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ORIGINAL ARTICLE

Comparative phylogeography reveals distinct colonization patterns of Cretan snakes

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ABSTRACT

Aim We assessed genetic relationships among populations for each of the four snake species found on Crete (*Zamenis situla*, *Hierophis gemonensis*, *Telescopus fallax* and *Natrix tessellata*), including conspecific populations from the Aegean area. Our aim was to reconstruct their phylogeographical histories, especially regarding their occurrence on Crete.

Location Crete, Aegean Sea and eastern Mediterranean.

Methods Genetic diversity and relationships were based on sequences of the mitochondrial marker cytochrome *b*, applying phylogenetic analyses (maximum likelihood, Bayesian inference and neighbour-joining), a median-joining network analysis and a molecular dating analysis.

Results The *Z. situla* phylogeny includes a clade with specimens from Crete, the Peloponnese and Thera, while specimens from Turkey, northern Greece and the eastern Aegean islands form a separate clade. The *H. gemonensis* tree also presents two clades: one comprising specimens from Crete and Kythera, and another representing the continental part of the species' distribution. For *N. tessellata*, Cretan populations are found as the sister clade to populations from Europe and western Turkey. A more complex genetic structure is found in *T. fallax*: specimens from Crete, Thera and Antikythera form a clade, which itself forms part of a 'western' clade, and an 'eastern' clade includes specimens from Turkey, the eastern Aegean islands and Cyprus. The splits resulting in the Cretan clades for *T. fallax* and *N. tessellata* occurred at the end of the Miocene and the Pliocene/Pleistocene boundary, respectively. The Cretan lineages of *H. gemonensis* and *Z. situla* diversified during the Pleistocene.

Main conclusions *Zamenis situla* and *H. gemonensis* exhibit a phylogeographical pattern that involves a transmarine dispersal from southern continental Greece to Crete (possibly by humans in the case of *Z. situla*). The occurrence of *T. fallax* on Crete is explained by a natural dispersal from the west and isolation by vicariance. Although these two patterns have also been inferred for other studied herptiles of Crete, the pattern in *N. tessellata* is unique and involves a transmarine dispersal from south-western Turkey to Crete.

Keywords

Biogeography, Colubridae, Crete, dispersal, divergence times, eastern Mediterranean, phylogeny, reptiles, vicariance.

INTRODUCTION

The main goal of comparative phylogeography is the search for concordant geographical distributions of lineages in different species, which would indicate the influence of common historical factors (Hickerson *et al.*, 2010). Several phylogeographical studies have been conducted for animal taxa in the Aegean archipelago, with reptiles and amphibians

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proving to be sensitive indicators of palaeogeographical and palaeoclimatic events (reviewed in Lymberakis & Poulakakis, 2010).

The Aegean area (Fig. 1) can be described as one of nature's most active laboratories. The biogeographical and evolutionary histories of many terrestrial animals inhabiting the Aegean have been influenced by (1) its position at the south-eastern margin of Europe, (2) its complex geological history, (3) global and regional climatic changes, and (4) intense human activities during the past 10,000 years (Lymberakis & Poulakakis, 2010, and references therein). The Aegean archipelago includes a large number of islands that vary in size, geological history and their palaeogeographical relationships with adjacent islands and continental regions. Thus, the observed distribution and differentiation of organisms in the Aegean can be explained by dispersal and vicariance events, their combination and by human activity.

Crete, the fifth largest Mediterranean island, lies at the southern edge of the Aegean. A major geological event that isolated Crete from the east was the formation of the mid-Aegean trench (east of Crete and west of Kasos–Karpathos; Fig. 1), which began some 12 million years ago (Ma) and was completed at 10–9 Ma (Dermitzakis & Papanikolaou, 1981). In the same period, Crete became isolated from the Cyclades (Dermitzakis, 1990) and was only connected in the north-west with the Peloponnese (Dermitzakis & Papanikolaou, 1981) (Fig. 1). During the Messinian Salinity Crisis (MSC; *c.* 5.9–5.3 Ma; Krijgsman *et al.*, 2010, and references therein), Crete was connected to southern Greece, but became permanently isolated after the re-flooding of the Mediterranean basin (5.3 Ma; Bache *et al.*, 2012). An earlier isolation from the Peloponnese, in the Tortonian (*c.* 8 Ma), has also been suggested (see maps in Lymberakis & Poulakakis, 2010).

From the end of the Miocene until the Pliocene, Crete consisted of several islands corresponding to the present major mountain peaks, whereas in the Pleistocene, these islands began to form a united landmass due to regional uplift (Dermitzakis, 1990).

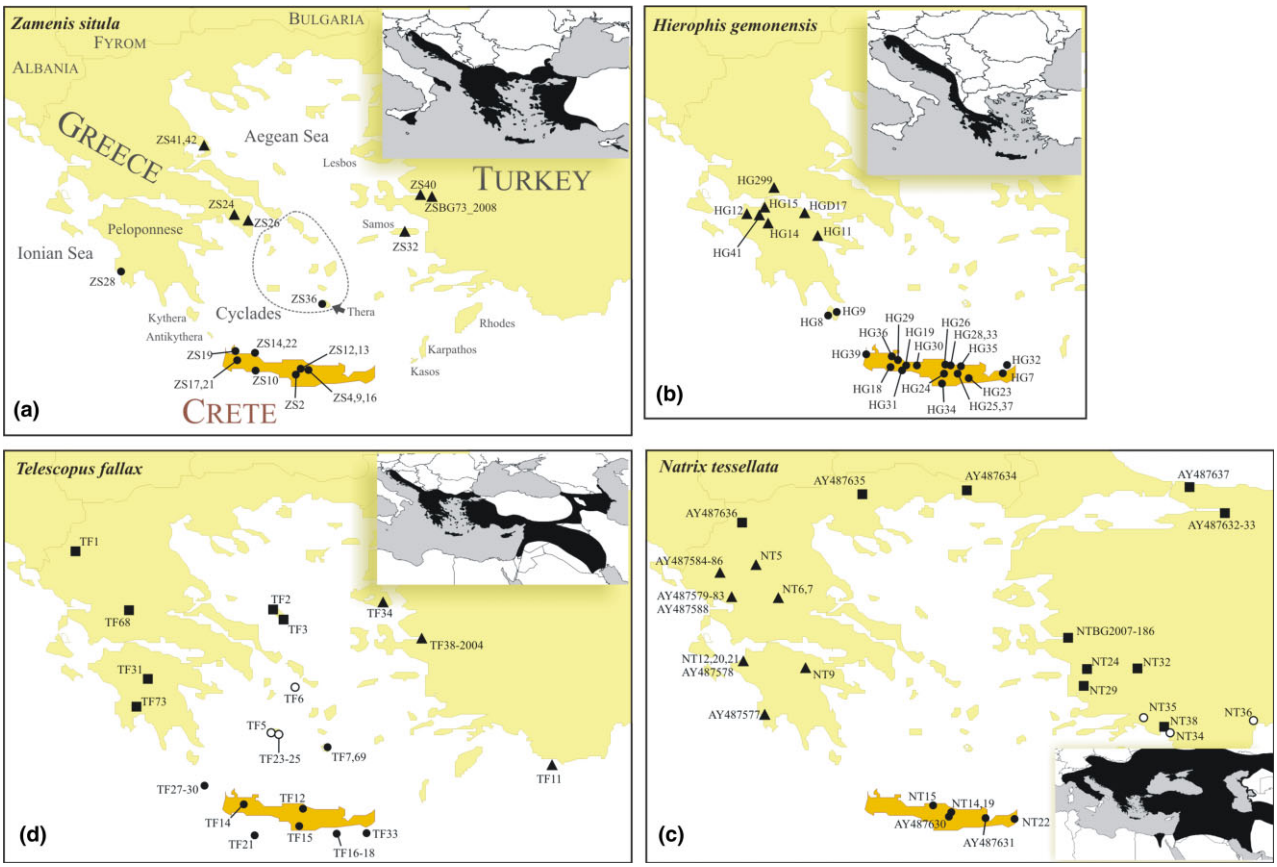

Figure 1 Maps showing sampling localities for the four studied species – (a) *Zamenis situla*, (b) *Hierophis gemonensis*, (c) *Natrix tessellata*, and (d) *Telescopus fallax* – in the circum-Aegean area (continental Greece, western Turkey, Crete and the Aegean islands). Sampling localities outside this area (Europe, Asia and Africa) are not shown, but the respective specimen codes and sampling localities are given in Appendix S1. For each species, the total geographical distribution is given as a separate inset. The map in panel (a) also shows several important regions and islands mentioned in the text. The distributions of mtDNA clades are shown with different symbols. *Z. situla*: closed circles = Crete, Peloponnese, Thera; triangles = Turkey, northern continental Greece, eastern Aegean islands. *H. gemonensis*: closed circles = Crete, Kythera; triangles = Croatia and continental Greece. *N. tessellata*: closed circles = Crete; triangles = Greece; squares = Europe, north-west Turkey; open circles = south-west Turkey. *T. fallax*: closed circles = Crete, Antikythera; squares = continental Greece, Skyros, Croatia; open circles = Cyclades; triangles = eastern clade.

Cretan herpetofauna

Although Crete's diverse geomorphology has played an important role in producing and sustaining biodiversity, its herpetofauna consists of a relatively small number of species, especially in comparison with other Mediterranean islands, comprising four species of snakes, seven lizards, one freshwater turtle and three anuran amphibians (15 species in total, two endemics). In comparison, the herpetofauna of other large Mediterranean islands (Corsica, Sardinia, Sicily, Cyprus, and even the smaller islands such as Lesbos and Rhodes) includes 21–27 species, with numbers of endemics reaching as high as 10 species in the case of Sardinia (Corti *et al.*, 1999).

Several phylogeographical studies have been conducted for Cretan herptiles. Only three of them are 'old inhabitants' of the island – *Pelophylax cretensis* (Lymberakis *et al.*, 2007; Akın *et al.*, 2010), *Mediodactylus kotschyi* (Kasapidis *et al.*, 2005) and *Podarcis cretensis* (Poulakakis *et al.*, 2005a; Lymberakis *et al.*, 2008) – whereas seven of them have colonized Crete recently (even in historical times) – *Hyla arborea* (Stöck *et al.*, 2008), *Bufo viridis* (Stöck *et al.*, 2006), *Mauremys rivulata* (Mantziou *et al.*, 2004), *Tarentola mauritanica* (Harris *et al.*, 2004), *Ablepharus kitaibelii* (Poulakakis *et al.*, 2005b), *Hemidactylus turcicus* (Carranza & Arnold, 2006) and *Chalcides ocellatus* (Kornilios *et al.*, 2010).

All Cretan snakes belong to the family Colubridae. Three of them – the leopard snake, *Zamenis situla* (Linnaeus, 1758), the Balkan whip snake, *Hierophis gemonensis* (Laurenti, 1768), and the European cat snake, *Telescopus fallax* (Fleischmann, 1831) – belong to the subfamily Colubrinae, whereas the dice snake – *Natrix tessellata* (Laurenti, 1768) – belongs to Natricinae.

To date, *N. tessellata* is the only species for which phylogeographical patterns have been investigated (Guicking *et al.*, 2006, 2009). It exhibits a well-resolved phylogeny, with Cretan populations having a sister-group relationship with populations from continental Europe. However, questions regarding the colonization of Crete by *N. tessellata* still need to be addressed.

In this study, we assess the genetic relationships among populations of each of the four snakes of Crete, and conspecific populations from the Aegean area (continental Greece, Aegean islands and Turkey), in a comparative phylogeographical manner. By using complete sequences of the mitochondrial marker cytochrome *b* (cyt *b*), we aim to: (1) depict their evolutionary and biogeographical history, especially regarding their occurrence on Crete, (2) explore similarities and differences in their phylogeographical structure, and (3) explore the roles of vicariance and dispersal and the influence of historical factors such as palaeogeography, palaeoclimate and human activities in shaping the phylogeographical patterns observed. This is the first phylogeographical study of *Z. situla*, *H. gemonensis* and *T. fallax*, while we revise the phylogeography of *N. tessellata* with the addition of new data from continental Greece and Crete and, most importantly, from western Turkey, that had not been previously assessed.

MATERIALS AND METHODS

Taxon sampling

In total, 117 cyt *b* sequences of the four studied species were generated in the current study (see Appendix S1 in Supporting Information). For *Z. situla*, all 21 analysed sequences were new, while for the *H. gemonensis* analyses, we produced 33 sequences and added another one from Nagy *et al.* (2003a). For *N. tessellata*, we analysed 27 new cyt *b* sequences and 78 additional ones from Guicking *et al.* (2009). For *T. fallax*, we analysed 36 new cyt *b* sequences and one from a previous study (Nagy *et al.*, 2003b). Sampling localities for the circum-Aegean area and the distributions of clades and species are shown in Fig. 1. As outgroups, we used both published sequences (Appendix S2) and others produced here, viz. *Hierophis cypriensis* and *Telescopus tripolitanus*. Finally, for the molecular dating, a large number of published sequences (Appendix S2) were combined with the ones from the present study, reconstructing a cyt *b* phylogeny of Colubrinae and Natricinae.

DNA extraction, amplification and sequencing

Tissue samples were preserved in ethanol. Total genomic DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). The entire cyt *b* gene was amplified with the primers L14910 and H16064 (Burbrink *et al.*, 2000; modified by de Queiroz *et al.*, 2002). Polymerase chain reaction (PCR) conditions were: 94 °C for 3 min; 40 cycles of 94 °C for 40 s, 46.5 °C for 30 s and 72 °C for 75 s; and 72 °C for 7 min. Products were purified using the NucleoFast 96 PCR Plate (Macherey-Nagel) and sequenced using the primers L14903 (the shorter version of L14910) and L-410 (Nagy *et al.*, 2003b). DNA sequencing was carried out on an ABI 3130xl automated capillary sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye v1.1 (Applied Biosystems) and following the manufacturer's instructions.

Phylogenetic and dating analyses

Sequences were aligned in ClustalX 2.0.12 (Larkin *et al.*, 2007) using the default parameters, and redundant haplotypes were removed using DnaSP 5.00.07 (Librado & Rozas, 2009). Nucleotide sequences of each unique haplotype have been deposited in GenBank (accession numbers: JX315462–JX315531; Appendix S1).

We employed three methods of phylogenetic analysis: maximum likelihood (ML), Bayesian inference (BI) and neighbour-joining (NJ). The best-fit model of DNA substitution was selected with jModelTest v.0.1.1 (Posada, 2008), under the Bayesian information criterion (BIC; Schwarz, 1978). The most suitable models for our data sets were TrN+I (Tamura & Nei, 1993) for *H. gemonensis* and *Z. situla*, TrN+I+G for *N. tessellata* and TrN+G for *T. fallax*. For

the molecular clock analyses, the most suitable model for the entire cyt *b* data set, and also for each of the three codon positions, was GTR+I+G (Tavaré, 1986).

Maximum-likelihood analyses (Felsenstein, 1981) were conducted in PhyML 3.0 (Guindon & Gascuel, 2003), applying the subtree pruning and regrafting (SPR) method (Hordijk & Gascuel, 2005) with 10 random starting neighbour-joining trees. Node support was assessed both with 1000 bootstrap replicates and the approximate likelihood-ratio test (aLRT), using the nonparametric Shimodaira–Hasegawa-like (SH-like) procedure (Anisimova & Gascuel, 2006).

Bayesian analysis was performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Four independent runs, with eight incrementally heated Markov chains and the default heating values, were used for 2×10^6 generations for *Z. situla*, *H. gemonensis* and *T. fallax*, and 6×10^6 generations for *N. tessellata*. The current tree was saved to file every 100 generations. We used Tracer 1.5 (Rambaut & Drummond, 2009) to confirm stationarity (all parameters had effective sample sizes > 200). The first 10% of the trees were discarded, and a majority-rule consensus tree was generated from the post-burn-in trees.

An NJ tree was calculated in mega v5 (Tamura *et al.*, 2011), based on genetic distance values under the Tamura–Nei (TrN) model of evolution. Node support was assessed with 1000 bootstrap pseudoreplicates.

We estimated divergence times, using 'external' calibration age constraints. For this purpose, we produced a phylogenetic tree of Colubrinae and Natricinae, as inferred by Burbrink & Lawson (2007) and Pyron *et al.* (2011), by combining our data with cyt *b* data from GenBank for a total of 170 sequences (Appendix S2). A similar approach was followed recently by Kuriyama *et al.* (2011) and Wood *et al.* (2011), who estimated divergence times for a colubrine (*Elaphe quadrivirgata*) and a natricine snake (*Thamnophis rufipunctatus*), respectively. For the calibration of the molecular clock, we used the age of six fossil records. Four of these are situated within the Colubrinae and were used in Kuriyama *et al.* (2011), while two are situated within the Natricinae and were used in Wood *et al.* (2011). These calibration points are: Cal1 (earliest *Coluber* and *Masticophis* fossils), Cal2 (earliest *Salvadora* fossil), Cal3 (earliest *Lampropeltis* fossil), Cal4 (earliest *Pantherophis* fossil), Cal5 (earliest *Thamnophis* fossil) and Cal6 (the first fossil appearance of the tribe Thamnophinii) [see Appendix S3, but also Burbrink & Lawson (2007), Kuriyama *et al.* (2011) and Wood *et al.* (2011) for further discussion of the composition and ages of these constraints]. We incorporated the fossil ages as in Kuriyama *et al.* (2011), by choosing prior age distributions so that the youngest age of the distribution corresponded to the youngest possible age at which that lineage existed. We chose a standard deviation so that 95% of the lognormal distribution was younger than the oldest age of appearance.

Finally, for the molecular clock analysis, we used Bayes factors to test two different partitioning strategies: employing either one substitution model for the entire cyt *b* segment, or three models of substitution for each of the three codon positions. We determined the Bayes factor by calculating the marginal likelihood for all analyses using Tracer and considered 2 ln Bayes factor > 10 as strong evidence for a hypothesis (Brown & Lemmon, 2007). The best partitioning strategy for the molecular clock analysis was the use of separate models for each codon position (2 ln Bayes factor = 1191.024).

All analyses were run in beast 1.6.2 (Drummond & Rambaut, 2007) under an uncorrelated lognormal relaxed molecular clock with a Yule prior on rates of cladogenesis. Four runs were conducted with a chain length of 3×10^7 iterations and a burn-in of 1.5×10^7 iterations. The four runs were analysed in tracer to check for convergence of the chains and combined in LogCombiner (Drummond & Rambaut, 2007). TreeAnnotator (Drummond & Rambaut, 2007) was used for the production of the chronogram.

Haplotype network analyses

Network approaches can be more effective than classical phylogenetic ones for representing intraspecific evolution (Posada & Crandall, 2001). In this sense, median-joining haplotype networks (Bandelt *et al.*, 1999) were calculated with Network 4.5.1 (available from http://www.fluxus-engineering.com/) for each of the studied species.

RESULTS

The *Z. situla* data set consisted of seven haplotypes, two of which, differing by one mutation, corresponded to the 12 specimens from Crete. All phylogenetic analyses produced trees of the same topology. Two major clades were found within *Z. situla* (Fig. 2a): one included specimens from Crete, the Peloponnese and Thera (Santorini), while specimens from Turkey, northern continental Greece and the eastern Aegean islands formed a separate clade. The network analysis showed that these two clades were separated by 16 substitutions (Fig. 2a).

Thirteen haplotypes were found in *H. gemonensis*. Three of them corresponded to the 18 Cretan specimens. The Cretan haplotype HapH3 was also shared by individuals from Antikythera and differed by a single substitution each of the haplotypes H1 and H2. The *H. gemonensis* phylogenetic tree (Fig. 2b) also presented two clades. One of them included specimens from Crete and Kythera while the other included all specimens from the continental part of the species' distribution (continental Greece and Croatia). Seven substitutions separated these two clades (Fig. 2b).

The six *N. tessellata* specimens from Crete correspond to four haplotypes, with the most common one (HapN16) differing by 1–4 substitutions from the other three. The phylogenetic tree of *N. tessellata* agreed with that of Guicking *et al.* (2009), also based on cyt *b* (Fig. 3): it distinguished the same major clades and exhibited similar values of nodal support. The Cretan clade was found to be sister to a clade that

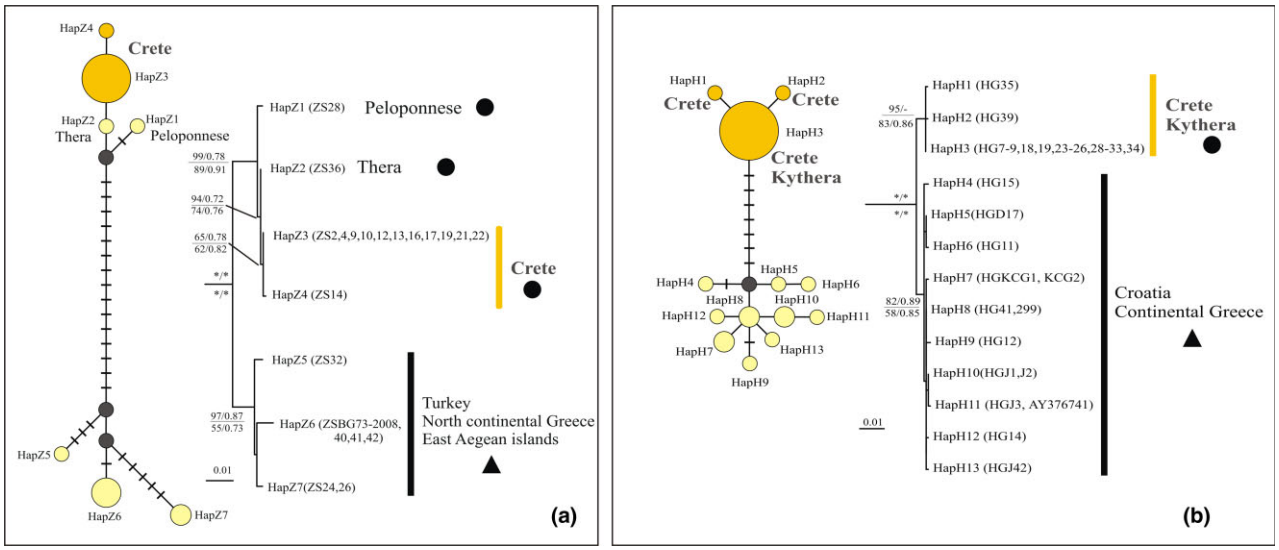

Figure 2 (a) Phylogenetic relationships among the studied *Zamenis situla* haplotypes/specimens. Right: maximum likelihood (ML) tree based on the cyt *b* haplotype data set, with Bayesian inference (BI) and neighbour-joining (NJ) methods resulting in trees of the same topology. *Zamenis lineatus* and *Z. longissimus* were used as outgroups (not shown). Numbers at terminal nodes refer to haplotype numbers presented in Appendix S1. Numbers in parentheses beside each haplotype correspond to specimen working codes, also shown in Appendix S1. Numbers above branches are NJ bootstrap values (BS)/BI posterior probabilities (PP), while numbers below branches are ML bootstrap values/values of PhyML approximate likelihood ratio test for branches. Only BS $\geq$ 50% and PP $\geq$ 0.50 are shown. Asterisks indicate full support (BS 100% or PP 1.00). Clade symbols are the same as those in Fig. 1. Left: a median-joining haplotype network based on the cyt *b* sequences. Each line (between two bars) represents a mutational step; dark yellow circles represent Cretan haplotypes; pale yellow circles represent haplotypes other than the Cretan ones sampled in the analysis; and dark grey circles represent missing haplotypes inferred using Network 4.5.1. The area of each circle is proportional to the number of individuals. (b) Phylogenetic relationships among *Hierophis gemonensis* haplotypes/specimens of the present study. *Hierophis cypriensis* and *H. viridiflavus* were used as outgroups (not shown). For a description of all information shown in this panel, see (a) above.

comprised specimens from Europe and western Turkey, consistent with the results of Guicking *et al.* (2009).

The number of haplotypes observed in *T. fallax* was 27. The eight *T. fallax* specimens from Crete and surrounding islets contained seven haplotypes. Together with four specimens from Antikythera and two specimens from Thera, these form a group of 11 haplotypes that are differentiated by 1–2 substitutions. Results from the phylogenetic and network analyses showed a more complex genetic structure within *T. fallax* than in *Z. situla* or *H. gemonensis*. As shown in the phylogenetic tree (Fig. 4), numerous clades and subclades occur within *T. fallax*, and in several instances their interrelationships are not well resolved. Specimens from Crete and surrounding islets, along with the specimens from the islands of Thera and Antikythera, formed a monophyletic clade which was resolved as sister to a clade comprising samples from continental Greece, the central and northern Aegean islands, and Croatia, albeit with low statistical support (Fig. 4). We refer to this larger clade as the 'western' clade. An 'eastern' clade was also resolved that included specimens from several parts of Turkey, the eastern Aegean islands and Cyprus. The haplotype network analysis showed that the Cretan clade is genetically closer to the continental Greece/Cyclades haplotypes than to the eastern clade (Fig. 4).

The chronogram from the molecular dating analysis for the entire Colubrinae and Natricinae phylogeny is shown in Appendix S3. A simplified version of this chronogram, mainly showing estimated dates for the Cretan lineages of the four studied species in a comparative manner, is presented in Fig. 5. The separation of Cretan *T. fallax* from all other populations probably occurred at the end of the Miocene (mean age: 5.4 Ma; 95% confidence intervals: 7.4–3.6 Ma), while for *N. tessellata*, the equivalent event occurred in the late Pliocene to early Pleistocene (2.3 Ma; 3.4–1.4 Ma). The Cretan lineages in *H. gemonensis* and *Z. situla* seem to have diversified from other conspecific populations recently, during the Pleistocene [0.9 Ma (1.7–0.4 Ma) for *H. gemonensis*; 0.3 Ma (0.7–0.0 Ma) for *Z. situla*].

DISCUSSION

Phylogenetic structure, relationships and biogeography

Zamenis situla

The phylogenetic tree of *Z. situla* (Fig. 2a) shows two distinct clades: one comprises specimens from Crete, Thera and the Peloponnese, while the other includes specimens from Turkey, northern parts of continental Greece, and the eastern Aegean island of Samos. Specimens from Turkey and northern Greece share the same haplotype, HapZ6 (Fig. 2a). This

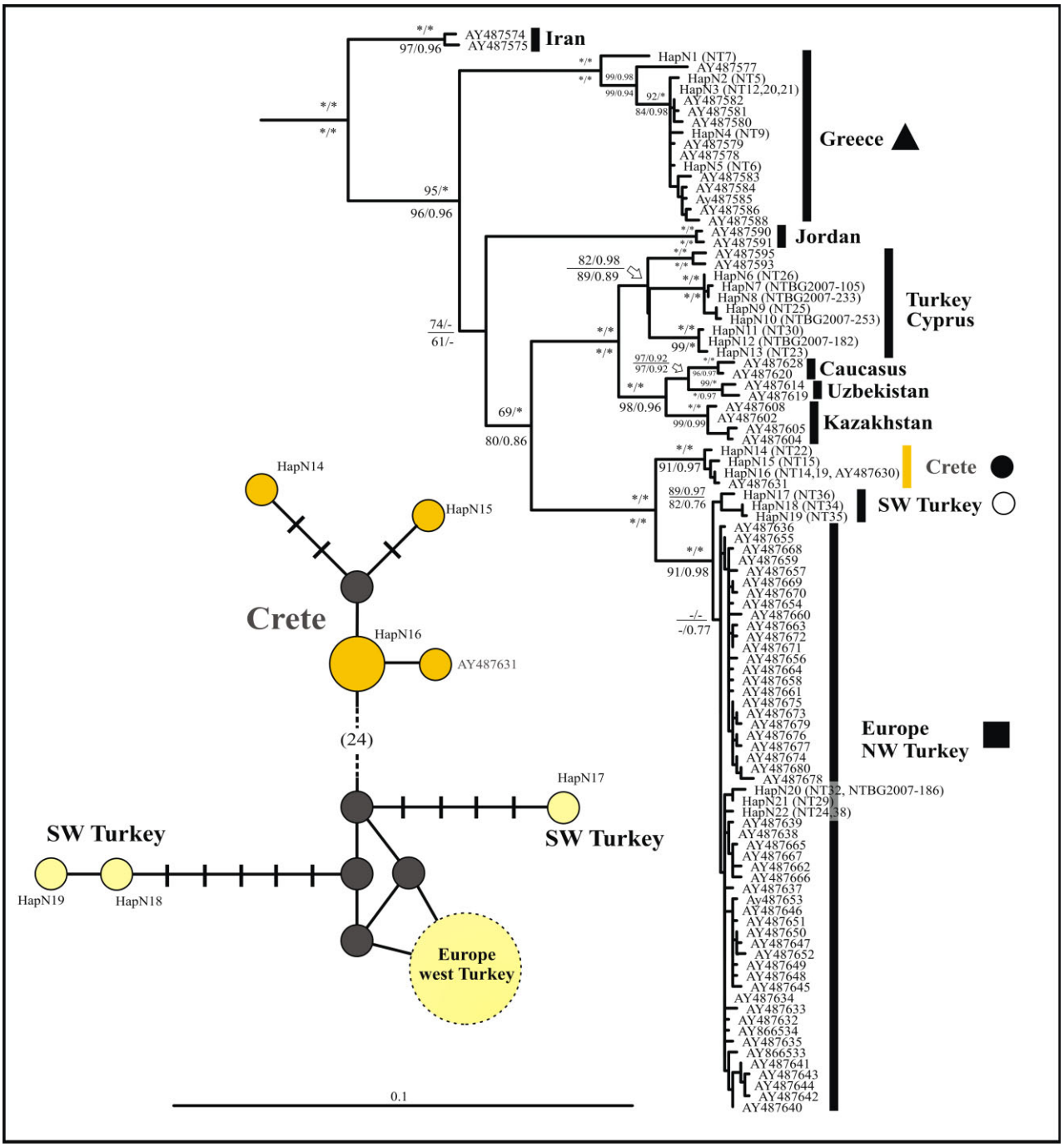

Figure 3 Phylogenetic relationships among *Natrix tessellata* haplotypes/specimens of the present study. *Natrix natrix* and *N. maura* were used as outgroups (not shown). For a description of all information shown in the figure, see Fig. 2(a).

close genetic relationship could represent a very recent dispersal or a human translocation. Our sampling was restricted to the Cretan populations and adjacent regions and, therefore, the overall phylogenetic structure and biogeographical history of *Z. situla* cannot be thoroughly inferred. Nevertheless, we can assume that its diversification occurred at least 0.8 Ma (Fig. 5). Better geographical sampling and analysis of further molecular markers could offer a clearer view of its phylogeography (D. Jandzik *et al.*, work in preparation).

Both the tree and the network (Fig. 2a) present a very close relationship between populations from Crete and the Peloponnese, which differ by very few substitutions. The clades on Crete and the Peloponnese probably diverged very recently, *c.* 0.3 Ma (0.7–0.0 Ma; Fig. 5). However, Crete has been permanently isolated since the Pliocene, with deep-sea barriers that very few animals (mostly large mammals) have managed to cross (Sondaar *et al.*, 1986; Dermitzakis & De Vos, 1987). It seems that *Z. situla* colonized Crete from the

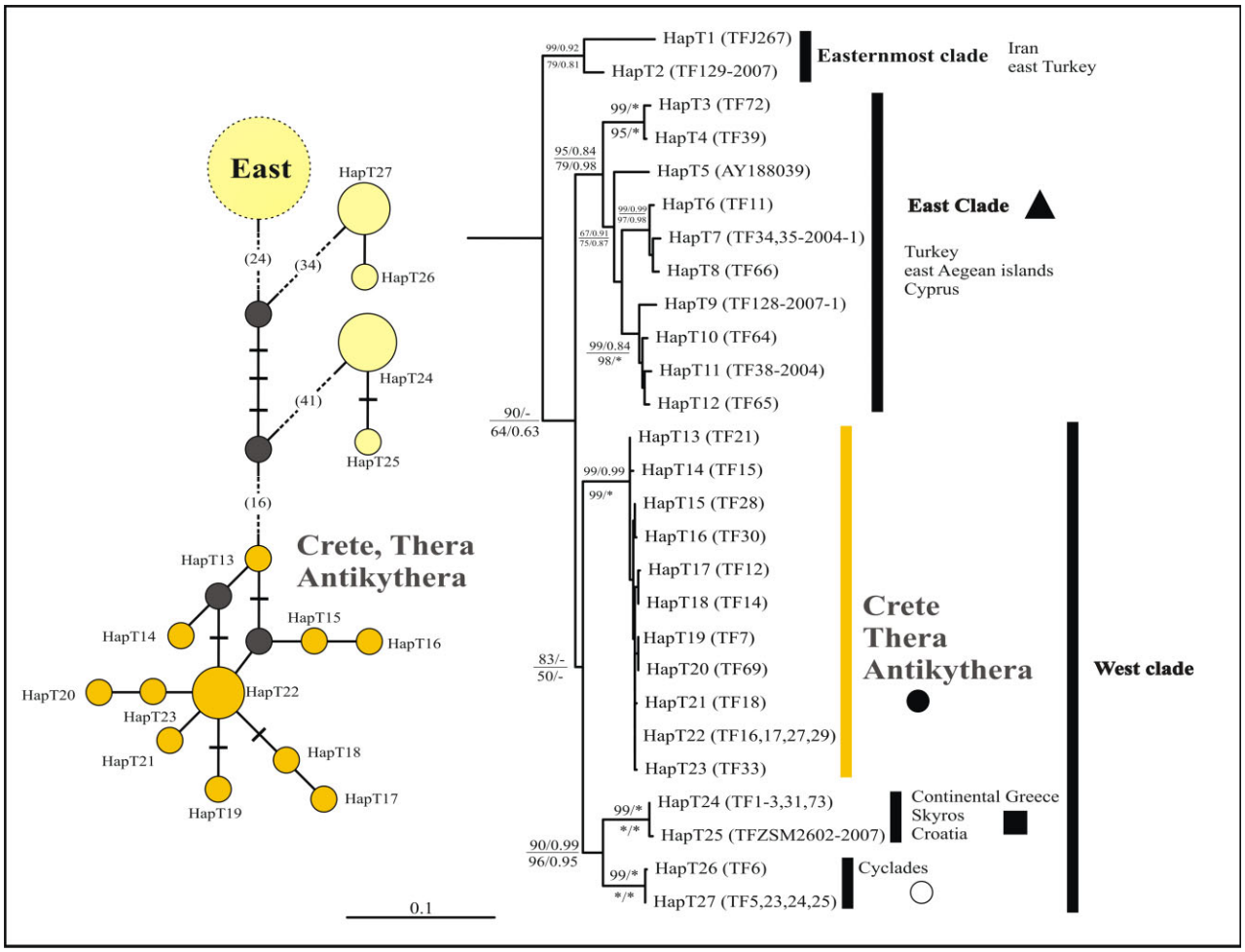

Figure 4 Phylogenetic relationships among *Telescopus fallax* haplotypes/specimens of the present study. *Telescopus tripolitanus* was used as outgroup (not shown). For a description of all information shown in the figure, see Fig. 2(a).

Peloponnese via transmarine dispersal during the Pleistocene or Holocene. The island of Kythera may have acted as a stepping stone for this colonization.

Alternatively, the colonization of Crete could have been anthropogenic. Valakos *et al.* (2004) remarked that the geographical distribution of *Z. situla* coincides with the ancient Greek colonies and that this snake could have been intentionally translocated in the past. This, in combination with our results, could suggest a scenario of a human-induced dispersal from the Peloponnese to Crete. Additionally, we hypothesize a similar situation for the colonization of Thera, with human-mediated dispersal from Crete. In fact, Gruber (1979) proposed that the volcanic eruption of Thera (1650 BC) resulted in species extinctions, with *Z. situla* and *T. fallax* succeeding in recolonizing the island by drifting or human transport.

Hierophis gemonensis

Hierophis gemonensis has a restricted geographical distribution, occurring in the western Balkan Peninsula and adjacent islands, and on Kythera, Antikythera and Crete (Fig. 1). Our study covers the greater part of this distribution, with specimens from the south (continental Greece and the islands of Crete and Kythera) and the north (Croatia). We distinguish two clades that correspond to two groups in the network analysis (Fig. 2b). These two groups differ by eight substitutions, while only one or two substitutions differentiate haplotypes within each group. The molecular-clock analysis suggests a recent diversification of *H. gemonensis*, probably occurring in the early to middle Pleistocene (Fig. 5).

The second clade (Fig. 2b) includes specimens from Crete and Kythera (haplotype HapH3 is shared by individuals from both islands). Diversification between the Crete/Kythera clade and the continental clade is dated at *c.* 0.9 Ma (1.7–0.4 Ma). As with *Z. situla*, *H. gemonensis* may have colonized Crete from the Peloponnese via transmarine dispersal during the early to middle Pleistocene.

Natrix tessellata

Our *N. tessellata* phylogeny confirms the conclusions of Guicking *et al.* (2009), in the identification of major

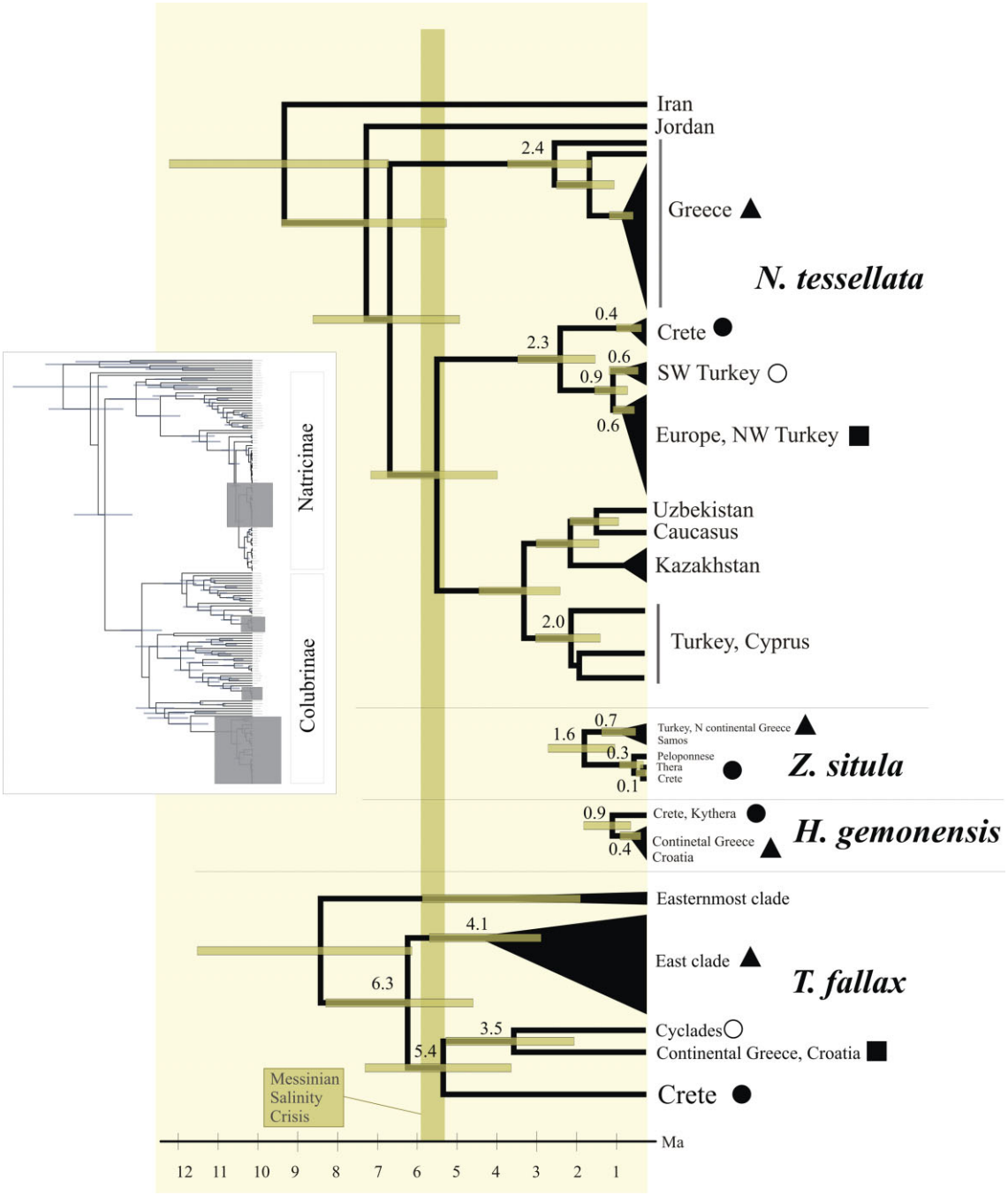

Figure 5 A simplified and collapsed version of the chronogram produced by BEAST, showing phylogenetic relationships and estimated divergence times of lineages of the four studied snake species: *Natrix tessellata, Zamenis situla*, *Hierophis gemonensis* and *Telescopus fallax*. Numbers at the nodes represent mean estimated times in Ma, while bars represent 95% confidence intervals. Clade names correspond to those of Figs 2–4. Clade symbols are the same as those in Fig. 1. The expanded chronogram is shown on the left of the figure.

phylogenetic/geographical groups, relationships and species' origin (Fig. 3). However, new information is revealed with the addition of new sampling localities and the molecular-clock analysis under a Bayesian framework.

In Guicking *et al.* (2009), diversification dates were estimated using a strict molecular clock approach and applying an evolutionary rate of 1.35% sequence divergence per million years (rate inferred in Guicking *et al.*, 2006). Our relaxed molecular clock approach, with an unlinked model of evolution for each codon position and the use of six independent calibration points, shifts the timeframe of the evolutionary history within *N. tessellata*. It seems that the strict-

clock approach (Guicking *et al.*, 2009) underestimated the age of older events, while the two approaches (strict and relaxed) agree when it comes to more recent phylogenetic splits. A similar result was shown for *N. natrix* in a recent study (Fritz *et al.*, 2012). Consequently, the most basal split, separating the Iranian clade from all other accessions, occurred some 9.6 Ma (12.6–6.8 Ma) in the Tortonian, possibly related to a major aridification/cooling climatic event (An *et al.*, 2001; van Dam, 2006), implicated in other phylogeographical studies of reptiles (Kapli *et al.*, 2008; Kornilios *et al.*, 2012). The Jordanian and Greek clades branch off next, indicating possible refugia, but the sequence of these events is not clear because they appear as a polytomy (Fig. 3; Guicking *et al.*, 2009). Nevertheless, the timing of these events is estimated at *c.* 7.4 Ma (9.6–5.3 Ma) and 6.7 Ma (8.8–4.9 Ma), during the late Miocene, although the end of the MSC (5.3 Ma) has been proposed as the agent that isolated Greek populations in the west (Guicking *et al.*, 2009). The estimated dates of these diversification events coincide with those of other studies on reptiles and amphibians in the same area (Weisrock *et al.*, 2001; Kyriazi *et al.*, 2008; Wielstra *et al.*, 2010; Kornilios *et al.*, 2012) and could relate to another major aridification event that occurred in the eastern Mediterranean (Eronen *et al.*, 2009).

All of the new specimens from continental Greece lie within the Greek clade, which includes three monophyletic subclades, revealing a complex phylogeographical structure for *N. tessellata* in continental Greece. Their diversification begins *c.* 2.4 Ma (3.7–1.5 Ma) at the Pliocene/Pleistocene boundary, and continues through the Pleistocene, probably corresponding to the glacial cycles and revealing the existence of subrefugia in the southern Balkan Peninsula. The Pliocene/Pleistocene transition has already been shown to have coincided with major diversification events in several ectothermic Mediterranean species (Gvoždík *et al.*, 2010a,b; Kornilios *et al.*, 2010; and references therein) because major climatic changes from hotter/wetter to colder/drier conditions (Bennett, 1990; Webb & Bartlein, 1992; Willis *et al.*, 1999) resulted in widespread extinctions outside refugia.

A similar pattern to that of continental Greece is observed within eastern Turkey, with the identification of three subclades that began to diverge *c.* 2.0 Ma (2.9–1.2 Ma). The specimen from Cyprus (NTBG2007_253, HapN10) lies within one of these subclades, implying a very recent – possibly human-mediated – dispersal from Turkey to Cyprus for *N. tessellata*, whose presence on Cyprus has been very recently confirmed (Göçmen *et al.*, 2008).

Finally, populations from western Turkey, not previously assessed, are found within the European clade of Guicking *et al.* (2009). Populations from Crete form a sister clade to the Europe + west Turkey clade. The divergence time of these two clades is *c.* 2.3 Ma (3.4–1.4 Ma) at the Pliocene/Pleistocene transition, a time also proposed by Guicking *et al.* (2009). Given that Crete has been isolated from the mainland since the end of the Messinian, the colonization of Crete may have occurred across water, and probably from south-western Turkey based on geographical proximity. This transmarine dispersal would have been possible because at the Pliocene/Pleistocene boundary the sea level was considerably lower due to eustatic movements caused by the earliest glaciations (Zachariasse *et al.*, 1990; Naish, 1997), and because *N. tessellata* is an exceptional swimmer and reasonably tolerant of saline water (Gruschwitz *et al.*, 1999).

Telescopus fallax

The *T. fallax* phylogenetic tree included three major clades, named the 'easternmost clade' (Iran, east Turkey), 'eastern' clade (Turkey, east Aegean islands, Cyprus) and 'western' clade (Croatia, continental Greece, Skyros, central Aegean islands, Crete and surrounding islets) (Fig. 4). Although the monophyly of the eastern clade is strongly supported, support values for the western clade are low. Additionally, the eastern clade shows a complex internal phylogenetic structure. The complex and, to some extent, unresolved phylogeny of *T. fallax* requires a more thorough geographical sampling and analysis of more mitochondrial and nuclear markers (Z.T. Nagy *et al.*, work in preparation). Concerning the phylogeography in the Aegean, the cyt *b* phylogenetic tree (Fig. 4) and the chronogram (Fig. 5) seem to imply an east–west dispersal event, occurring after the formation of the mid-Aegean trench, possibly through a path from Asia Minor to northern Greece, a scenario that needs further investigation.

During the MSC, Crete was probably connected with the Peloponnese with a land bridge that also included Antikythera, but was not connected to the Cyclades due to deep sea barriers. Therefore, *T. fallax* probably colonized Crete from the Peloponnese via that land bridge. It was subsequently isolated in Crete after the end of the MSC, 5.3 Ma. Indeed, the estimated time of divergence between the Cretan clade and the continental Greece/Cyclades clade is 5.4 Ma (7.4–3.6 Ma). Finally, the occurrence of *T. fallax* on Thera is probably the result of human dispersal, similar to that for *Z. situla* (Gruber, 1979). This snake has been intentionally translocated to other islands for religious purposes (Warnecke, 1988), and similar practices continue to this day.

Phylogeographical patterns and conclusions

There are several conclusions to be drawn from comparing the phylogeographical patterns of the four snakes of Crete, taking into consideration the source/direction of colonization, the time of colonization/isolation and the underlying biogeographical mechanisms.

As far as the colonization source is concerned, *Z. situla*, *H. gemonensis* and *T. fallax* probably colonized Crete from the west, i.e. southern continental Greece. On the other hand, *N. tessellata* colonized Crete from the east (Turkey). Animals with relatively restricted dispersal capacities, such as herptiles, may have found it more difficult to overcome the deep and older sea barriers between Crete and Kasos/Karpathos in the east (Fig. 1) or between Crete and the central

Aegean islands in the north, whereas there were opportunities of dispersal routes (transmarine or through land bridges) in the west. Considering the time of colonization and subsequent isolation on Crete, and the biogeogaphical mechanisms responsible for their current presence on the island, three unique biogeographical histories can be identified: recent (Pleistocene) over-sea dispersal from the west, with the possible role of islands as stepping stones, for *H. gemonensis* and *Z. situla* (human-mediated dispersal for *Z. situla* is possible) (pattern A), much older (Pliocene/Pleistocene boundary) over-sea dispersal from the east in *N. tessellata* (pattern B), and natural dispersal through a land bridge from the west and isolation by vicariance at the end of the MSC for *T. fallax* (pattern C). Anthropogenic dispersal has been suggested for three lizard species of Crete – *Tarentola mauritanica* (Harris *et al.*, 2004), *Hemidactylus turcicus* (Carranza & Arnold, 2006) and *Chalcides ocellatus* (Kornilios *et al.*, 2010) – and could also be the case for *Z. situla*. *Hyla arborea* (Stöck *et al.*, 2008) and *Bufo viridis* (Stöck *et al.*, 2006) may also correspond to pattern A. On the other hand, the lacertid *Podarcis cretensis* (Poulakakis *et al.*, 2005a; Lymberakis *et al.*, 2008) and the water frog *Pelophylax cretensis* (Lymberakis *et al.*, 2007) follow pattern C, but for the latter species an even older isolation on Crete has also been proposed (Akın *et al.*, 2010). The biogeographical pattern of *N. tessellata* (pattern B) is unique among the herptiles studied to date, partly comparable only to that of the freshwater terrapin *Mauremys rivulata*, which probably colonized Crete from the east very recently (Mantziou *et al.*, 2004).

Finally, no geographical structure among the identified haplotypes within Crete was found in any of the species examined, implying high levels of gene flow. The palaeogeography of Crete and its surrounding islets does not seem to have left a genetic imprint on the two assumed 'older' inhabitants, *N. tessellata* and *T. fallax*, at least on their mtDNA. The fine-scale genetic structure within Crete could be assessed with other markers, such as microsatellites. So far, the lizard *Podarcis cretensis* is the only Cretan herptile that exhibits a deep geographical structure within Crete. Its diversification into three groups on Crete (two in western Crete and nearby islets and one in the islets of eastern Crete) began *c.* 2.9 Ma, while the animal is absent from central and eastern Crete due to an unidentified extinction event (Poulakakis *et al.*, 2005a). We may hypothesize that this event did not affect Cretan snakes or that they managed to overcome this 'barrier' due to better dispersal abilities.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Samples used in this study.
Appendix S2 GenBank accession numbers and reference of other taxa.
Appendix S3 Chronogram showing a time-calibrated phylogeny of Colubrinae and Natricinae.

BIOSKETCH

The authors' main research interests lie in the fields of ecology, biogeography, genetics, systematics and evolution of amphibians and reptiles.

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