The role of Anatolian refugia in herpetofaunal diversity: an mtDNA analysis of *Typhlops vermicularis* Merrem, 1820 (Squamata, Typhlopidae)

Panagiotis Kornilios1,*,**, Çetin Ilgaz2,*,**, Yusuf Kumluata¸s3, Sinos Giokas1, Stella Fraguedakis-Tsolis1, Basil Chondropoulos1

**Abstract.** Anatolian mountains have played an important role in speciation and definition of biogeographical subregions and have been defined as “hotspots” of biodiversity. Because of its position and its long palaeogeographic and palaeoclimatic history, Anatolia acted in the past as a bridge or as a barrier for species’ dispersal, providing natural pathways or acting as a vicariant agent, respectively. In this study we investigated the phylogeny and biogeography of a small fossorial snake, *Typhlops vermicularis*, in Anatolia, using formalin-preserved specimens and following a special protocol. We inferred phylogenetic relationships using partial 12S and ND2 sequences, and estimated divergence times of major lineages. Our mtDNA analysis revealed a hidden genetic diversity within Anatolian *T. vermicularis*. Four well-supported lineages occur within our sampled populations corresponding to respective refugia, which represent humid areas with dense forest vegetation in high altitude. The remaining populations, from the western and southeastern Anatolia, are almost genetically identical, representing a recent geographic expansion. A distributional disruption and a following allopatric fragmentation for *T. vermicularis* possibly resulted from climatic oscillations that occurred during the Miocene and Pliocene. We propose that extreme and sudden aridification led to distribution shrinkage of *T. vermicularis*, with genetic lineages surviving in refugia.

**Keywords:** aridity, climate change, East Mediterranean, Greek blindsnake, molecular clock, phylogeography, Reptilia, Turkey.

**Introduction**

Phylogeography is the scientific field that studies the geographical distribution of genealogical lineages within a species, or closely related ones (Avise et al., 1987). Phylogenies are reconstructed and plotted geographically to display their spatial relationships and deduce the evolutionary origins and history of populations, subspecies and species (Avise, 2000; Hewitt, 2001). Analyses of intraspecific phylogeographical patterns have led to major advances in our understanding of historical biogeographical processes, with vicariance and dispersal as the most dominant ones (Avise, 2000 and references therein). The association of genetic patterns with environmental variation (Avise, 2000) and the advances in the fields of palaeogeography and palaeoclimatology reveal the past geological and environmental conditions and changes that have affected evolutionary processes (Hewitt, 2004). The use of DNA markers and the concomitant developments in analytical methods have made important biogeographical contributions (Brown, Suárez and Pestano, 2002; Hewitt, 2004).

Anatolia is a western Asia geographic region bounded by the Aegean, the Mediterranean and the Black Sea to the west, south, and north respectively, while to the northeast and the east it is confined by the Caucasus and the Armenian highlands. It is a predominantly mountainous area whose diverse geomorphology produces many different climatic regions and vegetation types. These characteristics and the geomorphology of Anatolia were described by Sin-
daco et al. (2000) in a checklist study of Anatolian herpetofauna. Because of its position and geological history through the Tertiary and Quaternary, Anatolia acted in the past as a bridge or as a barrier for species’ dispersal between Asia, Europe and the Ethiopian region via the Middle East, providing a natural pathway or acting as a vicariant agent (Tchernov, 1992). It has also played an important role as a refugium during the Quaternary ice ages, holding populations during glacial periods that could move out from there during the interglacials, to Europe via Thrace (European part of Turkey) and the Caucasus (see maps in Hewitt, 2001). Repetitive temperature fluctuations during these periods pushed Anatolian populations from south to north and vise versa (Çıplak, 2003). All these features render Anatolia as a biologically diverse region that has played an important role in producing and sustaining animal and plant diversity.

Anatolia, or its mother continent the Aegeid plate, has a long palaeogeographic history, closely related to that of the Tethys and Paratethys Seas. During the Paleocene-Eocene the Aegeid was an island-archipelago almost totally submerged under the Tethys. At the Eocene-Oligocene boundary (approx. 34 Ma), the formation of the Paratethys begun and Anatolia was only connected to Asia, while no connections with Europe or the Middle East occurred. From that point on, major tectonic events led to several connections and disconnections of Anatolia with Europe in the west, Asia in the east and Africa in the south, affecting animals’ geographic distribution and evolution. During certain periods (Aquitanian and Early Burdigalian – approx. 20-23 Ma), Anatolia was an island, while in others (Late Burdigalian – approx. 16 Ma and Early Serravallian – approx. 13.5 Ma), all three continents were connected, rendering Anatolia a bridge between Asia, Europe and the Ethiopian region (data from Rögl, 1999; Koufos, Kostopoulos and Vlachou, 2005).

Beside these major geological events, severe climatic changes must have also played a key role on the evolutionary and biogeographical history of animals inhabiting this area, since climatic oscillations between significantly wetter and drier conditions have produced repeated changes in habitat and periodic modifications of major biota (Rognon, 1993; Anhuf, 2000; Pren- tice and Jolly, 2000; Douady et al., 2003; Schuster et al., 2006).

Due to the limited dispersal capacities and temperature dependence, fossorial reptiles are sensitive indicators of palaeogeographic and palaeoclimatic events, and the study of their genomic markers contributes substantially to revealing biogeographical processes (Lenk et al., 1999). Of the two major divisions of snakes (Alethinophidia and Scolecophidia), scolecophidians are the most poorly known in terms of species diversity, phylogeny, biogeography and ecology (Greene, 1997). They include approximately 400 species, which, due to their fossorial lifestyle, present extreme morphological uniformity and include cryptic genetic lineages that are only revealed by DNA studies (Thomas and Hedges, 2007; Adalsteinsson et al., 2009).

Within scolecophidians, blindsnakes (Typhlopidae, ~240 species) occur in Africa (south of the Sahara desert), Madagascar, southern Asia, southern and central America and Australia, while only one representative, Typhlops vermicularis (Greek blindsnake) is found in southeastern Europe (fig. 1I) (Cox, Chanson and Stuart, 2006; Adalsteinsson et al., 2009; Vidal et al., 2010). The available research studies on T. vermicularis are very few and in fact, according to the IUCN, research actions towards its biology, ecology, population numbers and geographic range are needed.

This study is the first attempt to investigate the phylogeny and biogeography of T. vermicularis. In this context, we aim to infer the phylogenetic relationships of Anatolian T. vermicularis using mitochondrial DNA sequences, in order to evaluate its genetic diversity and assess the role played by the intense palaeogeographic and palaeoclimatic history of the studied area on the Greek blindsnake’s diversification.
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Figure 1. (I) Map showing the geographic distribution of T. vermicularis. (II) The sampling localities of this study and the putative distribution of mtDNA lineages, based on the analyzed samples. Numbers refer to specimen codes given in table 1.

Materials and methods

Samples, DNA extraction, amplification and sequencing

A total of 38 T. vermicularis specimens (collected from 1990 to 2009) were included in this study, while one Typhlops punctatus (Leach, 1819) and one Rhinotyphlops simoni (Boettger, 1879), were analyzed and employed as outgroups. All specimens were preserved in 10% formalin and 70% ethanol, except for the outgroups that were preserved in 95% ethanol. Specimen data and GenBank accession numbers are given in table 1. The sampling localities are shown in fig. III.

The use of formalin and 70% ethanol as a preservative stresses the need for a special treatment of the samples. More specifically, tissue samples (~20 mg) were incubated in 800 μl TE9 buffer (500 mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0) overnight at room temperature with shaking. During the next day, the buffer was removed every 2 h and the samples were again incubated overnight with 800 μl of TE9 buffer. This procedure has been proved to be quite efficient in ethanol removal in the past (Chakraborty, Sakai and Iwatsuki, 2006). Total genomic DNA was extracted according to the manufacturer’s instructions for 6 h, with the addition of an extra proteinase K aliquot after the first 3 h, in order to reinforce the digestion of the molecular complexes between proteins and formalin. The lysate was incubated in 90°C for 1 h, since heating in an alkaline pH solution seems to improve the efficiency of DNA extraction from formalin-fixed tissues by probable breakage of crosslinkages caused by formalin fixation (Shi et al., 2002). DNA extraction was then carried out according to the manual’s instructions.

We were obliged to amplify relatively small segments of mitochondrial DNA (350-400 bp) because of the use of formalin and 70% ethanol and the subsequent DNA degradation. Partial sequences of the 12S rRNA (12S) and the NADH dehydrogenase subunit 2 (ND2) were PCR-amplified using primers 12SaL and 12SbH (Kocher et al., 1989), and rND2-5L and rND2-2L (Amer and Kumazawa, 2005), respectively. Amplification of the target sequences involved an initial cycle of denaturation at 94°C for 3 min, and 35-40 subsequent cycles of 94°C for 1 min (12S) or 30 sec (ND2), 45°C for 1 min (12S) or 44°C for 45 sec (ND2) and 72°C for 1 min, in the presence of 2.0 and 1.5 mM MgCl2 for 12S and ND2, respectively.

PCR products were purified with the NucleoSpin Extract II DNA purification kit (MACHEREY – NAGEL) following the agarose-gel extraction protocol. Single stranded sequencing was conducted on an ABI PRISM 3100 capillary sequencer (VBC Biotech, Austria) using the primers of the amplification procedure.

Alignment and genetic divergence

DNA sequences were aligned using ClustalX v.2.0.12 (Larkin et al., 2007) with default parameters. No gaps were included or postulated in both gene alignments and no stop codons were observed when the ND2 sequences were translated into amino acids using the vertebrate mitochondrial code. The genetic divergences, as percentage of uncorrected p-distance values, between the clades of our phylogeny were estimated in MEGA (v 4.0, Tamura et al., 2007) (table 2).

Phylogenetic analyses

Two different methods of phylogenetic analysis were employed for each gene and the combined dataset: Maximum Likelihood (ML) and Bayesian Inference (BI).

The selection of the most suitable model of DNA substitution was done using Modeltest 3.7 (Posada and CRANDALL, 1998), under the Bayesian Information Criterion (Luo et al., 2010). The best fit model for the dataset comprising all genes, was HKY + G (α = 0.17). The most suitable model for 12S and ND2, was TVM + I (Pinvar = 0.65) and TrN + I (Pinvar = 0.58), respectively. For the ML analysis the nucleotide substitution model of the combined dataset was used, while BI was performed with the model parameters of each gene partition.

Maximum likelihood analysis (Felsenstein, 1981) was conducted in PHYML v.2.4.4. (Guindon and Gascuel, 2003), with the application of the SPR method and the
Table 1. Sample working codes (as in fig. 1), species names, sampling locations (locality/region) and museum numbers (ZDEU: Zoology Department, Ege University; NHMC: Natural History Museum of Crete; MVZ: Museum of Vertebrate Zoology, Berkeley) of the specimens used in the phylogenetic analyses. GenBank accession numbers of sequence data for the segments of 12S/ND2 respectively, are also shown.

<table>
<thead>
<tr>
<th>Working code</th>
<th>Species</th>
<th>Locality (District)</th>
<th>GenBank accession Nos:</th>
<th>Museum numbers</th>
</tr>
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<td>60</td>
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<td>Uzuncaburç/Silifke/Mersin</td>
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<td>ZDEU 27A</td>
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<td>T. vermicularis</td>
<td>Tire/Izmir</td>
<td>HQ113856/HQ113898</td>
<td>ZDEU 49A</td>
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<td>T. vermicularis</td>
<td>Örnekköy/Izmir</td>
<td>HQ113857/HQ113899</td>
<td>ZDEU 33F</td>
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<td>T. vermicularis</td>
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<td>HQ113858/HQ113900</td>
<td>ZDEU 33C</td>
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<td>T. vermicularis</td>
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<td>ZDEU 80B</td>
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<td>T. vermicularis</td>
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<td>HQ113860/HQ113902</td>
<td>ZDEU 77A</td>
</tr>
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<td>HQ113861/HQ113903</td>
<td>ZDEU 77B</td>
</tr>
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<td>Yamanlar/Izmir</td>
<td>HQ113862/HQ113904</td>
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<td>T. vermicularis</td>
<td>Çanakkale</td>
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<td>ZDEU C46/2008-1</td>
</tr>
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<td>T. vermicularis</td>
<td>Bozcaada island/Çanakkale</td>
<td>HQ113864/HQ113906</td>
<td>ZDEU C22/2008-1</td>
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<td>T. vermicularis</td>
<td>Tersane Cove, Kekova island, Kas/Antalya</td>
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<td>ZDEU D3/2009-1</td>
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<td>HQ113866/HQ113907</td>
<td>ZDEU D1/2009-1</td>
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<td>99</td>
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<td>Emiralen/Izmir</td>
<td>HQ113867/HQ113908</td>
<td>ZDEU D5/2009-1</td>
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<td>102</td>
<td>T. vermicularis</td>
<td>Kale, Kas/Antalya</td>
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<td>ZDEU D2/2009-1</td>
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<td>Çığır Village/Şırmak</td>
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<td>ZDEU C15/2008-1</td>
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<td>ZDEU D4/2009-1</td>
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<td>ZDEU 62A</td>
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<td>HQ113874/HQ113914</td>
<td>ZDEU 62B</td>
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<td>T. vermicularis</td>
<td>Siirt</td>
<td>HQ113875/HQ113915</td>
<td>ZDEU 65A</td>
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<td>151</td>
<td>T. vermicularis</td>
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<td>HQ113876/HQ113916</td>
<td>ZDEU 55A</td>
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<td>152</td>
<td>T. vermicularis</td>
<td>16 km NW of Birecik/Şanlıurfa</td>
<td>HQ113877/HQ113917</td>
<td>ZDEU 57A</td>
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<td>155</td>
<td>T. vermicularis</td>
<td>4 km E of Polateli/Kilis</td>
<td>HQ113878/HQ113918</td>
<td>ZDEU 70A</td>
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<td>159</td>
<td>T. vermicularis</td>
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<td>HQ113879/HQ113919</td>
<td>ZDEU 72B</td>
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<td>T. vermicularis</td>
<td>Örnekköy/Izmir</td>
<td>HQ113880/HQ113920</td>
<td>ZDEU 33B</td>
</tr>
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<td>201</td>
<td>T. vermicularis</td>
<td>32 km NE of Şanlıurfa</td>
<td>HQ113881/HQ113921</td>
<td>ZDEU 60A</td>
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<td>T. vermicularis</td>
<td>Bağpınar/Adıyaman</td>
<td>HQ113882/HQ113922</td>
<td>ZDEU 45A</td>
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<td>205</td>
<td>T. vermicularis</td>
<td>EskiSavaşan Village / Hafıteyi/Şanlıurfa</td>
<td>HQ113883/HQ113923</td>
<td>ZDEU 68A</td>
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<td>T. vermicularis</td>
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<td>ZDEU 59C</td>
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<td>218</td>
<td>T. vermicularis</td>
<td>Kemaliye Village, Kilis</td>
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<td>ZDEU 56A</td>
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<td>221</td>
<td>T. vermicularis</td>
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<td>ZDEU 46B</td>
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<td>T. vermicularis</td>
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<td>HQ113887/HQ113927</td>
<td>ZDEU 63A</td>
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<td>224</td>
<td>T. vermicularis</td>
<td>48 km W of Diyarbakir</td>
<td>HQ113888/HQ113928</td>
<td>ZDEU 63B</td>
</tr>
<tr>
<td>226</td>
<td>T. vermicularis</td>
<td>48 km W of Diyarbakir</td>
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<td>ZDEU C46/2008-2</td>
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<td>234</td>
<td>T. vermicularis</td>
<td>Around Karaköy/Çanakkale</td>
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<td>237</td>
<td>T. punctatus</td>
<td>Kirazli/Çanakkale</td>
<td>HQ113892/HQ113932</td>
<td>ZDEU C45/2007-4</td>
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<td></td>
<td>Rhinotyphlops simoni</td>
<td>4 km S of Al Mazar, al Janubi (Jordan)</td>
<td>HQ113894/HQ113934</td>
<td>NHMC80.3.21.8</td>
</tr>
</tbody>
</table>

model parameters fitted to the data by likelihood maximization. Statistical reliability was based on 1000 bootstrap replicates (Felsenstein, 1985).

The Bayesian analysis was performed in MrBayes (v3.1; Ronquist and Huelsenbeck, 2003). Four incrementally heated Markov chains, with the default heating values, were used for $2 \times 10^6$ generations. The current tree was saved to a file every 100 generations. After verifying that stationarity had been reached, both in terms of likelihood scores and parameter estimation, and using TRACER v1.5.0 (http://tree.bio.ed.ac.uk/software/tracer/) (Rambaut and Drummond, 2004), the first $2 \times 10^3$ trees (10% “burn-in” in Bayesian terms) were discarded, and a majority-rule consensus tree was generated from the remaining $1.8 \times 10^4$
Table 2. Mean p-distance genetic divergences (%) among the main mtDNA Clades of *T. vermicularis* for ND2 (above diagonal) and 12S (below diagonal). Values in diagonal are genetic divergences within each clade (ND2/12S).

<table>
<thead>
<tr>
<th>Main clades</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade A</td>
<td>0.1/0.0</td>
<td>5.8</td>
<td>6.8</td>
<td>7.6</td>
<td>7.7</td>
</tr>
<tr>
<td>Clade B</td>
<td>2.3</td>
<td>0.1/0.1</td>
<td>3.8</td>
<td>4.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Clade C</td>
<td>2.7</td>
<td>2.5</td>
<td>0.8/0.0</td>
<td>4.2</td>
<td>4.1</td>
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<tr>
<td>Clade D</td>
<td>2.2</td>
<td>2.0</td>
<td>2.0</td>
<td>0.9/0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Clade E</td>
<td>2.5</td>
<td>2.3</td>
<td>1.7</td>
<td>0.6</td>
<td>0.2/0.2</td>
</tr>
</tbody>
</table>

(post-burnin) trees. During tree search, full parameter estimation was performed, and the posterior probabilities (pp) were calculated as the percentage of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001), where pp ≥ 0.95 indicate significant support (Wilcox et al., 2002). Three 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.

A reciprocal 70% bootstrap proportion or a 0.95 pp threshold (Mason-Gamer and Kellogg, 1996) was used to test topological incongruence among partitions. Topological conflicts are considered significant if two different relationships for the same set of taxa were both supported with bootstrap values ≥ 70% or pp values ≥ 0.95. No such conflicts were observed in our analyses. Topological incongruence among partitions was also tested using the incongruence length difference (ILD) test (Michkevich and Farris, 1981; Farris et al., 1994), with 10,000 heuristic searches after removing all invariable characters from the data set (Cunningham, 1997). The two gene partitions (12S and ND2) were shown to be congruent (P = 0.80).

The genealogical relationships between groups were assessed with haplotype networks constructed using statistical parsimony (Templeton et al., 1992), implemented in the program TCS v1.21 (Clement et al., 2000), with a connection limit of 95%.

**Estimation of divergence times**

To estimate divergence times of *T. vermicularis* lineages, we first examined if the sequences of our ND2 dataset were evolving in a clocklike manner. To do so, we conducted a likelihood ratio test (LRT), comparing the log likelihood value of the obtained ML tree with that of a tree constructed from the same data under an assumption of regular change (Felsenstein, 1981). The LRT was positive for the hypothesis of a clocklike evolution of the analyzed sequences (LRT = 2 × [2436.89 − 2426.06] = 21.66, df = 38, α = 0.05, xcritical = 53.38). As it is not presently possible to calibrate clocks internally for Anatolian *T. vermicularis*, we employed evolutionary rates estimated for the ND2 genetic marker in other studies on ectotherm animal families (Agamidae, Anguidae, Gekkonidae, Lacertidae, Scincidae, Salamandridae, Bufonidae). More specifically, the rate for the ND2 marker used in our study, was estimated to be 0.57-0.70% per lineage per million years (Macey et al., 1998a, 1998b, 1999; Weisrock et al., 2001; Strasburg and Kearny, 2005; Brown et al., 2008; Liggins et al., 2008; Uruquhart, Wang and Fu, 2009; Gvoždík et al., 2010a). Divergence times were estimated using a Bayesian coalescence approach, as implemented in BEAST 1.6.0 (Drummond and Rambaut, 2007). We employed the Yule tree prior and the TrN + I model of substitution, as proposed by Modell for ND2. The prior for the mutation rate was specified as a uniform distribution, with a maximum value of 0.0057 and a minimum value of 0.0070. The search was started with an UPGMA tree. Five independent runs of 10^7 generations were conducted. The results were checked for convergence and stationarity of the different runs using Tracer 1.5.0 and combined with the BEAST module LogCombiner 1.6.0, after discarding a burn-in of 10^6 generations from each analysis.

**Results**

The combined data set, for the 38 individuals of Anatolian *T. vermicularis*, consisted of 747 bps of sequence (389 bps of 12S and 358 bps of ND2). The variable sites were 48 ($13.4\%$) for ND2 and 25 ($6.4\%$) for 12S, while the parsimony informative ones were 42 ($11.7\%$) and 18 ($4.6\%$) respectively.

Sequence divergence within the ingroup ranged from 0 to 3.4% for 12S, 0 to 8.4% for ND2 and 0 to 6.1% for the combined dataset. The values of genetic divergence, within and between defined units of *T. vermicularis*, are shown in table 2.

Both phylogenetic analyses (ML and BI) of each of the studied genes and the combined dataset gave very similar results, distinguishing the same monophyletic groups, and showed only minor differences, mainly concerning relationships between these groups and their respective support values. The phylogenetic tree of the ML analysis of the combined dataset is shown in fig. 2.

As shown in this figure, Anatolian populations of *T. vermicularis* form 5 major clades. These clades are well supported by the ML bootstrap values (the smallest value is 82, fig. 2) but are not that strongly supported by the BI posterior probabilities – values for clades A, B and D are 0.94, 0.90 and 0.92, respectively. On the other hand, the interrelationships among
Figure 2. Phylogenetic relationships among the *T. vermicularis* specimens of the present study, as produced from the combined dataset. Only the Maximum Likelihood (ML) phylogenetic tree is presented, while Bayesian Inference (BI) produced a tree with the same topology with regard to the major lineages. *T. punctatus* and *R. simoni* individuals were used as outgroups (not shown). Numbers in terminal nodes refer to the specimen numbers presented in table 1 and fig. 1III. Numbers above branches are ML bootstrap values, while numbers below branches are BI posterior probabilities. Only bootstrap values ≥ 50 and pp ≥ 0.50 are shown. Divergence times (in My) and their 95% intervals are indicated by arrows.

Major diversification events seem to have occurred during the Late Miocene (mean: 6.2 Mya, 95% intervals: 4.2-8.4 Mya), the Miocene/Pliocene boundary (mean: 4.5, 95% intervals: 2.9-6.2 Mya/mean: 3.8, 95% intervals: 2.3-5.4 Mya), the Pliocene/Pleistocene transition (mean: 2.5, 95% intervals: 1.4-3.5 Mya) and during the Pleistocene (mean: 1.2, 95% intervals: 0.7-1.8 Mya), as shown in fig. 2.

Finally, four independent networks, shown in fig. 3, were inferred based on the connection limit of 95%.

these clades are rather ambiguous, showing a polytomy. Clade A comprises specimens from the southwestern part of Turkey (support: 84 ML bootstrap values and 0.94 BI pp values). Clade B consists of specimens from the Nur or Amanos Mountain ridge (82 and 0.90). Clade C includes specimens from the Mersin Province (96 and 0.99). Clade D consists of specimens from the Şırnak Province (82 and 0.92). Finally, Clade E includes specimens from the northwestern and southeastern parts of Anatolia (88 and 0.95).
Figure 3. Parsimony networks corresponding to 12S/ND2 sequence variation calculated with TCS with a 95% connection limit. Lines represent a mutational step, black circles missing haplotypes and open circles haplotypes. The circle area is proportional to the number of individuals. Dashed lines represent probable ancestral haplotypes. For correspondences of sample codes and locations see table 1 and fig. 1. (A) Clade A – southwestern Anatolia, (B) Clade B – Nur Mountain ridge, (C) Clade C – southern parts of the central Taurus Mountains, (D) Clades D and E – western and eastern Anatolia.

Discussion

Anatolian refugia

Our mtDNA analysis revealed four well-supported lineages within our sampled populations that correspond to respective refugia within this area (fig. III). All four refugia represent humid areas with high altitude and dense forest coverage.

More specifically, southwest Anatolia is a mountainous and densely forested area that reaches altitudes of 3000 m, and represents the western edge of the Taurus Mountain ridge. This region corresponds to Clade A of our phylogenetic tree (fig. III). Southeast Anatolia must have acted as a “biodiversity pocket” for *T. vermicularis*, due to its geomorphological and ecological characteristics. Several reptiles with restricted geographic distribution in Anatolia seem to expand in the same area. Such cases are *Anatololacerta oertzeni* (Werner, 1904), *Anatololacerta danfordi* (Günther, 1876), *Vipera anatolica* (Eiselt and Baran, 1970), *Ophiomorus punctatissimus* (Bibron and Bory, 1833) and *Zamenis hohenackeri* (Strauch, 1873).

Clade B corresponds to the Nur Mountain ridge. The Nur Mountains represent another forested and humid area that reaches an elevation of 2300 m and coincide with the geographic distribution regions of several Anatolian snakes and lizards, e.g. *Eirenis barani* Schmidtler, 1988, *Phoenicolacerta cyanisparsa* (Schmidtler and Bischoff, 1999), *Zamenis hohenackeri* (Strauch, 1873), and also the typhlopid *Rhinotyphlops episcopus* Franzen and Wallach, 2002.

Clade C corresponds to the Mersin Province, situated at the southern parts of the central Taurus Mountains with a highest peak of 3500 m. *Eirenis aurolineatus* (Venzmer, 1919), *Lacerta pamphylica* Schmidtler, 1975 and *Zamenis hohenackeri* (Strauch, 1873) are reptiles that are geographically distributed in this region.

The last refugium, according to our results (Clade D), is the Şırnak Province at the eastern part of Anatolia. On the other hand, populations from the western part and the southeastern parts of Anatolia are almost genetically identical, forming Clade E, which most probably represents a more recent geographic expansion of the Greek blindsnake. This recent expansion scenario is also supported by the star-like topology of the haplotype network corresponding to this Clade (fig. 3D). As seen in this figure, there is no geographic pattern within this network, and even the presumable ancestral haplotype includes specimens both from the eastern and the western parts of Anatolia.

The genetic divergence values within each of the Clades of our phylogenetic analysis are very low, especially compared to the ones between...
them (table 2). This could be explained either by high levels of gene flow between the respective populations and/or the occurrence of a genetic bottleneck or even founder effects, although one cannot exclude restricted sampling as another possible explanation. On the other hand, the high values of genetic divergence between the main clades, especially between Clade A and the others probably indicate restricted gene flow between them due to physical or ecological barriers.

It seems that several vicariant events may have occurred in Anatolia diminishing formerly larger geographic distributions across this region, and isolating populations in several areas. This distributional disruption and the following allopatric fragmentation possibly resulted from climatic oscillations that occurred during the Miocene, Pliocene and Pleistocene, at least in the case of *T. vermicularis*. Evolutionary lineages could have originated within populations in habitat refugia during that time, as shown in several studies of Anatolian and the Mediterranean reptiles and amphibians (Plötner et al., 2001; Weisrock et al., 2001; Fritz et al., 2007; Kyriazi et al., 2008; Akın et al., 2010; Gvoždík et al., 2010a, 2010b; Kornilios et al., 2010; Wielstra et al., 2010).

Although several phylogenetic studies have been conducted so far for Anatolian amphibians and reptiles that include two or more morphologically recognized species, the respective studies that focus on the intraspecific level are very few. The occurrence of cryptic genetic lineages within reptilian or amphibian species that have a continuous geographic distribution in Anatolia is confirmed in some of them. *Ophisops elegans* shows four distinct genetic lineages in Anatolia (Kyriazi et al., 2008). The Anatolian water frogs exhibit a similar pattern with various lineages (Plötner et al., 2001; Akın et al., 2010). Fritz et al. (2007) revealed the existence of several mtDNA varieties of *Testudo graeca* in Anatolia that were assigned to the specific level, some of which were also confirmed by a thorough morphological approach (Türkozan et al., 2010).

Anatolian mountains, especially in the south, have played an important role in speciation and definition of biogeographical subregions. These mountains have been defined as “hotspots” of biodiversity for many different organisms (Çiçek, 2003, 2004 and references therein). In fact, Çiçek (2003) in a study of Anatolian Orthoptera, proposes that a special conservation perspective needs to be developed for these mountainous habitats, since each mountain or range (especially in southern Anatolia) harbours endemic species and important subspecific genetic diversity. Anatolia has been defined as an important endemism area for animals and plants (Çiçek, 2004 and references therein). However, there are still unknown aspects of Anatolian biogeography, especially in terms of the geological and climatic history of this area but also in terms of its plant and animal biodiversity (Çiçek, 2004).

**Molecular dating and diversification events**

Populations from southwest Anatolia (Clade A) diverged from the other Anatolian *T. vermicularis* in the Late Miocene (mean: 6.2 Mya, 95% intervals: 4.2–8.4 Mya) (fig. 2). Palaeontological data from Spain, Italy, Sicily, Greece and Cyprus suggest that a major isochronous palaeoenvironmental change at 6.8–6.7 Ma affected the entire Mediterranean (Krijgsman et al., 2002). It is believed that during the Tortonian-Messinian transition, there was an increase in summer drought, while during the Messinian, high evaporation, low rainfall and lower seasonality, because of increased duration of summer aridity, is reported, resulting in more arid-adapted faunas in the eastern Mediterranean (Eronen et al., 2009 and references therein). This time-period coincides with several faunal episodes, such as the drastic decline of the middle Turonian large mammal fauna known as the “Pikermian” event, which occurred around 6.9 Mya (Kostopoulos, 2009) and seems to be correlative with the
early Messinian glaciation (7.0-6.9 My), which marked the worldwide expansion of C4 ecosystems (Hodell et al., 1994; Quade, Solounias and Cerling, 1994; Cerling et al., 1997; Barry et al., 2002; Krijgsman et al., 2002; Strömberg et al., 2007 and references therein).

Phylogenetic studies of herpetofauna with adequate sampling in Anatolia are scarce. Kyriazì et al. (2008) showed that the snake-eyed lizard’s major diversification took place in the eastern Mediterranean some 7.65-7 Ma, almost simultaneously. The authors correlate this radiation to the late Miocene aridification. Additionally, the six major lineages identified within Lyctiasalamandra luschani (Steindachner, 1891), distributed in southwest Anatolia, seem to have diverged approximately between 5.9 and 7.9 Ma (Weisrock et al., 2001), while the basal split of the mtDNA clades in the Triturus karelinii group is placed around 7 Ma (Wielstra et al., 2010). In the latter two studies, the researchers attribute these vicariant events to tectonic activity and orogenic isolation caused by the Arabia-Eurasia collision (5-10 Ma) (Weisrock et al., 2001; Wielstra et al., 2010). This could also be the case for other amphibian and reptilian taxa, including T. vermicularis, but since amphibians are also humidity-depended, the Late Miocene aridification-scenario is also quite plausible.

Clades B and C seem to have diverged roughly during the Miocene/Pliocene boundary (mean: 4.5, 95% intervals: 2.9-6.2 Mya/mean: 3.8, 95% intervals: 2.3-5.4 Mya) (fig. 2). In the Late Miocene, significant global climatic changes from wetter to more arid environments occurred (Fortelius et al., 2002, 2006; Janis, 1993). Aridification of the Asian inland during the Late Miocene has been shown (e.g. Guo et al., 2004), while in Europe, it is referred to as the Messinian salinity crisis (e.g. Hsü et al., 1977; Krijgsman et al., 1999). In several other studies, a global cooling and drying trend around the Miocene/Pliocene boundary has also been well-documented (e.g. García-Alix et al., 2008). This climatic change, and the following vegetation and habitat turnover (Cerling et al., 1997), may be one of the most important reasons for a wave of species radiation (He et al., 2010 and references therein) and also responsible for the split of the central and western clades in the T. karelinii group is (Wielstra et al., 2010) and the species radiations within Anguis (Gvoždík et al., 2010a) that occurred roughly in the same area.

This time-period of divergence of Clade D dates back to the Pliocene/Pleistocene transition (mean: 2.5, 95% intervals: 1.4-3.5 Mya), during which, worldwide major climatic changes also occurred. These were followed by widespread extinctions, except within refugia where climate remained within tolerance-limits for a species (Hewitt, 1996, 2000). The hotter and wetter climate of Late Pliocene (3.6-2.5 Ma) (Willis, Kleczkowski and Crowhurst, 1999) suddenly became colder and drier during the Early Pleistocene (2.5-1.8 Ma) (Webb and Bartlein, 1992), inducing fragmentation of plant and animal populations (Bennett, 1990). This transition has already been shown to coincide with major diversification events in several animal species (Kornilios et al., 2010 and references therein). Roughly in the same study area, the estimated time of major intraspecific splits within Anguis colchica (Gvoždík et al., 2010a) and Hyla savageyi and H. felixarabica (Gvoždík et al., 2010b) coincide with this climate transition.

The diversification of Clade E seems to occur during the Pleistocene (mean: 1.2, 95% intervals: 0.7-1.8 Mya), probably relating to the oscillating glacial cycles. The climate of the studied area included warm/humid interglacial periods and cold/dry glacial ones (Joannis et al., 2010 and references therein). The last four glacial periods of the Pleistocene probably had the greatest impact on faunal composition in Anatolia and related areas (Çiplak, 2004). Typhlops vermicularis’ biogeography and divergence were most probably affected by the glacial periods, during which aridity increased dramatically, since warm and humid interglacials probably had a positive effect on its life cycle. Human-induced dispersal is also a
quite plausible scenario for Clade E. Six major pathways have been described for the introduction of most reptile and amphibian species: intentional pathways include introductions for biocontrol, food use, the pet trade, and personal release of animals for aesthetic self-indulgence, while accidental pathways include transport as aquacultural contaminants and cargo stowaways (Kraus, 2003). Hitch-hikers in nursery plant materials is a subcategory of cargo stowaways. If human-mediated dispersal is the case for *T. vermicularis*, we can hypothesize that its fossorial lifestyle could render it a cargo stowaway or a hitch-hiker in nursery plant material, as has been shown in other cases of typhlopids in roughly the same study area (Afroosheh et al., 2010). This is not surprising given that human presence and commercial activities have occurred for a long time in the Mediterranean area, affecting animals’ geographic distribution (Kornilios et al., 2010 and references therein).

The estimated diversification dates and the fact that the discovered refugia represent humid areas of high altitude, lead us to the assumption that extreme aridity and sudden aridification are factors that act negatively on the reproduction and survival of the Greek blindsnake. *Typhlops vermicularis* is a strictly fossorial snake associated with moist, sparsely vegetated, open areas and it can only be found under stones in grassy fields and slopes during April and May (Cox et al., 2006; personal observations of the authors). It shows aggregation behaviour either for mating or moisture conservation (Cox et al., 2006). During summer it retreats deeper in the ground to avoid heat and ground-surface aridity. Additionally, its distribution is restricted to mesic habitats and is not found in arid or semi-arid ones (fig. 1). Although humidity, and consequently aridity, seem to play an important role on its life-cycle, low temperatures do not seem to affect it, at least in the long term and compared to other reptilian taxa, as *T. vermicularis* occurs in regions of high altitude (1700 m) and/or latitude, where low temperature and heavy snow-coverage are present for a significant period of time throughout the year.

Although with limited sampling, the present study has uncovered genetic divergences within *T. vermicularis*, but further sampling of this species is needed, not only from Anatolia (especially the northern parts) but also throughout the animal’s whole geographic distribution, in order to reveal additional evolutionary lineages. Moreover, our results cannot be used to propose a biogeographic scenario, since unsampled lineages may affect the results of the phylogenetic analyses and divergence-time estimation, and better geographic representation could explain the Greek blindsnake’s present distribution and diversification.

Finally, mitochondrial DNA markers, that have traditionally been used in phylogenetic and phylogeographic studies (Avise, 2000), might not be sufficient, so that the mtDNA phylogenetic tree alone might not reflect the true evolutionary history of the species (Zhang and Hewitt, 2003; Ballard and Whitlock, 2004). Therefore, independent nuclear markers should also be analyzed to better address the phylogeny of *T. vermicularis*.

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