Map Code	Species	Locality	Voucher number	Accession number		
				Cyt *b*	12S	16S
1	*O. punctatissimus*	Kithira Island, Greece	NHMC 80.3.83.4	EF581254	EF581267	EF581280
2	*O. punctatissimus*	Kithira Island, Greece	NHMC 80.3.83.5	EF581255	EF581268	EF581281
3	*O. punctatissimus*	Kithira Island, Greece	NHMC 80.3.83.9	EF581256	EF581269	EF581282
4	*O. punctatissimus*	Eurotas, Peloponnesos, Greece	NHMC 80.3.83.1	EF581249	EF581262	EF581275
5	*O. punctatissimus*	Eurotas, Peloponnesos, Greece	NHMC 80.3.83.2	EF581250	EF581263	EF581276
6	*O. punctatissimus*	Eurotas, Peloponnesos, Greece	NHMC 80.3.83.3	EF581251	EF581264	EF581277
7	*O. punctatissimus*	Dokos Isl, Peloponnesos, Greece	NHMC 80.3.83.10	EF581252	EF581265	EF581278
8	*O. punctatissimus*	Dokos Isl, Peloponnesos, Greece	NHMC 80.3.83.11	EF581253	EF581266	EF581279
9	*O. punctatissimus*	Stoupa, Peloponnesos, Greece	NHMC 80.3.83.7	EF581247	EF581260	EF581273
10	*O. punctatissimus*	Bouliari, Peloponnesos, Greece	NHMC 80.3.83.8	EF581248	EF581261	EF581274
11	*O. punctatissimus*	Kastelorizo Island, Greece	NHMC80.3.83.13	EF581257	EF581270	EF581283
12	*O. punctatissimus*	Kastelorizo Island, Greece	NHMC80.3.83.14	EF581258	EF581271	EF581284
13	*O. punctatissimus*	Kekova, Adasi, Turkey	MVZ 230221	n/c	AY649127	AY649168
14	*O. latastii*	Buraq SW Syria	NHMC 80.3.78.1	EF581259	EF581272	n/c
Outgroup	*E. egrerius*	—	—	NC000808	NC000808	NC000808
Outgroup	*C. ocellatus*	—	—	Z98040	AY649106	AY308184

Individuals from two closely related species were used as outgroup taxa: *Eumeces egrerius*, *Chalcides ocellatus*. Acronyms: NHMC, Natural History Museum of Crete, Greece; MVZ, Museum of Vertebrate Zoology.
n/c: Failed to amplify.

Criterion (AIC) (Akaike, 1974), whereas BI was performed under mixed models (the data were partitioned by gene), and full parameter estimation was performed during tree search for each gene. Heuristic ML searches were performed in PAUP* with 10 replicates of random sequence addition and TBR branch swapping using the search strategy of successive-approximations (Sullivan et al., 2005; Swofford et al., 1996) and significance was estimated by 100 repartitions. BI analysis was performed in MrBayes v.3.1 (Ronquist and Huelsenbeck, 2003), with four runs and four chains for each run for 10^7 generations and the current tree saved every 100 generations. A 50% majority rule consensus tree was produced from the posterior distribution of trees, and the posterior probabilities calculated as the percentage of samples recovering any particular clade, with posterior probabilities $\geqslant 95\%$ indicating significant support. Two further independent Bayesian analyses were run so that global likelihood scores, individual parameter values, topology and nodal support could be compared to check for local optima.

2.4. Molecular-clock hypothesis test

A likelihood ratio test (LRT) was used to examine the clock-like evolution of sequences in the combined data set by calculating a χ^2 statistic (Likelihood RatioTest, LRT) based on estimated ML log-likelihood values with and without rate constancy enforced $[\chi^2 = -2[(\ln L_{\mathrm{CLOCK}} - \ln L_{\mathrm{UNCONSTRAINED}})]$, df = number of terminal nodes -2; (Felsenstein, 1981)].

3. Results

Of the 1282 sites examined, there were 142 variable sites (339 with outgroups included). Sequence divergence within *O. puntcatissimus* ranged from 0 to 13.4% for *cyt b*, 0 to 6.3% for *16S*, and 0 to 6.8% for *12S*, whereas the genetic distance between *O. puntcatissimus* and *O. latastii* is 18.5% for *cyt b* and 9.0% for *12S* (for *16S* the PCR amplification was unsuccessful for *O. latastii*). Genetic distances using the GTR model of evolution from the entire data set are given in Table 2. Saturation analysis did not reveal any kind of saturation (figure now shown).

A partition homogeneity test indicated no conflicting phylogenetic signals between the datasets ($p = 0.971$) (the mtDNA genes were analyzed together). Maximum parsimony analysis of the 176 parsimony-informative characters produced three equally parsimonius trees ($L = 499$ steps; CI = 0.853; and RI = 0.823). ML analysis under the GTR+G model produced a single optimal topology ($\ln L = -4091.141$), which is consistent with the parsimony results (final parameter estimates: base frequencies A = 0.32, C = 0.27, G = 0.17, T = 0.24, a = 0.2596, Pinv = 0, and A/C = 2.56, A/G = 6.25, A/T = 1.82, C/G = 0.48 and C/T = 13.01).

Bayesian inference under the GTR+G model for *cyt b* and *16S*, and HKY+G model for *12S* produced a topology with mean $\ln L = -3933.686$. The $-\ln L$ stabilized after approximately 5×10^5 generations and the first 5×10^3 trees (10% "burn-in", chain had not become stationary) were discarded as a conservative measure to avoid the possibility of including random, sub-optimal trees. Identical topologies were recovered for each of the 4 runs with the full dataset. The 50% majority-rule consensus tree of the 95×10^3 trees remaining after burn-in are presented in Fig. 2.

In all phylogenetic analyses, *O. latastii* (from SW Syria) is the sister taxon of *O. punctatissimus*, and two very well supported allopatric clades of *O. punctatissimus* were identified (Fig. 2). Clade A comprised *O. punctatissimus* specimens from the western part of its distribution (Peloponnesos and Kythira Island) (100/98/1.00 = MP bootstrap value/ML bootstrap value/BI posterior probability, respectively), while clade B consists of *O. punctatissimus* populations from eastern Mediterranean localities (the island of Kastelorizo and southwest Turkey). (99/97/1.00)

The likelihood-ratio test did not reject the null hypothesis of a homogeneous clocklike rate for the tree produced by the *Ophiomorus* sequences [LRT = 2(3936.839 − 3927.415) = 18.848, df = 14, $\chi^2_{0.05} = 23.68$]. This suggests that the genetic distances between populations inhabiting different geographical regions can be used to estimate the divergence times among the major lineages of *O. punctatissimus*. The net nucleotide divergence (*Da*), which estimated in MEGA (v. 3.1) (Kumar et al., 2004) and corresponds to the between group variation corrected for the within-group variation in haplotypes (Nei, 1987), was used for this reason. Even though the absence of any accurate paleogeographical event didn't permit us to calibrate a "local" molecular clock, the divergence rates for the genes fragments used in this study have been calculated for other lizard species, such as *Ablepharus* (Scincidae) (*cyt b*: 1.33% and *16S*: 0.457% My⁻¹) and *Lacerta* (Lacertidae) (*12S*:

Table 2
Sequence divergences (%, below diagonal) among the main mtDNA lineages of *Ophiomorus* for *cyt b*/*16S*/*12S* computed by the GTR model (Rodriguez et al., 1990), and uncorrected net nucleotide divergences (*Da*) for the same genes fragments (above diagonal)

Population	1	2	3	4
1. Clade A (West, *O.p.*)	—	11.2/5.1/5.3	14.7/nc/7.6	19.2/16.3/17.5
2 Clade B (East, *O.p.*)	12.9/6.1/6.1	—	16.2/nc/7.8	19.4/15.0/17.6
3. Syria (*O.l.*)	17.7/nc/8.6	18.5/nc/8.6	—	21.1/nc/18.0
4. Outgroup	21.9/19.3/22.9	23.0/17.5/21.3	24.9/nc/22.0	—

No values were calculated (n/c) where no data was available.
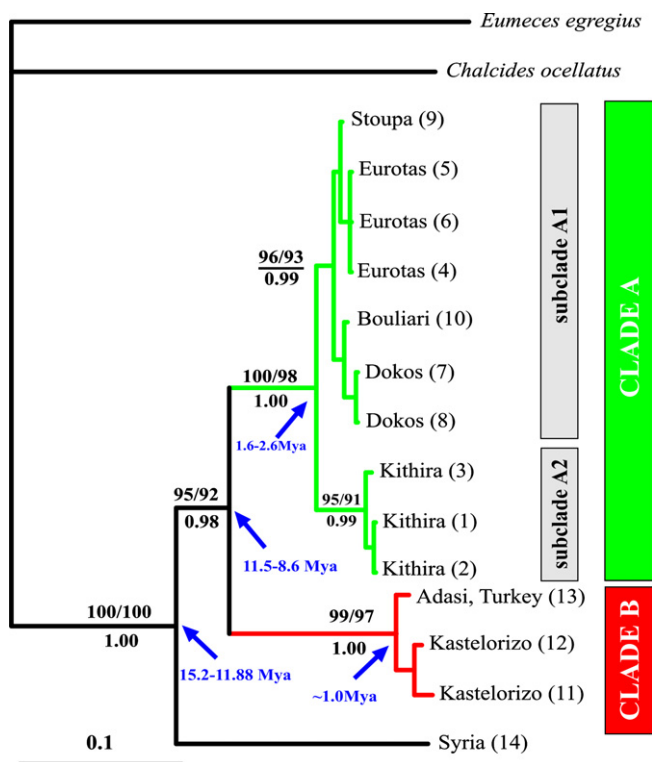*O.p.*, *O. punctatissimus*; *O.l.*, *O. latastii*.

Fig. 2. The 50% majority-rule consensus BI phylogenetic tree of *O. punctatissimus*. The numbers in the names of clades correspond to the map code of Table 1. Individuals from two closely related species were used as outgroup taxa: *E. egrerius*, *C. ocellatus*. Phylogenetic analyses (all the methods used: MP, ML, and BI) produced trees with the same topology. Numbers above branches indicate bootstrap values on MP and ML analysis respectively (MP/ML). Numbers below branches indicate posterior probabilities of Bayesian analysis (BI).

0.5% My^{-1}) (Carranza et al., 2004; Poulakakis et al., 2005a). Based on these rates and the net nucleotide divergences (uncorrected pairwise distances, Table 2), we estimated the divergence times of the major *Ophiomorus* lineages (Fig. 2).

4. Discussion

The results of this study revealed a well-resolved phylogeny and identified two major haplotype clades which, based on the observed levels of sequence divergence, indicate long-separated lineages within *O. punctatissimus*. These clades correspond to separate geographic regions: clade A west of the mid-Aegean trench and clade B east of the mid-Aegean trench (Figs. 1 and 2). Clade A could be further subdivided into two lineages (A1 and A2), which are in accordance with the geographical origin of the specimens. The strongly supported subclades A1 and A2 include populations from Peloponnesos and the island of Kithira, respectively. On the other hand, the inferred relationships among populations within clade B are weakly supported, and thus uncertain.

In our analyses, *O. punctatissimus* is a monophyletic species, since the two major clades of this species cluster together with strong statistical support and they are distant from the lineage of *O. latastii*, which Greer and Wilson (2001) identified as its closest relative. The genetic distance between the two clades of *O. punctatissimus* is 7.35% (all genes included), while the corresponding distance between *O. punctatissimus* and *O. latastii* is 11.9% (Table 2).

Anderson and Leviton (1966), Nilson and Andren (1978), and Greer (2002) suggested the Asian origin of the genus *Ophiomorus*, speculating a hypothetical ancestral form of *Ophiomorus* that was less specialized for fossorial habits and might have existed in the central plateau and upland areas of Iran and contiguous countries. The evidence thus suggests that the ancestral form of *O. punctatissimus* that invaded Greece is of Anatolian origin and that its former distribution was perhaps much wider than today.

In the Aegean region, the formation of the mid-Aegean trench has been hypothesized before to have acted as a major factor determining biogeographical patterns in reptiles (Poulakakis et al., 2003, 2005a,b,c), land snails (Douris et al., 1995; Parmakelis et al., 2005), coleopterans (Fattorini, 2002), isopods (Sfenthourakis, 1996), and scorpions (Parmakelis et al., 2006a,b). During the early and middle Miocene (23–12 Mya), the Aegean was part of a continuous landmass, also known as Agäis (Creutzburg, 1963; Dermitzakis and Papanikolaou, 1981). During the late Seravallian to early Tortonian (12–8 Mya) intense tectonic movements probably initiated the modern history of the Aegean region and the surrounding areas, causing the break up of the southern Aegean landmass. At the end of the middle Miocene (12 Mya), the formation of the Mid-Aegean trench (east of Crete and west of Kasos–Karpathos) (Fig. 1) began and was fully completed during the early late Miocene (~9 Mya) (Creutzburg, 1963; Dermitzakis and Papanikolaou, 1981), resulting in the separation of west Aegean from east Aegean islands.

The uncorrected net nucleotide divergence between *O. punctatissimus* and *O. latastii* is 15.45%% for the *cyt b*, and 7.6% the *12S* rRNA (we failed to amplify the *16S* fragment for the Syrian specimen), whereas within *O. punctatissimus* the divergence between Clade A and Clade B is 11.2% for the *cyt b*, 5.1% for the *16S*, and 5.3% the *12S* rRNA genes (Table 2). On the basis of the evolutionary rates that we mentioned in results (1.33% for *cyt b*, 0.457% for *16S*, and 0.5% My^{-1} for *12S*), we infer that the divergence of *O. punctatissimus* from *O. latastii* occurred at middle Miocene (*cyt b*: 11.88 Mya and *12S*: 15.2 Mya), while the divergence of Clade A (west) from Clade B (east) at late Miocene (*cyt b*: 8.6 Mya, *16S*: 11.5 Mya, and *12S*: 10.2 Mya).

These dates fit well with the geological information for the formation of mid-Aegean trench and led us to consider this geological phenomenon as the major factor that determined the biogeographical pattern of *O. punctatissimus* and probably caused the separation of the western (Clade A) and eastern (Clade B) *O. punctatissimus* lineages.

Thus, considering the molecular phylogeny of Fig. 2, the homogeneous clocklike rate, the estimated divergence times, and the fact that *Ophiomorus spp.* are species with poor dispersal abilities (Anderson and Leviton, 1966), we hypothesize that the most parsimonious scenario that could explain the present distribution of *O. punctatissimus* involves the formation of the mid-Aegean trench as the major vicariant event that has shaped the evolutionary history of *O. punctatissimus* in the Aegean region (vicariant scenario). This suggests a long history of *O. punctatissimus* in this area, in which the ancestral form of this species invaded the area from Anatolia before the complete formation of the Mid-Aegean trench, when the Aegean was still a uniform landmass. The formation of this barrier led to the basal clades of *O. punctatissimus* (clade A in the west and clade B in the east of the trench), which probably remained isolated and have not come into contact with each other for the last 8.6 million years. Within each clade, additional vicariant and dispersal events have produced the present-day distribution of the evolutionary lineages of *O. punctatissimus* [e.g., the isolation of Kythira islands during the Plio-Pleistocene (1.6, 2.6, and 2.2 Mya from *cyt b*, *16S*, and *12S*) or Kastelorizo Island during the Pleistocene (1.0, and 0.8 Mya for *16S*, and *12S*]. These dates are consistent with the paleogeographic events, since the island of Kythira was submerged in the early Pliocene, and did not reemerge until the transition from middle to late Pliocene (~2.5 Mya; Meulenkamp, 1985) (it was probably an island in the Pleistocene). The island of Kastelorizo, on the other hand, was part of mainland Turkey until very recently (middle Pleistocene).

In addition, an unknown fact led to a restriction in the distribution of the ancestral form of *O. punctatissimus* in two isolated areas. One of these isolates occurred, perhaps, in southern continental Greece, and gave rise to the clade that in our phylogenetic tree is identified as clade A. The other isolated population evolved in southwest Anatolia and produced the present complex of clade B.

Phylogenetic information can now be added to the knowledge of their morphology and distribution, producing a more accurate taxonomy for *O. punctatissimus*. The present results also confirm that the molecular information in conjunction with geological data can be used to resolve questions about the paleogeography of a region or the phylogeography of a species. It is worth noting that this peculiar distribution of *O. punctatissimus* (east and west of the Aegean Sea) corresponded to the age of one of the major geological events of the Aegean region, the formation of the mid-Aegean trench, which caused the separation of west Aegean from east Aegean islands.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.10.014.

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