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Mitochondrial DNA reveals the genealogical history of the snake-eyed lizards (*Ophisops elegans* and *O. occidentalis*) (Sauria: Lacertidae)

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ABSTRACT

The snake-eyed lizards of the genus *Ophisops* (Lacertidae) have been through a series of taxonomical revisions, but still their phylogenetic relationships remain uncertain. In the present study we estimate the phylogeographic structure of *O. elegans* across its distributional range and we evaluate the relationships between *O. elegans* and the sympatric, in North Africa, species *O. occidentalis*, using partial mtDNA sequences (16S rRNA, COI, and cyt *b*). All phylogenetic analyses produced topologically identical trees where extant populations of *O. elegans* and *O. occidentalis* were found polyphyletic. Taking into account all the potential causes of polyphyly (introgressive hybridization, incomplete lineage sorting, and imperfect taxonomy) we suggest the inaccurate taxonomy as the most likely explanation for the observed pattern. Our results stress the need for re-evaluation of the current taxonomical status of these species and their subspecies. Furthermore, our biogeographic analyses and the estimated time of divergences suggest a late Miocene diversification within these species, where the present distribution of *O. elegans* and *O. occidentalis* was the result of several dispersal and vicariant events, which are associated with climatic oscillations (the late Miocene aridification of Asia and northern Africa) and paleogeographic barriers of late Miocene and Pliocene period.

1. Introduction

Lacertids (Lacertidae)—the predominant reptile group in Europe (Arnold, 1989; Arnold and Burton, 1978)—have been the subject of scientific interest for a long time, due to their astonishing phenotypic variability, rendering their taxonomy complex and unstable (Arnold, 2004). The high phenotypic variation within some lacertid species as well as the occurrence of cryptic species and species complexes (Carranza et al., 2004; Castilla et al., 1998; Harris and Sá-Sousa, 2001; Poulakakis et al., 2003), complicated morphological diagnosis of species. Recent phylogenetic analyses using genetic data, show discrepancies from the actual systematic grouping based on morphological characters [i.e., conspecific populations often do not form monophyletic clades (Harris and Sá-Sousa, 2001; Poulakakis et al., 2003)], indicating that the molecular markers could be used as an independent means to clarify species diagnoses and phylogenetic relationships within the taxa of this family.

Mitochondrial DNA have served as the marker of choice for phylogeographic and species-level phylogenetic analyses of animals (Avise, 2000), because it shares a number of favorable properties such as matrilineal inheritance, a general lack of recombination, a high mutation rate, reduced effective population size, and availability of universal primers (McGuire et al., 2007 and references therein). Although, genealogical histories, inferred from mtDNA, will often provide robust phylogenetic and phylogeographic estimates (Avise, 2000), it is widely recognized that, under certain circumstances, phylogeographic inferences within and between closely related species can be misleaded by introgression and retention of ancestral polymorphism (incomplete lineage sorting) (Funk and Omland, 2003). Thus, interpretations based on mtDNA genes are susceptible to errors and caution is needed in the case of lack of other independent molecular markers (i.e., nuclear markers).

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The snake-eyed lizards of the genus *Ophisops* (Lacertidae) are distributed in southeast Europe, North Africa, and Asia with 8 species currently recognized. Two of them (*O. elegans* and *O. occidentalis*) could be considered as Mediterranean species. *O. elegans* is widely distributed across the east Mediterranean region and south–west Asia (Fig. 1), with seven uncertain morphological subspecies (Baran and Atatur, 1998; Darevsky and Beutler, 1981; Disi, 2002; Disi et al., 2001; Lymberakis and Kaliontzopoulou, 2003; Moravec, 1998). Both Mediterranean species have patchy distributions in North Africa (Fig. 1). The classic view was that the two species—*O. occidentalis* in the west and *O. elegans* in the east—are separated by a distribution gap of about 400 km in the Gulf of Sirte (N. Libya) (Schleich et al., 1996). However this standpoint was already doubted by Calabresi (1923) and Bons and Geniez (1996) who argued that both are members of one species. New evidences shed light on the distributional patterns of the North African *Ophisops*. First, the discovery of a case of sympatric occurence of these species in Cyrenaia (present study), and another one in NE Algeria (Chirio and Blanc, 1993). Second, the findings of Baha El Din (2006) expanded the range of *O. occidentalis* till Egypt. Consequently, *Ophisops* populations from the western Egyptian Mediterranean coast should be referred to as *O. occidentalis* rather than *O. elegans* and the populations of *O. elegans* in Cyrenaica and NE Algeria can now be considered as relicts (Baha El Din, 2006; Chirio and Blanc, 1993; Frynta et al., 2000; Schleich et al., 1996). Hence, the evolutionary history and taxonomy of this small reptile species need to be re-evaluated.

It is known that analysis of intraspecific phylogeographic patterns has led to major advances in our understanding of historical biogeographical processes (Avise, 2000), where the natural forces of vicariance and dispersal are used to explain the biogeographical pattern of organisms. Although, vicariance is considered by many to have been the dominant force underlying biogeographical patterns of modern taxa, neither dispersal nor vicariance seems to be especially favored (Austin et al., 2003). Within Lacertidae, one of the most impressive dispersal events was that of the subfamily of Eremiadinae (in the Saharo-Sindian assemblage which includes the genus *Ophisops*) from Eurasia to Africa (Arnold et al., 2007). The Lacertidae probably arose in the European area, with the Gallotiinae later reaching Northwest Africa and the Canary Islands, and the ancestor of the Eremiadini invading Africa in the mid-Miocene (Arnold et al., 2007). Mayer and Benyr (1995) proposed a colonization of Africa by lacertids 17–19 Mya, immediately after the first Neogenic contact between Eurasia and Africa (Steininger and Rögl, 1984). Arnold (2004) and Mayer and Pavličev (2007), in agreement with the former scenario, suggested a secondary recolonization of southwest Asia from Africa by an ancestor of the 'Saharo-Sindian' group across a land connection that existed until the early Pliocene between the Horn of Africa and Arabia. This could have probably happened during the middle Miocene, since the separation of the "Saharo-Sindian" lineage within Eremidianae and its first radiation occurred at ~13 Mya (Mayer and Pavličev, 2007). The invasion of this ancestor gave rise to the xeric forms of *Eremias, Mesalina, Acanthodactylus*, and *Ophisops*, the last three of which later colonized dry areas of North Africa (Arnold, 2004; Arnold et al., 2007; Mayer and Pavličev, 2007). In other words, *Ophisops* evolved in Southwest Asia and dispersed into North Africa later.

Given the incomplete knowledge of the evolutionary history of *O. elegans* and *O. occidentalis*, the present study has two objectives. First, to estimate the phylogeographic structure of *O. elegans* across most of its distributional range. We are particularly interested in exploring whether the phylogeographic pattern of *O. elegans* fits in with the model of the origin of *Ophisops* by estimating times of divergence among the major lineages within this group. Second, to evaluate the phylogenetic relationships between *O. elegans* and, the sympatric in North Africa species, *O. occidentalis*. In order to address these issues, we compared patterns of divergence in mitochondrial DNA (mtDNA) sequence of two protein-encoding [cytochrome *c* oxidase subunit 1 (COI), and cytochrome *b* (cyt *b*)] and one non-protein-encoding [16S rRNA (16S)] genes.

2. Materials and methods

2.1. DNA extraction, amplification, and sequencing

A total of 93 *Ophisops* specimens were used in this study: 87 *O. elegans*, five *O. occidentalis* and one *O. jerdonii* (Appendix A and Fig. 2). For all samples, voucher specimens were deposited in the Natural History Museum of Crete (NHMC). Total genomic DNA was extracted from small pieces of either the tail or the liver using standard methods (Sambrook et al., 1989). Partial segments of three mtDNA genes (16S, cyt *b*, and COI) were selected for the molecular phylogenetic analysis. Primers used in the polymerase chain reaction (PCR) amplifications and in the cycle sequencing reactions are shown in Table 1.

Amplification of all targeted genes involved an initial cycle of denaturation at 94 °C for 5 min, and 40 subsequent cycles of 94 °C for 1 min, annealing temperature ranging from 42 to 55 °C (Table 1) for 1 min and 72 °C for 1 min. PCR products were purified with the NucleoSpin PCR purification Kit (Macherey-Nagel). Single stranded sequencing of the purified PCR products was performed using a Big-Dye Terminator Cycle sequencing Kit (v. 3.1) on MJ Base Station automated sequencer following the manufacturer's protocol. Both strands of the amplified PCR products were sequenced for all specimens.

Three additional sequences (one for each gene) of *O. elegans* (Fu, 2002) were retrieved from GenBank, and were included in the phylogenetic analyses. In addition, sequences of two other lacertid

Fig. 1. Map showing geographic distributions of (a) *O. elegans* and (b) *O. occidentalis* in the Mediterranean region (Cox et al., 2006; present study).

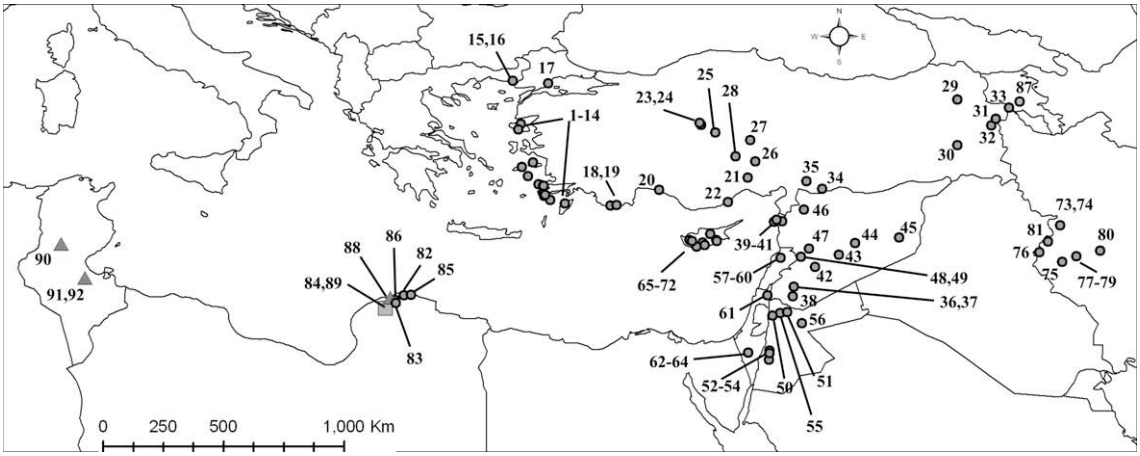

Fig. 2. Species and localities of specimens examined. Numbers (1–92) correspond to codes in Appendix A, (●) *O. elegans*, (▲) *O. occidentalis*, (■) both species in sympatry.

Table 1
Primers used for amplification and sequencing of the mitochondrial 16S rRNA, COI, and cyt *b* genes

Gene	Primer	Sequence	Annealing temperature (°C)	Reference
16S rRNA	16SAR-L	5′-CGC CTG TTT ATC AAA AAC AT-3′	47	Palumbi et al. (1991)
	16SBR-H	5′-CCG GTC TGA ACT CAG ATC ACG T-3′		
	Pod16S-L	5′-TGT CCC CTA AAT AGG GAC BRG-3′	55	Poulakakis (2005)
	Pod16S-H	5′-GGT GTC CTG ATC CAA CAT CG-3′		
COI	LCO 1498	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	42	Folmer et al. (1994)
	HCO 2198	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′		
	C1-J-1718	5′-GGA GGA TTT GGA AAT TGA TTA GTT CC-3′	42	Simon et al. (1994)
	C1-J-2191	5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′		
Cyt *b*	L14841	5′-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3′	42	Kocher et al. (1989)
	H15149	5′-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3′		
	Pod Cytb L	5′-AAA ACA TCA CCC CAT SAT WA-3′	42	Poulakakis (2005)
	Pod Cytb R	5′-GGA CTC CAA TGT TTC ATG TT-3′		

species were used as outgroup taxa: *Eremias velox* (16S: AF206604; COI: AF206576; cyt *b*: AF206549; Fu, 2002) and *Gallotia galloti* (16S: AF019651; Harris, 1999, COI: AF206561; Fu, 2002, cyt *b*: AY151841; Carranza et al., 2004) (Appendix A).

2.2. Alignment and genetic divergence

The alignment of the sequences was performed separately for each gene with Clustal X (Thompson et al., 1997) and manually corrected by eye. Alignment gaps were inserted to resolve length differences between sequences, and positions that could not be unambiguously aligned were excluded. Cytochrome *b* and COI sequences were translated into amino acids prior to analysis and did not show any stop codons. Software MEGA (v.3.1; Kumar et al., 2004) was used to determine the number and type of nucleotide substitutions in pairwise comparisons of sequences and to measure the degree of divergence between sequences using the Tamura–Nei model of evolution (Tamura and Nei, 1993). The resulting alignment is available on request from the authors. A saturation analysis was performed in DAMBE (Xia and Xie, 2001).

2.3. Phylogenetic analyses

Phylogenetic analyses were conducted on the concatenated dataset including all three phylogenetic markers (16S, COI, and cyt *b*) using neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods. Nucleotides were used as discrete, unordered characters.

The best-fit model of DNA substitution and the parameter estimates used for tree constructions were chosen according to the Akaike Information Criterion (AIC, Akaike, 1974; see Posada and Buckley, 2004) as implemented in Modeltest (v. 3.7; Posada and Crandall, 1998). This test indicated that the TrN+I+G, HKY+I+G, HKY+G, and TVM+I+G models showed a significantly better fit than the other less complicated models for the 16S, COI, cyt *b*, and the concatenated datasets, respectively.

NJ and MP analyses were performed with PAUP* (v.4.0b10; Swofford, 2002). MP was carried out (heuristic searches) using stepwise addition (with 100 replicates) and performing tree-bisection-reconnection (TBR) branch swapping (Swofford et al., 1996). Confidence in the nodes of NJ and MP trees was assessed by 1000 bootstrap replicates (Felsenstein, 1985).

Maximum likelihood analyses (Felsenstein, 1981) were conducted using PAUP* and RAxML-VI-HPC (v. 4.0.0) (Stamatakis, 2006). In PAUP, heuristic ML searches were performed with 10 replicates of random sequence addition and TBR branch swapping, based on the successive-approximations strategy (Sullivan et al., 2005; Swofford et al., 1996). Since a ML tree search with such a complex model (TVM+I+G) would be computationally excessive in PAUP, the confidence of the nodes was assessed only in RAxML based on 100 bootstrap replicates, computed with the parallel message-passing-interface-based version of RAxML-VI-HPC.

BI analysis was performed with the software MrBayes (v3.1; Ronquist and Huelsenbeck, 2003) using the ability of MrBayes to handle a wide variety of data types and models, as well as any mix of these models, based on the procedure described in MrBayes manual. In the BI analysis we partitioned the dataset according to the corresponding

molecular markers, and the analysis was run by implementing the respective model of evolution to each one of the mtDNA genes. The analysis was run with four chains for 10^7 generations, sampling from the chain every 100 generations. This generated an output of 10^5 trees. In order to confirm that the chains had achieved stationarity, we evaluated "burn-in" plots by plotting log-likelihood scores and tree lengths against generation number using the software Tracer (v. 1.4; Rambaut and Drummond, 2007). After determining convergence, which generally occurred within the first one million generations (10%) of each analysis, we discarded all samples obtained during the first million generations as "burn-in". The percentage of samples recovering any particular clade in a BI analysis represents that clade's posterior probability (Huelsenbeck and Ronquist, 2001). A majority rule consensus tree ('Bayesian' tree) was then calculated from the posterior distribution of trees, and the posterior probabilities calculated as the percentage of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001), where probabilities $\geqslant$95% indicate significant support.

Congruence among methods (NJ, MP, ML, and BI) was assessed qualitatively by comparing similarity of topologies and corresponding nodal support. Quantitative examination of congruence was done using the Shimodaira–Hasegawa (SH) test, which statistically compares topology likelihoods among multiple competing hypotheses (Shimodaira and Hasegawa, 1999). The SH test was implemented in PAUP* using the RELL resampling method of Kishino et al. (1990), with 10,000 bootstrap replicates. Additionally, the alternative hypothesis of *O. elegans* monophyly was also tested.

2.4. Tempo of diversification

Unresolved evolutionary relationships are considered soft polytomies in that they are multiple dichotomous branching events occurring in rapid succession. To differentiate between poorly supported clades (soft polytomies) vs. zero-length branches (hard polytomies), we used the likelihood ratio test $[-2(\ln L_{Ha} - \ln L_{Ho})]$, proposed by Slowinski (2001), where L_{Ha} is the likelihood under the alternate hypothesis (the length of branch in question is nonnegative) and L_{Ho} is the likelihood under the null hypothesis (branch has zero-length). Using the 'describe trees' command following our ML run (with 'Perform likelihood-ratio test for zero branch lengths' selected in the likelihood settings menu), PAUP* calculated the probability for each likelihood ratio under the χ^2 distribution with one degree of freedom. However, the χ^2 distribution with one degree of freedom is inapplicable (see Slowinski, 2001 and references therein). Goldman and Whelan (2000) have shown that a statistically rigorous distribution is a 50:50 mixture of the χ^2 with zero degrees of freedom and with one degree of freedom. Significance for the likelihood ratio test for each branch in the phylogeny was determined using the percentage point values under the Goldman and Whelan (2000) mixed model (their Table 2). We used a conservative significance level ($a = 0.01$) to account for possible Type I error.

To estimate temporal divergence, a log-likelihood ratio test was used to examine the clock-like evolution of sequences of the ingroup in the combined data set by calculating a χ^2 statistic (Likelihood Ratio Test, LRT) based on ML values with and without rate constancy enforced ($\chi^2 = 2 \times [(-\ln L_{CLOCK}) - (-\ln L_{UNCONSTRAINED})]$, df = number of terminal nodes-2) (Felsenstein, 1981). However, the LRT was negative ($p < 0.001$), therefore a clock-like evolution of the involved sequences could not be assumed. Thus, the divergence times of *Ophisops* lineages were estimated using the nonparametric rate smoothing (NPRS) analysis with the recommended Powell algorithm as implemented in the software r8s (v.1.7.1 for Mac) (Sanderson, 1997, 2003), which relaxes the assumption of a molecular clock. As calibration points we used the previously estimated time of divergence between the clade of Gallotinae (*G. galloti* in our study) and the rest of Lacertidae (Lacertinae: Eremiadini and Lacertini) at 19.5 Mya (Arnold et al., 2007) and setting up the maximum age of divergence in Eremiadini (*Ophisops, Eremias* in our study) at 16 Mya (Arnold et al., 2007).

3. Results

For the phylogenetic analyses, a data set of 95 sequences was used. Eighty-eight different haplotypes were recovered among the 93 ingroup sequences. Of the 1248 sites examined, there were 311 (24.91%) variable sites of which 285 (22.83%) were parsimony-informative [438 (35. 1%) and 346 (27.7%), respectively, when outgroups were included in the analysis]. The level of divergence within and between *O. elegans* and *O. occidentalis* was extremely high. The ingroup (*O. elegans–O. occidentalis*) sequence divergence ranged from 0% to 14.1%, 18.4%, and 22.3% for 16S, COI and cyt *b*, respectively, while it increased to 23.2% and 30.8% for COI and cyt *b*, respectively, when *O. jerdonii* was included (*O. jerdonii* failed to amplify with the 16S primers). Saturation analysis did not reveal any kind of saturation (figure now shown).

All phylogenetic analyses (NJ, MP, ML, and BI) produced trees of the same overall (general large-scale structure) topology (Fig. 3). It is worth noticing that the tree topology is in congruence with the major lineages of *Ophisops*. Equally weighted parsimony analysis of the 346 parsimony-informative characters produced more than 10,000 most parsimonious trees with a length of 1144 steps (Homoplasy Index, HI = 0.462, Retention Index, RI = 0.891). The large number of equally parsimonious solutions was largely due to terminal branch swapping, particularly among specimens originating from the same or geographically proximal populations.

Maximum likelihood analyses under the same model of evolution resulted in topologies with $\ln L = -6822.5437$ in PAUP and $\ln L = -6733.9655$ in RAxML, which were identical to the BI tree. Bayesian inference under the TrN+I+G model for 16S rRNA, the HKY+I+G model for COI and the HKY+G model for cyt *b* resulted in a topology with mean $\ln L = -6949.2$. Posterior probability values from the BI were highly congruent with ML bootstrap support. To-

Table 2
Mean sequence divergences (%) among the main mtDNA subclades of *Ophisops* for COI (below diagonal) and 16S rRNA (above diagonal), based on the Tamura and Nei model of evolution

Clades/Subclades	1	2	3	4	5	6	7	8	9	10
A1 (Greece-W Turkey)	**1.9/0.5**	6.4	7.6	7.7	6.7	9.3	7.9	10.9	n/c	30.0
A2 (Syrian Jordan Libya SE Turkey)	10.6	**1.5/0.4**	4.7	6.7	4.5	5.5	6.3	9.3	n/c	28.0
A3 (Turkey)	12.9	6.4	**2.4/1.3**	8.3	6.6	8.4	7.5	11.4	n/c	30.2
A4 (Iran)	11.9	8.3	8.6	**0.3/0.0**	5.5	10.0	8.9	12.2	n/c	35.8
A5 (Cyprus	12.8	8.9	11.1	11.7	**0.3/0.3**	6.7	6.1	8.8	n/c	27.2
B1 (Libya–Tunisia)	13.7	11.0	11.6	14.2	15.3	**2.5/1.1**	4.4	10.2	n/c	29.7
B2 (Israel)	14.1	12.6	11.6	14.2	14.0	7.5	**0.5/0.7**	9.9	n/c	27.1
C (NE Turkey Armenia Iran)	15.4	16.7	17.6	17.0	15.6	16.6	16.3	**0.7/n/c**	n/c	28.1
O. jerdonii	21.1	21.5	21.6	19.2	21.9	23.2	20.3	19.0	**n/c**	n/c
Outgroup	27.5	26.8	27.9	26.7	28.4	28.1	28.1	26.4	25.4	**23.5/19.7**

No values were calculated (n/c) where no data was available. Values in diagonal are within subclade sequence divergences, COI/16S rRNA.

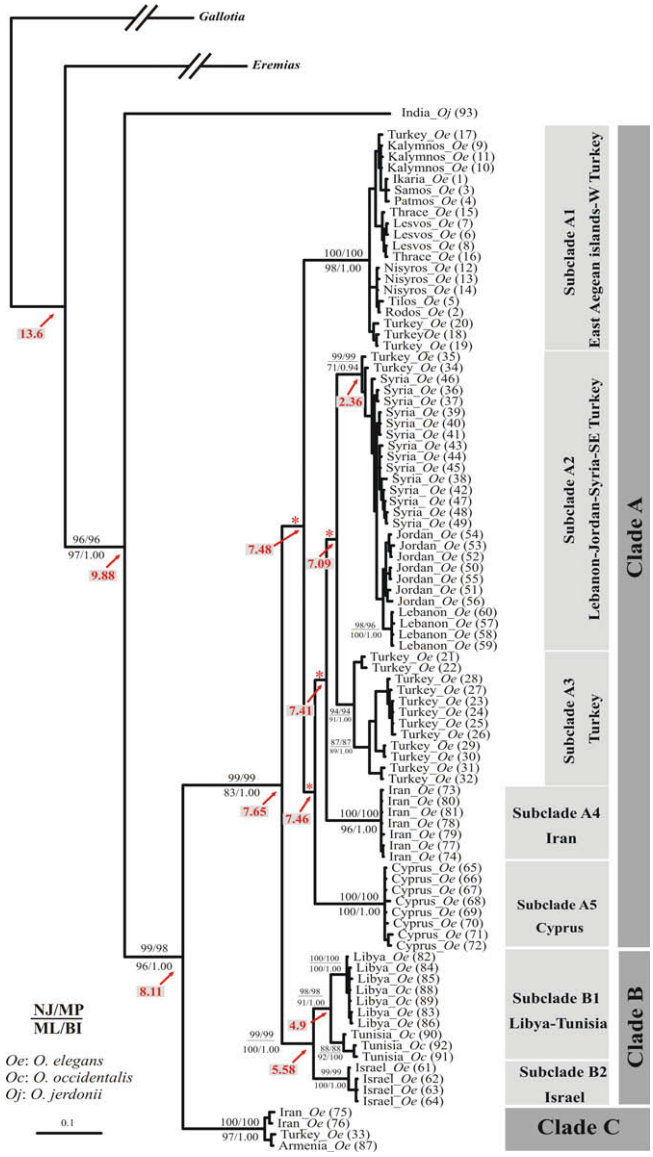

Fig. 3. Phylogenetic relationships among the two Mediterranean species (*O. elegans–O. occidentalis*) included in the analyses. Individuals of *E. velox* and *G. galloti* were used as outgroup taxa. Phylogenetic analyses of neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) produced trees with the same topology with regard to the major lineages. Only the BI tree is presented. The statistical support (bootstrap values and posterior probabilities) for the major clades and subclades are presented in the tree. Asterisks indicate branches that are not significantly different from zero-length (only the zero-length branches of the major lineages are indicated). Red arrows indicate the estimated time of divergences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

gether, similarity in ln*L* values and nodal support suggest the two methods successfully converged on the same tree space. Moreover, the results of multiple comparisons of log-likelihoods using Shimodaira–Hasegawa test indicated no statistical difference between the NJ, MP, ML, and BI trees.

Although this study focused on the two Mediterranean species (*O. elegans* and *O. occidentalis*), we also included one individual of *O. jerdonii* collected from India, the eastern range of this genus distribution. The single sample of *O. jerdonii* separated first from all other *Ophisops* specimens and was highly divergent from all other clades (Tables 2 and 3).

Apart from the *O. jerdonii* lineage, all analyses identified three very well-supported clades of *Ophisops* (Fig. 3). Clade A consisted of *O. elegans* specimens from southeastern Europe (Greece) and south–west Asia (excluding Israel). Clade B comprises *O. elegans* and *O. occidentalis* from North Africa and Israel. Finally, four individuals (one from Armenia, one from NE Turkey and two from Iran) form another distinct clade (C), which is placed as the sister group to all other *Ophisops* specimens.

Clade A could be further subdivided into five subclades that host *O. elegans* specimens from separate geographic regions as follows: (a) northeastern Greece and West Turkey (subclade A1), (b) SE Turkey, Syria, Jordan, and Lebanon (subclade A2), (c) central, east, and southern regions of Turkey (subclade A3), (d) Iran (subclade A4), and (e) Cyprus (subclade A5). Although the monophyly of each of these subclades was supported by high bootstrap values and posterior probabilities, the relationships among them are considered unresolved.

Clade (B) was divided into two well-supported subclades (B1, B2). Within subclade B1 two subgroups of specimens, corresponding to two geographically distinct sites of samples, were recognized. The first consisted of five *O. elegans* and two *O. occidentalis* specimens from Libya and the second of three *O. occidentalis* specimens from Tunisia. Suclade B2 consisted entirely of specimens from Israel.

Phylogenetic analyses using NJ, MP, ML, and BI methods supported a pattern of polyphyly among lineages of *O. elegans and O. occidentalis*. The Shimiodaira–Hasegawa test rejected the monophyly of *O. elegans* ($p < 0.001$). Furthermore, *O. elegans* haplotypes do not reflect subspecies designations.

The branch length separating *O. jerdonii* and clade C (*O. elegans* based on the current literature, but see discussion) from the more terminal nodes were significantly greater than zero. The relationship between the major lineages (subclades in Fig. 3) within *O. elegans* and *O. occidentalis* remains unresolved. All four phylogenetic methods resulted in a polytomy where bootstrap and posterior probabilities are low and branch lengths not significantly larger than zero. In particular, for four interior branches among the branches which lead to the major lineages within clades A and B the null hypothesis was not rejected at $\alpha = 0.01$ using Table 2 in Goldman and Whelan (2000) and hence the lack of support may correspond to molecular polytomy.

Approximate dates for some diversification events were inferred using the NPRS method. They are shown in Fig. 3 and yield an age of approximately 10 Mya for the common ancestor of the mitochondrial lineages of *Ophisops* examined in this study. *O. elegans* and *O. occidentalis* diverged approximately 7.65 Mya (late Miocene), whereas both species underwent rapid splitting during the late Miocene period about 7.48–7.0 Mya.

4. Discussion

The main objective of this study was to compare the mtDNA population history between closely related snake-eyed species (*O. elegans* and *O. occidentalis*) with overlapping distributions in northern Africa. The mitochondrial gene tree recovered substantially conflicts with the current phylogenetic (i.e., taxonomic status) views of the two Mediterranean *Ophisops* species predicted from the morphological data. Distance analyses revealed high genetic divergences among *Ophisops* specimens investigated in this study (Tables 2 and 3), which are among the higher genetic distances within the family of Lacertidae (Carranza et al., 2004; Kapli et al., 2005; Poulakakis et al., 2005b), indicating a deep biogeographical history of these species.

4.1. Mitochondrial polyphyly in O. elegans

The mtDNA haplotypes of *O. occidentalis* are phylogenetically nested within the haplotypes of *O. elegans*, indicating that

Table 3
Mean sequence divergences (%) among the main mtDNA subclades of *Ophisops* for cyt *b* (below diagonal) and concatenated data set (above diagonal) based on the Tamura and Nei model of evolution

Clades/subclades	1	2	3	4	5	6	7	8	9	10
A1 (Greece-W Turkey)	**1.5**	9.9	11.5	10.9	10.2	12.8	13.3	15.9	24.2	27.4
A2 (Syrian Jordan Libya SE Turkey)	13.4	**1.4**	6.6	7.7	6.8	9.2	11	17.2	23.3	27
A3 (Turkey)	12.5	10.4	**2.3**	9.5	9.2	11.2	11.7	17.7	23.8	27.6
A4 (Iran)	12.3	12.5	12.4	**0.0**	8.9	12	12.9	18.2	21.5	29.1
A5 (Cyprus	n/c	n/c	n/c	n/c	**n/c**	11.7	11.9	14.8	22	27.6
B1 (Libya–Tunisia)	15.0	9.9	13.1	9.9	n/c	**0.1**	6.3	16.1	23.9	27.5
B2 (Israel)	14.9	10.7	13.8	12.3	n/c	5.0	**0.2**	15.3	22.2	26.5
C (NE Turkey Armenia Iran)	17.6	19.9	18.4	19.7	n/c	15.8	13.9	**1.2**	19.6	26.9
O. jerdonii	29.8	27.0	27.2	24.5	n/c	25.3	24.6	20.2	**n/c**	25.4
Outgroup	25.5	27.3	26.2	25.9	n/c	24.8	24.8	27.1	25.3	**26.9**

No values were calculated (n/c) where no data was available. Values in diagonal are within subclade sequence divergences.

O. elegans is polyphyletic (Fig. 3). This result was further reinforced using non-parametric bootstrapping (Shimodaira–Hasegawa test, SH), where we were able to reject the alternative (null) hypothesis that *O. elegans* is a monophyletic species.

Polyphyly and paraphyly are observed in many species (Funk and Omland, 2003 and references therein) and have multiple potential causes, including: (a) incomplete lineage sorting due to recent speciation events, (b) introgressive hybridization through inter-specific mating followed by backcrossing of hybrids into parental populations, and (c) imperfect taxonomy caused by misidentification of intra- and inter-specific variation.

Lineage sorting eliminates ancestral polymorphism over time so that sister species eventually become reciprocally monophyletic with respect to mtDNA, but this is expected to be incomplete when the rate of lineage splitting or speciation exceeds the rate of stochastic sorting of allelic polymorphisms within lineages (Sullivan et al., 2002). In these cases the phylogeny of alleles sampled will differ from larger species phylogeny and a gene genealogy may be misleading (Pamilo and Nei, 1988). In our case, some *O. elegans* and *O. occidentalis* share identical or weakly identical mitochondrial haplotypes despite the fact these taxa are morphologically distinct. If this is due to incomplete lineage sorting of haplotypes present in their common ancestor, then the speciation events that gave rise to their distinct morphologies must have occurred recently enough so that the mitochondrial genomes retained in the descendent species have yet to accumulate independent changes. Thus, if *O. elegans* and *O. occidentalis* diverged recently, the probability that the taxa would be reciprocally monophyletic is quite low, even if they do not exchange any genes. Additionally, if multiple alleles, present in a common ancestor, have been retained in descendent species, the alleles are expected to be randomly distributed in the descendent populations (Masta et al., 2002). This scenario seems unlikely for *O. elegans*, especially in the light of the facts that (1) the estimated time of divergence is very old (~7 Mya, late Miocene), (2) other populations of this species are characterized by mitochondrial haplotypes that are very divergent from those of *O. occidentalis*, and (3) the populations of *O. elegans* that carry mitochondrial haplotypes identical or slightly divergent from those of *O. occidentalis* are not randomly distributed throughout the range of these species.

Alternatively, our findings may be accounted for by introgressive hybridization between species after they diverged (Avise et al., 1994). It is worth noticing that no morphological hybrids have been observed in the contact zone of *O. elegans* and *O. occidentalis* (Chirio and Blanc, 1993). Although hybridization could account for the presence of common haplotypes in *O. elegans* and *O. occidentalis* in North Africa, where these species occur in sympatry (Cyrenaica; Libya), it seems unlikely to justify the presence of *O. elegans* haplotypes in Israel that are phylogenetically closer to (1) *O. occidentalis* from Libya and Tunis and (2) *O. elegans* from Libya than to *O. elegans* from the rest of its distributional range.

Hence, the most likely explanation for the observed polyphyletic pattern is inaccurate taxonomy caused by the fact that the high intraspecific morphological variation occurring both within *O. elegans* and *O. occidentalis* populations from North Africa may have been misidentified as species level-variation. Calabresi (1923) and Bons and Geniez (1996) argued that *O. elegans–O. occidentalis* of NE Libya belong to the same species. Moreover, Arnold (1986) pointed out that the hemipenis structure of *O. elegans* and *O. occidentalis* from NE Libya is similar, but elsewhere *O. elegans* is different. These observations and our results explain the contradictions of the species status of *Ophisops* populations in North Africa (Baha El Din, 2006; Lantz, 1930) and the presence of two species in sympatry in Algeria (Chirio and Blanc, 1993) and Cyrenaica (present study). Thus, the currently defined taxonomic status of *O. elegans* and *O. occidentalis* is doubtful. However, in order to avoid any taxonomic confusion, at least until further input (nuclear genes and morphology) becomes available, we suggest that the North Africa and Israeli *Ophisops* specimens be referred to as the "northern African lineage of *Ophisops*", which includes populations belonging to two currently recognized species.

Interestingly, the phylogenetic position of four *O. elegans* collected from eastern Turkey, Armenia and Iran represents an unexpected problem for its taxonomy. These specimens form a distinct clade (C), which branches off first from all other *O. elegans* and *O. occidentalis* specimens (Fig. 3). Their phylogenetic position as a sister clade to all remaining specimens of *O. elegans* and *O. occidentalis*, raise questions regarding the phylogenetic and taxonomic affinities of this lineage. However, there is no doubt that a more robust hypothesis could be proposed once all missing taxa from Afghanistan, Pakistan, and India are included in the analysis.

4.2. Historical biogeography

It is worth noticing that caution is needed in interpreting the molecular phylogeny of *O. elegans* and *O. occidentalis*. There are more *Ophisops* species that have not been included in the current analyses. Considering the Asiatic origin (southwest Asia) of the genus *Ophisops* (Arnold, 2004; Arnold et al., 2007; Mayer and Pavličev, 2007), our mtDNA advocate a middle Miocene diversification of the two members of "Saharo-Sindian" assemblage used in this study, since the divergence of the lineages of *Eremias* and *Ophisops* is estimated to have occurred around 13.6 Mya. This is in agreement with Mayer and Pavličev (2007) who argued that the radiation of the "Saharo-Sindian" lineage occurred approximately at 13 Mya. The speciation within *Ophisops* in southwest Asia has been estimated to have occurred during late Miocene. Later on the species expanded into North Africa. The first two lineages branched off before 9.88 and 8.11 Mya and led to the *O. jerdonii* and the clade C

of our analysis, respectively. The next major divergence events took place at 7.65–7 Mya and resulted in all other major clades of our analysis (clades A and B). This almost simultaneous divergence resulted in the observed molecular polytomy (Fig. 3) and might explain the inadequacy in resolving the relationships among the major subclades. The biological reality of polytomies is a topic of debate (Walsh et al., 1999). Most commonly, researchers assume that polytomies are "soft" and can be resolved into sequential bifurcations given sufficient data and proper treatment of characters (DeSalle et al., 1994; Walsh et al., 1999). On the other hand, several researchers argue that an ancestral lineage sometimes can generate three or more descendent lineages at one time, resulting in multiple simultaneous speciation events represented by a "hard" polytomy (Maddison, 1989). Although polytomies can often be resolved by an increase in data, some remain uncertain, even after the analysis of large datasets (see Rokas and Carroll, 2006). Our statistical analysis indicates that four interior branches, which lead to the major lineages (subclades) of *O. elegans* and *O. occidentalis,* are not significantly larger than zero (Fig. 3) and hence the lack of support may correspond to a "hard" molecular polytomy. However, a molecular polytomy is a polytomy on a gene tree. A series of independent gene trees are necessary to test the null hypothesis of a real species polytomy (Slowinski and Page, 1999). Although in our case the molecular polytomy is produced by three mitochondrial genes, the fact that mtDNA functions effectively as a single locus (inherited as a unit), stresses the need for the incorporation of a nuclear gene to test whether the molecular polytomy coincides with the species polytomy.

Consequently, our data supports a scenario of simultaneously geographic dispersal of an ancestral lineage that occurred somewhere in southwest Asia towards the areas that *O. elegans* and *O. occidentalis* are distributed today, resulting in the distinct lineages of Fig. 3. This radiation may have been correlated to the late Miocene aridification (Duellman and Trueb, 1986; Fortelius et al., 2006; Guo et al., 2004), caused by the retreat of the Paratethys (~7–8 Mya; Ramstein et al., 1997). Palaeobotanical data suggest that northern Africa was occupied by a subtropical woodland savanna with a sclerophyllous evergreen forest until the late Miocene (Caujape-Castells et al., 2001; Quezel, 1978). However, in late Miocene it has become progressively more arid (Duellman and Trueb, 1986), when the incipient aridification of the Sahara began to settle biotypes favorable to the expansion of xerophytic organisms. In fact, a long-lasting arid period during the upper Miocene with only minor climatic oscillations should have allowed for range expansion of any xeric group, including *Ophisops*. This is evidenced by the fact that the greatest divergence of Saharo-Sindian lacertids is associated with adaptations to arid habitats (Fu, 2000).

The close relationship of Israeli and northern African populations indicates that they originated from the same ancestral stock, which diverged from the common ancestor that we mentioned before, approximately 7.65 Mya. The invasion of North Africa is dated at 5.6 Mya, before the flooding of the Nile (early Pliocene) due to the uplift of the Mediterranean sea-level (Goudie, 2005). The aridification of the Sahara in Mid-Upper Pliocene (Le Houerou, 1997) and the significant climatic changes of Pliocene and Pleistocene glaciation cycles (Caujape-Castells et al., 2001) would have broken the distribution area of *Ophisops* populations in northern Africa, leading to the present allopatric distribution with a gap of about 400 km in the Gulf of Sirte (Fig. 1).

All specimens from Syria, Jordan, Lebanon and SE Turkey form a subclade (A2) with no obvious phylogeographic pattern and relatively low intraspecific sequence variation (Tables 2 and 3). Syria shares a considerable number of reptile species with the surrounding countries (Disi, 1996; Disi and Bohme, 1996), where the lack of geographical boundaries between these countries probably prevents the isolation and the distinctiveness of their populations. The absence of any accordance between the haplotypes grouping and the geographic origin of the specimens could be attributed to the late Pliocene and Pleistocene diversification of this subclade and changes in the geographical distribution of the respective forms due to the current aridification of the Near East, such as *M. brevirostris* (Mayer et al., 2006).

Subclades A1 and A5 reveal aspects of the dispersal ability of *O. elegans*. The first consists of *O. elegans* specimens from SE Europe and W Turkey (Fig. 2) and its isolation could be due to Manavgat River that separates the populations of W Anatolia (Kumlutas et al., 2004). The estimated time of divergence (~7.5 Mya) indicates that the colonization of the east Aegean islands occurred after the formation of the Mid-Aegean trench (9–12 Mya, Dermitzakis and Papanikolaou, 1981), justifying the absence of *O. elegans* from any central Aegean island or continental area west of this trench. The unexpected absence of this species from the islands of Kasos and Karpathos (east of trench) (Wettstein, 1953) could be attributed to its inexplicable extinction from these islands or to the fact that *O. elegans* settled on Rodos Island, which served in the past as the connection of Kasos/Karpathos to the mainland, after the geological isolation of these islands (3.5 Mya; Daams and Van de Weerd, 1980).

Subclade (A5) includes *O. elegans* specimens from the oceanic island of Cyprus, which is geologically one of the most isolated Mediterranean islands (Moores et al., 1984). Although previous phylogeographic studies on frogs and lizards (Lymberakis et al., 2007; Poulakakis et al., 2005a) support the strong relationship of Cyprus with Syria, there is no solid evidence to confirm this in our study. Some authors noted that Cyprus has never been connected to any mainland (Sondaar, 1977; Hadjisterkotis, 1993) while others suggested that the animals could have arrived by means of a land bridge, when the island was joined to the nearby mainland (Küss, 1973; Hsü, 1983; Palikarides, 1997). Although *O. elegans,* like the other terrestrial reptiles, is a species with poor over-water dispersal ability, its sea-crossing ability could be assumed in the case of the volcanic island of Nisyros (east Aegean), which was never connected to continental areas (Papanikolaou and Lekkas, 1991). Consequently, the presence of *Ophisops* on Cyprus is unclear and either overseas dispersal or the existence of a land bridge is equally conceivable explanations.

5. Conclusions

This study revealed that *O. elegans* and *O. occidentalis* may be viewed as a species complex, and the current phylogenetic information can be added to the knowledge of their morphology and distribution, producing a more accurate taxonomy for these species. Our results suggest possible future lines of research in *Ophisops*. The inclusion of the remaining *Ophisops* species from Asia and other data (i.e., nuclear genes) in molecular analyses will be critical for understanding the evolutionary history of the whole genus. We expect this study to initiate new research efforts with the ultimate goal of yielding a stable picture of the evolutionary history of *Ophisops*.

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Appendix A

Specimens used in molecular analyses

Code	Species	Locality	Museum no.		Accession No.		
					16S rRNA	COI	Cyt *b*
1	*O. elegans*	Greece (Ikaria)		NHMC 80.3.70.121	n/c	EU081519	EU081611
2	*O. elegans*	Greece (Rodos–Salakos)		NHMC 80.3.70.2	EU081682	EU081520	EU081612
3	*O. elegans*	Greece (Samos)		NHMC 80.3.70.120	n/c	EU081521	EU081613
4	*O. elegans*	Greece (Patmos)		NHMC 80.3.70.19	EU081683	EU081522	EU081614
5	*O. elegans*	Greece (Tilos)		NHMC 80.3.70.162	EU081684	EU081523	EU081615
6	*O. elegans*	Greece (Lesvos)		NHMC 80.3.70.180	EU081685	EU081524	n/c
7	*O. elegans*	Greece (Lesvos)		NHMC 80.3.70.181	EU081686	EU081525	n/c
8	*O. elegans*	Greece (Lesvos)		NHMC 80.3.70.205	EU081687	EU081526	EU081616
9	*O. elegans*	Greece (Pserimos)		NHMC 80.3.70.213	EU081688	EU081527	EU081617
10	*O. elegans*	Greece (Pserimos)		NHMC 80.3.70.187	EU081689	EU081528	EU081618
11	*O. elegans*	Greece (Simenia)		NHMC 80.3.70.217	EU081690	EU081529	EU081619
12	*O. elegans*	Greece (Nisyros - Giali)		NHMC 80.3.70.222	EU081691	EU081530	EU081620
13	*O. elegans*	Greece (Nisyros - Lies)		NHMC 80.3.70.220	EU081692	EU081531	EU081621
14	*O. elegans*	Greece (Nisyros)		NHMC 80.3.70.218	EU081693	EU081532	EU081622
15	*O. e. macrodactylus*	Greece (Thrace)	BEV 4797	NHMC 80.3.70. 346	EU081694	EU081533	EU081623
16	*O. e. macrodactylus*	Greece (Thrace)	BEV 4799	NHMC 80.3.70.348	n/c	EU081534	EU081624
17	*O. elegans*	Turkey, Guzelkoy	NMP6V 70567	NHMC 80.3.70. 307	n/c	EU081535	n/c
18	*O. elegans*	Turkey 15 km E Kas	NMP6V 71316	NHMC 80. 3 .70. 313	EU081695	EU081536	EU081625
19	*O. elegans*	Turkey, Kas,	NMP6V 705649	NHMC 80.3.70. 308	EU081696	EU081537	EU081626
20	*O. e. macrodactylus*	Turkey (Manavgat)	BEV 8948	NHMC 80.3.70.349	EU081697	EU081538	EU081627
21	*O. e. basoglui*	Turkey (Gulek)	BEV 1665	NHMC 80.3.70.230	n/c	EU081539	n/c
22	*O. e. cf. basoglui*	Turkey (Icel delta du Goksu)	BEV 8203	NHMC 80.3.70.231	n/c	EU081540	EU081628
23	*O. e. centralanatoliae*	Turkey (Kayseri)	BEV 1444	NHMC 80.3.70.233	EU081698	EU081541	EU081629
24	*O. e. centralanatoliae*	Turkey (Karahamzeli)	BEV 1469	NHMC 80.3.70.234	EU081699	EU081542	EU081630
25	*O. e. centralanatoliae*	Turkey (Tuz Golu)	BEV 1470	NHMC 80.3.70.235	EU081700	EU081543	EU081631
26	*O. e. centralanatoliae*	Turkey (Demirkazig)	BEV 1480	NHMC 80.3.70.236	EU081701	EU081544	EU081632
27	*O. elegans*	Turkey, Goreme,	NMP6V 70826	NHMC 80.3.70.310	EU081702	EU081545	EU081633
28	*O. elegans*	Turkey, Ulukisla,	NMP6V 70827	NHMC 80.3.70.311	EU081703	EU081546	EU081634
29	*O. e. cf. centralanatoliae*	Turkey (Karakurt)	BEV 1367	NHMC 80.3.70.238	EU081704	EU081547	EU081635
30	*O. e. cf. centralanatoliae*	Turkey (lac Van)	BEV 1642	NHMC 80.3.70.240	n/c	EU081548	EU081636
31	*O. e. cf. centralanatoliae*	Turkey (Saz Golu)	BEV 1290	NHMC80.3.70.237	EU081705	EU081549	EU081637
32	*O. e cf. centralanatoliae*	Turkey (Kuskukiran Gecidi)	BEV 8241	NHMC 80.3.70.242	EU081706	EU081550	EU081638
33	*O. e. cf. centralanatoliae*	Turkey (Aralik)	BEV 7686	NHMC 80.3.70.241	n/c	EU081551	EU081639
34	*O. e. cf. ehrenbergii*	Turkey (Gaziante)	BEV 8182	NHMC 80.3.70.243	EU081707	EU081552	EU081640
35	*O. e. cf. ehrenbergii*	Turkey (Gaziante)	BEV 8204	NHMC 80.3.70.244	EU081708	EU081553	EU081641
36	*O. e. blanfordi*	Syria (Buraq)		NHMC 80.3.70.23	EU081709	EU081554	n/c
37	*O. e. ehrenbergii*	Syria (Buraq)		NHMC 80.3.70.25	EU081710	EU081555	n/c
38	*O. e. ehrenbergii*	Syria (Nizran)		NHMC 80.3.70.29	EU081711	EU081556	EU081642
39	*O. e. ehrenbergii*	Syria (Alawit mount. range)		NHMC 80.3.70.36	EU081712	EU081557	n/c
40	*O. e. ehrenbergii*	Syria (Maquam Assayedh)		NHMC 80.3.70.37	EU081713	EU081558	n/c
41	*O. e. ehrenbergii*	Syria (Lattakia beach)		NHMC 80.3.70.40	EU081714	EU081559	n/c
42	*O. e. blanfordi*	Syria (Al Badiah desert)		NHMC 80.3.70.67	EU081715	EU081560	EU081643
43	*O. e. blanfordi*	Syria (Rocky desert)		NHMC 80.3.70.70	EU081716	EU081561	EU081644
44	*O. e. ehrenbergii*	Syria (As Suhhnah)		NHMC 80.3.70.72	EU081717	EU081562	EU081645
45	*O. e. blanfordi*	Syria (Qal' at Al Rahbeh castle)		NHMC 80.3.70.73	EU081718	EU081563	EU081646
46	*O. e. ehrenbergii*	Syria (Kanaten Taflanaz)		NHMC 80.3.70.74	EU081719	EU081564	EU081647
47	*O. e. ehrenbergii*	Syria (40 km A.Homs)		NHMC 80.3.70.76	EU081720	EU081565	EU081648
48	*O. e. ehrenbergii*	Syria (Preij, 40 km N.Homs)		NHMC 80.3.70.82	EU081721	EU081566	n/c
49	*O. e. blanfordi*	Syria (Preij, 40 km N.Homs)		NHMC 80.3.70.83	EU081722	EU081567	EU081649
50	*O. elegans*	Jordan (Jerash)		NHMC 80.3.70.93	EU081723	EU081568	EU081650
51	*O. elegans*	Jordan (Thygratal Jubb)		NHMC 80.3.70.94	EU081724	EU081569	EU081651
52	*O. elegans*	Jordan (Dana Natural Reserve)		NHMC 80.3.70.95	EU081725	EU081570	EU081652
53	*O. elegans*	Jordan (Al Tafila)		NHMC 80.3.70.98	EU081726	EU081571	EU081653
54	*O. elegans*	Jordan (Al Manshiyva)		NHMC 80.3.70.101	EU081727	EU081572	EU081654
55	*O. elegans*	Jordan (Zai park)		NHMC80.3.70.108	EU081728	EU081573	EU081655
56	*O. elegans*	Jordan (Azrap)		NHMC 80.3.70.119	EU081729	EU081574	EU081656
57	*O. elegans*	Lebanon, Bsharri	NMP6V 35724	NHMC 80.3.70.306	n/c	EU081575	n/c
58	*O. elegans*	Lebanon, Bsharri	NMP6V35724	NHMC 80.3.70.315	n/c	EU081576	EU081657
59	*O. elegans*	Lebanon, Bsharri,	NMP6V35724	NHMC 80.3.70.316	n/c	EU081577	EU081658

Appendix A (*continued*)

Code	Species	Locality	Museum no.		Accession No.		
					16S rRNA	COI	Cyt *b*
60	*O. elegans*	Lebanon, Bsharri,	NMP6V35724	NHMC 80.3.70.317	EU081730	EU081578	EU081659
61	*O. e. ehrenbergii*	Israel (Mezudat Nimrod)	BEV 8495	NHMC 80.3.70.246	EU081731	EU081579	EU081660
62	*O. e. ehrenbergii*	Israel (Nahal Zin)		NHMC 80.3.70.226	n/c	EU081580	EU081661
63	*O. e. ehrenbergii*	Israel (Nahal Zin)		NHMC 80.3.70.227	n/c	EU081581	EU081662
64	*O. e. ehrenbergii*	Israel (Nahal Zin)		NHMC 80.3.70.228	EU081732	EU081582	EU081663
65	*O. elegans*	Cyprus (Larnaka Kamares)		NHMC 80.3.70.124	EU081733	EU081583	n/c
66	*O. elegans*	Cyprus (Larnaka)		NHMC 80.3.70.127	EU081734	EU081584	n/c
67	*O. elegans*	Cyprus (Lemesos)		NHMC 80.3.70.128	EU081735	EU081585	n/c
68	*O. elegans*	Cyprus		NHMC 80.3.70.131	EU081736	EU081586	n/c
69	*O. elegans*	Cyprus (Arkouda)		NHMC 80.3.70.132	EU081737	EU081587	n/c
70	*O. elegans*	Cyprus (Pareklisia)		NHMC 80.3.70.133	EU081738	EU081588	n/c
71	*O. elegans*	Cyprus		NHMC 80.3.70.135	EU081739	EU081589	n/c
72	*O. elegans*	Cyprus Roudia		NHMC 80.3.70.139	EU081740	EU081590	n/c
73	*O. elegans*	Iran (Kurdistan-Sarvabad)	*	NHMC 80.3.70.323	EU081741	EU081591	EU081664
74	*O. elegans*	Iran (Kurdistan- Sarvabad)	*	NHMC 80.3.70. 384	EU081742	EU081592	EU081665
75	*O. elegans*	Iran (Eslam Abade- Gharb)	*	NHMC 80.3.70. 340	n/c	EU081593	EU081666
76	*O. elegans*	Iran (Ghasr-e-shirin)	*	NHMC 80.3.70.341	n/c	EU081594	EU081667
77	*O. elegans*	Iran (Kermanshah)	*	NHMC 80.3.70.337	EU081743	EU081595	EU081668
78	*O. elegans*	Iran (Kermanshah)	*	NHMC 80.3.70.338	EU081744	EU081596	EU081669
79	*O. elegans*	Iran (Kermanshah)	*	NHMC 80.3.70.339	EU081745	EU081597	EU081670
80	*O. elegans*	Iran (Kngavar)	*	NHMC 80.3.70.324	EU081746	EU081598	EU081671
81	*O. elegans*	Iran (Harsin)	*	NHMC 80.3.70.325	EU08147	EU081599	EU081672
82	*O. elegans*	Libya (Kyrinis-Apolonias)		NHMC 80.3.70.3	EU08148	EU081600	EU081673
83	*O. elegans*	Libya (canyon after NatPark)		NHMC 80.3.70.5	EU08149	EU081601	EU081674
84	*O. elegans*	Libya (semi-desert Igdeida)		NHMC 80.3.70.11	n/c	EU081602	EU081675
85	*O. elegans*	Libya (waterfall)		NHMC 80.3.70.16	EU081750	EU081603	EU081676
86	*O. elegans*	Libya (Conduka-Al Beida)		NHMC 80.3.70.20	EU081751	EU081604	EU081677
87	*O. elegans*	Armenia (Chosrov)		—	AF206605	AF206556	AF206532
88	*O. occidentalis*	Libya (lagoon)		NHMC 80.3.101.1	EU081752	EU081605	EU081678
89	*O. occidentalis*	Libya (Desert Igdeida)		NHMC 80.3.101.2	EU081753	EU081606	EU081679
90	*O. occidentalis*	Tunis (Quled Maeur)		NHMC 80.3.101.3	n/c	EU081607	EU081680
91	*O. occidentalis*	Tunis (Matmata)		NHMC 80.3.101.8	EU081754	EU081608	n/c
92	*O. occidentalis*	Tunis (Matmata)		NHMC 80.3.101.9	EU081755	EU081609	n/c
93	*O. jerdonii*	India		NHMC 80.3.125.1	n/c	EU081610	EU081681

n/c: Failed to amplify.

The identification of species and subspecies was conducted by the authors on the basis of the morphology. Map code, species name, samples localities, museum numbers, and GenBank accession numbers of sequence data in our analysis. (NHMC: Natural History Museum of Crete; BEV: EPHE/CEFE-CNRS in Montpellier; NMP: National Museum Prague; *: personal collection Nasrullah Rastegar-Pouyani, Iran).

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