Multilocus phylogeny and coalescent species delimitation in Kotschy's gecko, *Mediodactylus kotschyi*: Hidden diversity and cryptic species

Panayiota Kotsakiozi[a,b], Daniel Jablonski[c], Çetin Ilgaz[d], Yusuf Kumlutaş[d], Aziz Avcı[e], Shai Meiri[f,g], Yuval Itescu[f,g], Oleg Kukushkin[h,i], Václav Gvoždík[j,k], Giovanni Scillitani[l], Stephanos A. Roussos[m,n], David Jandzik[c,o], Panagiotis Kasapidis[p], Petros Lymberakis[a], Nikos Poulakakis[a,b,*]

[a] *Natural History Museum of Crete, School of Sciences and Engineering, University of Crete, Knosos Avenue, Irakleio 71409, Greece*
[b] *Department of Biology, School of Sciences and Engineering, University of Crete, Vassilika Vouton, Irakleio 70013, Greece*
[c] *Department of Zoology, Comenius University in Bratislava, Mlynská dolina, Ilkovičova 6, 84215 Bratislava, Slovakia*
[d] *Dokuz Eylül University, Faculty of Science, Department of Biology, 35160 Buca/İzmir, Turkey*
[e] *Adnan Menderes University, Faculty of Science and Arts, Department of Biology, 09010 Aydın, Turkey*
[f] *School of Zoology, Tel Aviv University, 6997801 Tel Aviv, Israel*
[g] *The Steinhardt Museum of Natural History, Tel Aviv University, 6997801 Tel Aviv, Israel*
[h] *Department of Biodiversity Studies and Ecological Monitoring, T.I. Vyazemski Karadagh Research Station – Nature Reserve of Russian Academy of Sciences, Nauki Srt. 24, stm. Kurortnoe, Theodosia 298188, Republic of the Crimea, Russia*
[i] *Department of Herpetology, Institute of Zoology of Russian Academy of Sciences, Universitetskaya Emb. 1, Saint Petersburg 199034, Russia*
[j] *Institute of Vertebrate Biology of the Czech Academy of Sciences, 603 65 Brno, Czech Republic*
[k] *National Museum, Department of Zoology, 193 00 Prague, Czech Republic*
[l] *Department of Biology, Section of Animal and Environmental Biology, Laboratory of Histology and Comparative Anatomy, University of Bari Aldo Moro, via Orabona 4/a, Bari, Italy*
[m] *Department of Biological Sciences, Texas Tech University, MS 43131, Lubbock, TX, 79409-3131, USA*
[n] *Department of Biological Sciences, University of North Texas, Union Circle 1155, 76203 Denton, TX, USA*
[o] *Department of Zoology, Charles University in Prague, Viničná 7, Prague, 12844 Czech Republic*
[p] *Hellenic Centre for Marine Research, Institute of Marine Biology, Biotechnology and Aquaculture, P.O. Box 2214, 71003 Irakleio, Greece*

A R T I C L E   I N F O

A B S T R A C T

Kotschy's Gecko, *Mediodactylus kotschyi*, is a small gecko native to southeastern Europe and the Levant. It displays great morphological variation with a large number of morphologically recognized subspecies. However, it has been suggested that it constitutes a species complex of several yet unrecognized species. In this study, we used multilocus sequence data (three mitochondrial and three nuclear gene fragments) to estimate the phylogenetic relationships of 174 specimens from 129 sampling localities, covering a substantial part of the distribution range of the species. Our results revealed high genetic diversity of *M. kotschyi* populations and contributed to our knowledge about the phylogenetic relationships and the estimation of the divergence times between them. Diversification within *M. kotschyi* began approximately 15 million years ago (Mya) in the Middle Miocene, whereas the diversification within most of the major clades have been occurred in the last 5 Mya. Species delimitation analysis suggests there exists five species within the complex, and we propose to tentatively recognize the following taxa as full species: *M. kotschyi* (mainland Balkans, most of Aegean islands, and Italy), *M. orientalis* (Levant, Cyprus, southern Anatolia, and south-eastern Aegean islands), *M. danilewskii* (Black Sea region and south-western Anatolia), *M. bartoni* (Crete), and *M. oertzeni* (southern Dodecanese Islands). This newly recognized diversity underlines the complex biogeographical history of the Eastern Mediterranean region.

1. Introduction

The Palearctic "naked-toed geckos" include ~100 species distributed from North Africa across the southern Balkans and southwestern and central Asia to northern India, western China, and southern Mongolia (Agarwal et al., 2014; Bauer et al., 2013). The taxonomy of these geckos has been unstable and remains unresolved, because qualitative characters have been used for generic delimitation
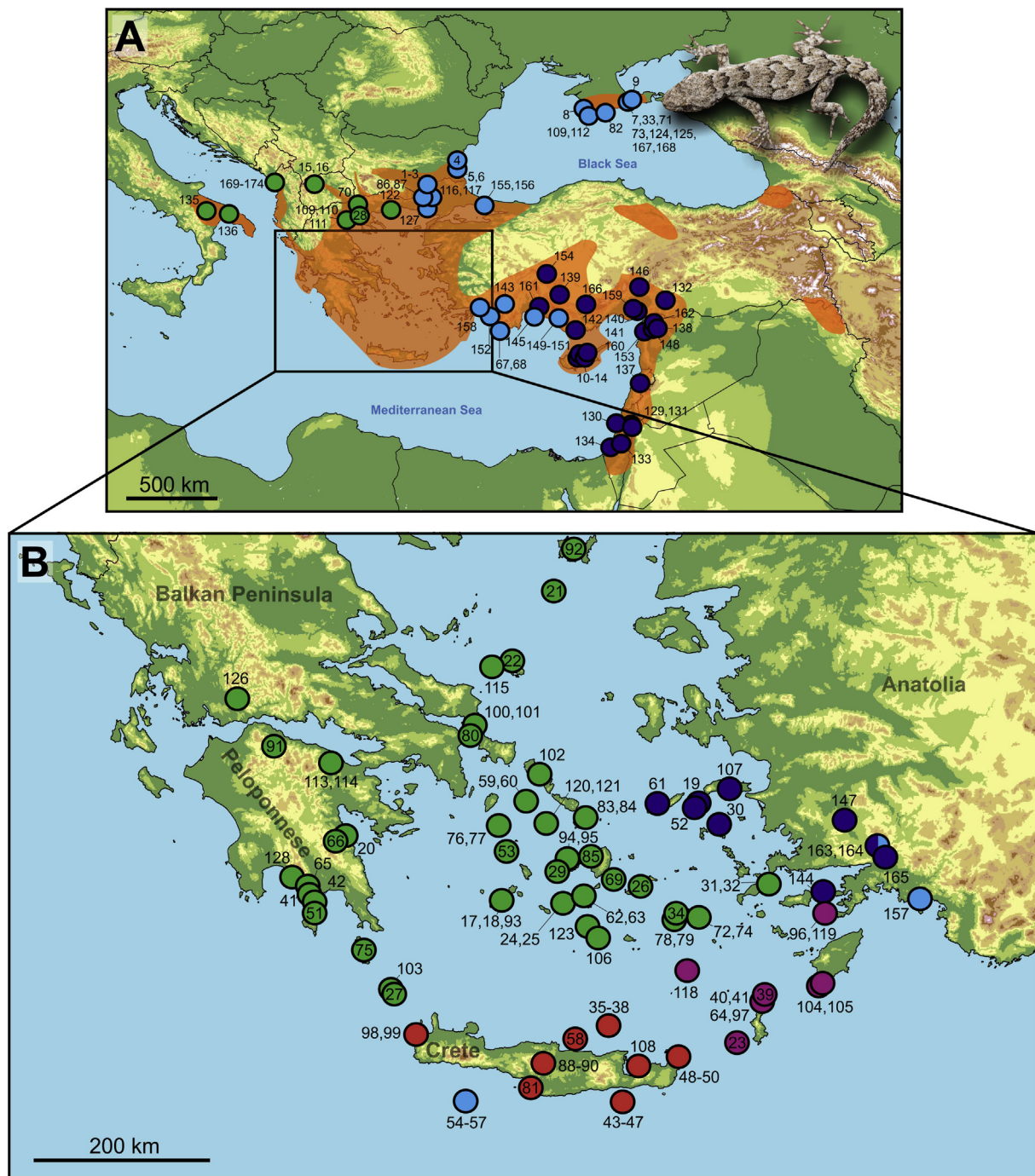

Fig. 1. The study area and the sampling localities of the samples used in this study. Numbers correspond to the sampling localities codes given in Table S1. The map is redrawn based on the *Mediodactylus kotschyi* distribution according to Sindaco and Jeremčenko (2008) in order for the shaded area (A) to indicate the distribution range of the species. For clarity reasons, the specimens from the Aegean region and south continetntal Greece are presented in B. Populations of the different phylogenetic clades as presented in Fig. 2 are indicated with differently colored dots. Inset: *Mediodactylus kotschyi* (Vlorë, Albania); photo: Daniel Jablonski. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Bauer et al., 2013 and references therein). Indicative of the great confusion is that the species of the genus *Mediodactylus* were initially assigned to the genus *Gymnodactylus,* later on to *Cyrtodactylus* and then removed to *Tenuidactylus* as a distinct subgenus (Szczerbak and Golubev, 1984). At present, *Mediodactylus* has been recognized as a full monophyletic genus (Bauer et al., 2013; Macey et al., 2000).

The genus *Mediodactylus* comprises 13 species (Uetz, 2018) with *M. kotschyi* (Steindachner, 1870) being its type species. *Mediodactylus kotschyi* is a thermophilic and xerophilic species inhabiting the Mediterranean and sub-Mediterranean landscapes and associated mainly

with stony habitats at heights up to 1700 m a.s.l. (Böhme et al., 2009). It is a small gecko distributed in the Eastern Mediterranean and Black Sea regions from southeastern Italy in the west to Turkish Transcaucasian provinces in the east, and from south Greece, Cyprus and Israel in the south to the Danube River valley at the central and eastern Balkans (in the limits of Serbia and Bulgaria) and Crimea in the north (Böhme et al., 2009; Fig. 1). It is considered introduced in Italy, Serbia and Hungary (Böhme et al., 2009; Urosevic et al., 2016).

Mediodactylus kotschyi displays great morphological variation which is reflected in the at least 26 (Baran and Gruber, 1982; Szczerbak, 1960;

Szczerbak and Golubev, 1986) described subspecies (but see also Uetz (2018) who mentioned 30 subspecies), 17 of which are found in Greece (Valakos et al., 2008). The species shows very high inter-population variability in external morphology (Beutler and Gruber, 1977; Kukushkin and Sharygin, 2005), making morphological differentiation at the subspecific level difficult due to the overlap of morphological characters used in their taxonomy (Ajtić, 2014; Szczerbak and Golubev, 1986). The subspecies of *M. kotschyi* have been classified into four groups (Beutler, 1981): (i.) the *kotschyi* group in Italy, Albania, FYROM, southwestern and central Bulgaria, mainland Greece, northern and eastern Aegean islands, Cyclades islands, northern Sporades islands, Argosaronic islands, Ionian islands and Kythira; (ii.) the *danilewskii* group, which includes all the Asiatic subspecies and those from Crimea, western Thrace (NE Greece), eastern Bulgaria and Cyprus; (iii.) the *bartoni* group in Crete and its satellite islets, excluding Gavdos; and (iv.) the *oertzeni* group in the Kasos-Karpathos island group and Gavdos island. A recent study, based on 18 morphological characters in *M. kotschyi* samples from the Balkans and the Middle East, showed that although the discrimination between the *kotschyi* and the *danilewskii* groups is feasible, there are no explicit characters that can clearly discriminate the subspecies within each group (Ajtić, 2014). Furthermore, the taxonomic classification at the subspecific level is not in congruence with the results of the only available phylogeographic study for this species (Kasapidis et al., 2005), which was based on a single mitochondrial marker and focused only on a part of the species distribution range (mainly on the Aegean islands and the neighboring mainland). However, these authors concluded that Beutler's (1981) subspecies groups are phylogenetically robust, and suggested that a taxonomic revision of *M. kotschyi* was necessary.

High levels of "cryptic" diversity in common species is not an unusual phenomenon [e.g. see (Garcia-Porta et al., 2017)]. Thus, a taxonomic revision of the taxon is important since, as it has been underlined by the IUCN (Böhme et al., 2009), "it may be a species complex containing a number of threatened species". If there are unrecognized species within *M. kotschyi*, some of them may be endangered or vulnerable. It is also important from a nature conservation perspective given that the species is distributed in numerous protected areas and is protected by national legislation in some countries (e.g., Greece and Israel (Valakos et al., 2008)). Thus, the knowledge of the actual number of species and their phylogenetic relationships will largely contribute to the design of possible conservation strategies. *Mediodactylus kotschyi* shows an exceptionally complex picture in terms of morphological variation and biogeographic patterns and it is an excellent example highlighting that "the systematic crisis" existing in some taxa severely affect their proper study and conservation. Additionally, the study of the phylogenetic relationships and the estimation of the possible number of cryptic species within this taxon, will contribute to our understanding of faunogenesis and connections between regions in the geological past - especially in cases of lack or inconsistency of paleogeographic data. The largest part of *M. kotschyi's* distribution is located around the Eastern Mediterranean basin which is considered as one of the world's top 25 "biodiversity hotspots" (Myers et al., 2000) and especially in Anatolia and the Aegean archipelago. This region has a complicated and rich geological history that has left its imprint on the biogeography of many other taxa, especially reptiles (Lymberakis and Poulakakis, 2010; Poulakakis et al., 2015).

Here, we explore the phylogenetic relationships within *M. kotschyi* covering almost its entire distribution (Fig. 1). We sequenced six gene fragments from three mitochondrial (mtDNA) and three nuclear (nuDNA) markers to assess its taxonomy and biogeographic history.

2. Material and methods

2.1. Samples, DNA extraction, amplification and sequencing

In total, 174 individuals were used (Table S1) from 129 sampling localities (Fig. 1) ranging from Italy to eastern Turkey and from Crimea to Israel covering almost the whole distribution range of the species (Sindaco and Jeremčenko, 2008). Exact sampling localities, specimen codes and GenBank accession numbers of all markers included in the phylogenetic analyses are shown in Table S1.

Total genomic DNA was isolated from tissue samples (tail or tongue) of specimens that were either frozen ($-80\,°C$) and/or preserved in ethanol. DNA was isolated using an Ammonium Acetate based DNA extraction procedure (Bruford et al., 1998).

Three mtDNA (COI, cyt *b*, 16S rRNA) and three nuDNA (RAG2, MC1R, c-mos) markers were amplified. For the nuDNA dataset a subset of specimens that had been sequenced for the mtDNA markers, was sequenced for the three nuDNA markers. The samples for the nuDNA dataset were selected in order to represent all the major clades revealed by the phylogenetic analyses on mtDNA data based on the results of a Poisson Tree Processes (PTP) analysis (for details see below). Thus, the samples included in the nuDNA dataset and the concatenated mtDNA & nuDNA dataset is a subset of those included in the mtDNA dataset. The primers' sequences and the conditions used for the PCR amplification of each fragment are presented in Table S2 (Supplementary File 1). Each PCR was performed in a 20 μL volume using a range of 50–150 ng of template DNA. The PCR products were purified with the Nucleospin PCR purification kit (Macherey-Nagey). Automated sequencing of each amplicon was performed in an ABI3730XL automated sequencer (using Big-Dye terminator chemistry). The primers used in the sequencing reactions were the same as in the PCR amplifications.

Sequences of four *Tarentola* species were used as outgroups (see Table S1 for sequences information). Although *Tarentola* is quite distant from *Mediodactylus* (Bauer et al., 2013), it was chosen as an outgroup due to three major reasons. First, we performed preliminary phylogenetic analyses (Neighbor Joining as implemented in MEGA, 1000 bootstraps) using different geckos lizards [*Hemidactylus*, *Cyrtopodion*, *Stenodactylus*] closely related to *Mediodactylus* as outgroups (sequences downloaded from GenBank) to test the effect of the outgroup selection on the tree topology (see Supplementary File 1; Fig. S1). The topology of the ingroup was slightly affected by the outgroup choice (Fig. S1), mainly regarding the relationship of Crete (clade D) and sometimes that of clade E, with the remaining clades. However, these changes do not affect the main results of the study since regardless the outgroup selection, the exact same five major clades are formed in all the analyses and within each clade the exact same *Mediodactylus* samples are included. Thus, given that the primary aim of this study is to discuss the relationships within the respective phylogenetic clades (not the interclade relationships), we consider that the use of a relatively distant taxon as an outgroup does not affect the main results of the study. Second, all six gene fragments amplified in our study were also available for the *Tarentola* species used as outgroups and third the published data for *Tarentola* (Carranza et al., 2002) allowed us to use three external calibration points for the divergence time estimations (see divergence time estimation section).

2.2. Sequences alignment and genetic data analysis

Sequences were viewed and edited using CodonCode Aligner v. 4.1.1 (Codon code Corporation). The authenticity of the obtained sequences and the homology to the targeted mtDNA and nuDNA markers were evaluated with a BLAST search in the NCBI genetic database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment of the sequences was performed separately for each gene with MAFFT v.7 (Katoh and Standley, 2013) with default parameters and auto strategy. The protein-coding gene fragments (cyt *b*, COI, RAG2, c-mos, and MC1R) were translated into amino acids prior to analysis, and did not show any stop codons. The heterozygotic positions in the nuclear markers were given ambiguity codes and were treated as unknown for the phylogenetic analyses (see 2.3. phylogenetic analyses section). Sequence divergences (uncorrected *p*-distances) were estimated in MEGA

v.6.06 (Tamura et al., 2013).

2.3. Phylogenetic analyses

Prior to the phylogenetic analyses, we used the Bayesian Information Criteria (BIC) as implemented in PartitionFinder v.1.1.1 (Lanfear et al., 2012) to calculate and select the best-fit partitioning scheme and DNA substitution models for each analysis. The partitions for the PartitionFinder (PF) were by codon, for each one of the five protein-coding genes (cyt *b,* COI, RAG2, c-mos, and MC1R). Thus, the alignment was partitioned into 16 blocks in total, including 15 blocks for the 1st, 2nd, and 3rd codon positions for the protein-coding genes and one block for the 16S rRNA gene fragment (for details see Supplementary File 1; Details on the Phylogenetic analyses).

Phylogenetic trees were constructed using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. Maximum likelihood analyses were conducted with RAxML v. 8.1.21 (Stamatakis, 2014) using RAxMLGUI v.1.5 (Silvestro and Michalak, 2011) under the partition scheme and the models of evolution proposed by PF and parameters estimated independently for each partition (Supplementary File 1; Table S3). The best ML tree was selected from 500 iterations and the confidence of the branches of the best ML tree was assessed based on 1000 thorough bootstrap replicates. Bayesian Inference (BI) was performed in MrBayes v.3.2.6 (Ronquist et al., 2012). The partition scheme and the model applied in the analysis were indicated by PF and are presented in Table S3. All nucleotide substitution model parameters were unlinked across partitions and the different partitions were allowed to evolve at different rates using the "prset ratepr = variable" command. We ran eight concurrent chains (one cold and seven heated) for 5×10^7 generations and recorded samples every 5000 generations. The first 25% of the samples were discarded as burn-in, and the remaining samples were used to summarize the posterior probability distributions for parameters ($\geq 95\%$ indicate significant support) (Huelsenbeck and Ronquist, 2001). Results were analyzed in Tracer v.1.6 (Drummond and Rambaut, 2007) to assess convergence and effective sample sizes (ESS) for all parameters (for details on the parameters assessed through Tracer see Supplementary File 1; Details on the Phylogenetic analyses).

The aforementioned analyses were performed separately in three different datasets: (i) a concatenated dataset containing the three mtDNA markers; (ii) a concatenated dataset containing the three nuDNA markers; and (iii) a concatenated dataset containing both mtDNA and nuDNA markers.

2.4. Divergence times estimation

The divergence time estimation analysis was performed on the concatenated mtDNA and nuDNA dataset. This dataset, as mentioned above, consists of a subset of the samples of the mtDNA dataset. The samples were chosen to be sequenced for the nuDNA markers based on a preliminary Poisson Tree Processes (PTP) analysis performed on the mtDNA dataset. In the PTP analysis the sequence variation was divided into intra- and interspecies groups using the PTP model for delimiting species on a rooted phylogenetic tree (Zhang et al., 2013). This preliminary analysis was considered necessary since the extrapolation of rates across different timescales (i.e. across the population-species boundary) might result in non-valid dates (Ho et al., 2011). The PTP model was used to identify "independently evolving" entities in the mtDNA dataset, considering only the ingroup sequences. This method identifies the location of the switches from speciation to coalescent nodes on a tree and delimits independently evolving entities. In PTP, speciation or branching events are modeled in terms of number of substitutions (represented by branch lengths), considering that the number of substitutions between species is significantly higher than the number of substitutions within species. Analyses were conducted on the web server for PTP (http://species.h-its.org/ptp/) using the produced

by MrBayes BI phylogenetic tree (see above). Divergence times of *Mediodactylus* were estimated using the *BEAST package of BEAST2 v2.4.5 on the dataset comprised of all the mtDNA and nuDNA sequences. The nuDNA data were not phased and the few heterozygotic positions were treated as unknown (N). This dataset consists of one representative per PTP entity and using unique specimen identifiers as the species trait, i.e. without a priori species definitions. In the BI analysis implemented in BEAST2, the data were partitioned according to gene and the substitution model indicated by the PartitionFinder (Table S3). For its greater accuracy we used the relaxed uncorrelated lognormal clock model (Drummond et al., 2006) in all partitions. A Yule process was chosen, as recommended for species-level phylogenies (Drummond and Rambaut, 2009). The analysis was run for 10^9 generations with a sampling frequency of 1 per 5000 trees from which 25% were discarded as burn-in. Adequate sampling and convergence of the Markov chain was confirmed by inspection of the MCMC samples in Tracer v.1.6.0 (Drummond and Rambaut, 2009). The effective sample size (ESS) values of all parameters were well above 200, which is usually considered a sufficient level of sampling (Drummond and Rambaut, 2009). The sampled posterior trees were summarized using TreeAnnotator v.2.4.5 to generate a maximum clade credibility tree (maximum posterior probabilities) and calculate the mean ages and 95% highest posterior density (HPD) intervals for each node. The species tree inferred with *BEAST was visualized using FigTree v1.3.1 (Rambaut, 2012). The divergence times for the phylogenetic clades of *M. kotschyi* were estimated using three external calibration age constraints based on the estimated range of the ages of the *Tarentola* species used as outgroups (Carranza et al., 2002). More specifically, the divergence of *Tarentola boehmei* from *T. deserti* has been estimated between 5 and 8.4 Mya (Carranza et al., 2002) so we used the normal distribution and set the mean divergence time at 6.6 Mya in order to keep these age limits. Similarly, for the divergence of *T. mauritanica* from *T. angustimentalis* we set a mean estimate at 8.8 Mya, and for the divergence of *boehmei/deserti* group from *mauritanica/angustimentalis* group at 10.3 Mya.

2.5. Species delimitation

Species delimitation was performed using the Species Tree And Classification Estimation, Yarely - STACEY v.1.2.1 (Jones, 2015), in BEAST2 (Bouckaert et al., 2014), which is an extension of the multispecies coalescence model used in *BEAST (Heled and Drummond, 2010), in which a birth-death-collapse model is used to estimate the species tree. For this analysis, we used the concatenated dataset including both the nuDNA and mtDNA fragments and unique specimen identifiers as the species trait, i.e. without a priori species definitions (the 43 PTP entities were used as minimal clusters). Here we note that, the tree created by STACEY is not exactly a 'species tree' but more a 'species or minimal clusters tree' [SMC-tree, since the minimal requirement of the program is one individual in each minimal cluster (tip of the tree)]. So, in STACEY the possible number of species ranges from one (merge all the clades of the tree) to the number of individuals (PTP entities in our case). The input files (.xml) were created using BEAUti. The nucleotide substitution models were not given *a priori* but instead, the BEAST Model Test option was selected. As for other priors the Birth Death Model was used to estimate the species tree [priors: Collapse Height = 0.001, Collapse Weight = 0.5 using a beta prior (1.1) around [0.1], which is a flat prior on the number of species, Ploidy: equal to 2 for nuclear genes and 0.5 for mtDNA genes] and the Uncorrelated Lognormal Model to describe the relaxed molecular clock. The MCMC analysis was run for 10^9 generations, saving the result every 10,000 generations. The obtained log files were analyzed with Tracer to verify that the convergence of the analysis had been achieved and that satisfactory effective sample sizes had been obtained. The value of –ln L was stabilized after 10^7 generations and the first 25% were discarded. Following the completion of STACEY analysis we used the

SpeciesDelimitationAnalyser to process the log files and find out the distribution over species assignments (Jones et al., 2015).

3. Results

3.1. Alignment and genetic distances

A total of 1688 base pairs (bp) of concatenated mtDNA alignment (cyt *b*: 359 bp, 16S rDNA: 640 bp, COI: 689 bp) revealed 148 haplotypes with 738 bp (43.7%) variable and 641 bp (38.0%) parsimony informative sites (841 bp and 707 bp including the outgroup sequences). Based on the results of PTP analysis, 43 independent evolving entities were revealed and one specimen from each one was sequenced for the three nuDNA markers and used for the concatenated phylogenetic analyses and the estimation of times of divergence. The concatenated nuDNA alignment consisted of 1437 bp (MC1R: 685 bp, RAG2: 386 bp and c-mos: 366 bp), 97 bp (6.7%) and 80 bp (5.6%) of which were variable and parsimony informative sites, respectively (222 bp and 186 bp including the outgroup sequences).

The pairwise uncorrected *p*-distances between single sequences of *M. kotschyi* varied up to 24.4% in cyt *b*, 23.1% in 16S rRNA, 26.1% in COI, 3.3% in MC1R, 1.8% in c-mos, and 3.1% in RAG2 (pairwise distance matrix not shown). However, the mean genetic distances among the main lineages of *M. kotschyi* are ~17–18%, ~15–20% , ~11–16% for COI, cyt *b* and 16S rRNA respectively and 0.4–2% for the nuDNA markers. The mean genetic distance between and within the main lineages are presented in detail in Table 1.

3.2. Phylogenetic analyses

The best-fit partitioning schemes for each downstream analysis (ML and BI as performed in MrBayes and BEAST2) and the selected nucleotide substitution models are summarized in Table S3.

Bayesian Inference (ln L = –23,377.0) and Maximum Likelihood (ln L = –23,066.9) analyses of the concatenated mtDNA data set produced trees with similar topologies as shown in Fig. 2 (BI tree topology) and Fig. S2 (ML tree topology) respectively. The phylogenetic reconstruction from both analyses highlights the presence of five major monophyletic units (A–E), each one with high statistical support [PP = 1.00 and BS ≥ 95]. Three of them (A, B, and C) can be divided further into four, six and four well-supported subclades (PP ≥ 0.99 and BS > 80), respectively. More specifically, the populations originating from Albania, FYROM, Italy, northern/central Greece and the Cyclades islands cluster together (PP = 1.00 and BS = 91) forming one major phylogenetic clade (clade A; Fig. 1; green). Samples originating from the Cyclades islands form two distinct lineages separating the northern (subclade A1) from the southern (subclade A2) islands. Clade B hosts populations from Anatolia, Cyprus and eastern Aegean islands (Fig. 1; dark blue). Within Clade B, *Mediodactylus kotschyi* populations from Cyprus and those from Syria, Lebanon, and Israel form two distinct subclades (B3 and B5, respectively). However, the phylogenetic relationships between these subclades and the remaining ones of the Clade B, are not resolved. Clade C (Fig. 1; light blue) hosts individuals originating from a broad geographic region (from Crimea through

eastern Greece, Bulgaria and southern Turkey to an isolated and probably introduced population on Gavdos island). Clade D includes populations from Crete and its neighboring islets (Fig. 1; red), whereas clade E comprises populations from southeastern Aegean islands (Fig. 1; violet).

Both phylogenetic (ML and BI-MrBayes) analyses of the nuDNA dataset produced phylogenies (ln L = −3500.5 for ML and ln L = −3635.5 for BI) that are in agreement with the mitochondrial topologies but with incongruences in statistical support (Supplementary File 1; Fig. S3). Similarly, both ML and BI (as implemented in MrBayes) analyses of the concatenated mtDNA and nuDNA dataset (Supplementary File 1; Fig. S4) produced similar phylogenetic trees (ln L = −16,964.8 for ML and ln L = −16,988.0 for BI) to the concatenated mtDNA trees (see Fig. 2).

3.3. Divergence times

In the estimation of the divergence times as implemented in BEAST2, high ESSs were observed (−ln L = −35,004.14; ESS values > 242) and assessment of convergence statistics in Tracer indicated that analyses had converged. According to the estimated divergence times of *M. kotschyi* lineages, based on all 3 calibration points, the diversification of *M. kotschyi* lineages dates back to Middle Miocene at ~15 Mya (Fig. 3). The mean (as well as the 95% HPD interval) estimated divergence times between the main lineages of *M. kotschyi* are presented in detail in Fig. 3.

3.4. Species delimitation

The species delimitation analysis as implemented in STACEY produced high ESSs values (> 300) with ln L = −14,254.STACEY indicated five different lineages at 68% of the samples run in this analysis (total number of samples run; 108,003). A percentage of 10% of the analysis run, indicated 6 taxa while other possible number of taxa (between 4 and 8 taxa) were defined in a very low percentage of the runs (4–6% each).

Each one of the five defined taxa corresponds to one major clade (A to E) of the phylogenetic tree (Fig. 3). The uncorrected *p*-distance between the five STACEY-defined taxa is presented in Table 1.

4. Discussion

4.1. Phylogenetic relationships and biogeography of Mediodactylus kotschyi species complex

A comprehensive and robust assessment of the phylogenetic relationships of *M. kotschyi* populations covering the largest part of its distributional range and using both mitochondrial and nuclear data is presented here. The traditional phylogenetic reconstructions (BI and ML) highlight the presence of five strongly supported clades, which correspond to three major geographic regions (Figs. 1 and 2); the "Western" (Fig. 1; green), the "Southern" (Fig. 1; red and violet) and the "Eastern" (Fig. 1; blue and light blue) regions.

The first region (Western; clade A) consists of specimens from

Table 1

Percentage (%) of sequence divergences (*p*-distance between groups) among the main lineages of *M. kotschyi*, for the COI/cyt*b*/16S rRNA (below diagonal) and cmos/RAG2/mc1 (above diagonal). Values in diagonal in bold characters represent the within lineages sequence divergences [COI/cyt*b*/16S rRNA-cmos/RAG2/mc1]. The names of the main lineages referring to the clades of the phylogenetic tree presented in Fig. 2.

Clade	A	B	C	D	E
A	**11.8/10.0/6.1–0.1/0.7/0.7**	1.1/2.1/1.6	0.5/1.1/1.5	0.7/1.8/2.1	0.4/1.3/1.6
B	17.8/17.8/12.3	**13/11.8/8.1–0.3/1.0/1.2**	0.9/2.0/1.3	0.5/2.4/2.0	0.9/2.0/1.4
C	16.8/18.0/10.9	17.4/16.9/11.7	**7.1/8.1/4.7–0.1/0.4/0.6**	0.4/1.6/1.9	0.8/1.2/1.3
D	18.2/17.7/15.5	17.0/19.7/16.6	17.5/17.4/14.5	**3.4/4.7/3.0–0.0/0.0/0.1**	0.4/1.6/1.5
E	17.8/15.6/12.9	18.1/16.9/14.9	17.0/15.1/13.3	17.2/17.3/16.4	**7.4/5.0/5.5–0.2/0.2/0.0**
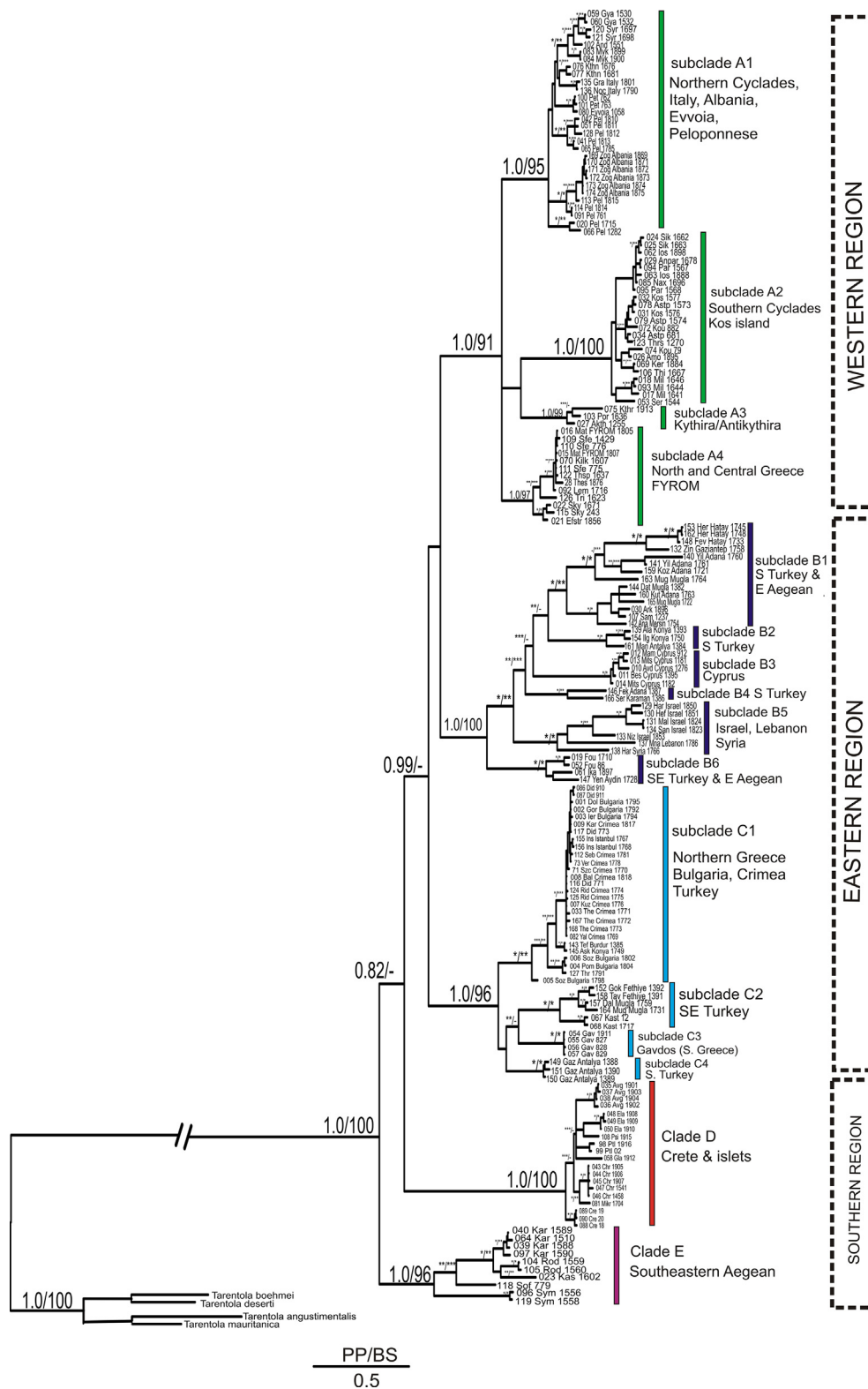
Fig. 2. Bayesian Inference (BI) tree reconstructed from the mtDNA dataset. Posterior probabilities (PP) and bootstrap (BS) support values resulted from the Bayesian Inference and Maximum Likelihood analyses respectively, are indicated with asterisks on the branches of the tree. BS < 70 and PP < 70, as well as statistical support of the very external clades are not presented. Individual codes are consistent with those in Table S1. Symbols used: *: PP ≥ 0.99 or BS ≥ 99, **: 0.90 < PP < 0.99 and 90 < BS < 99, ***: 0.70 < PP < 0.89 and 70 < BS < 89.

Greece (mainland Greece, Peloponnese, Evvoia, Kythira/Antikythira islands, Cyclades, and northern Aegean islands), FYROM, Italy and Albania. A similar grouping has also been recorded in other lizard species, such as the green lizards of the genus *Lacerta* and the snake-eyed skinks of the genus *Ablepharus* (Sagonas et al., 2014; Skourtanioti et al., 2016). However, the clustering of the Peloponnese and Cyclades populations with those Albania and Italy (where the species is introduced) may also suggest human-mediated dispersal for Albanian
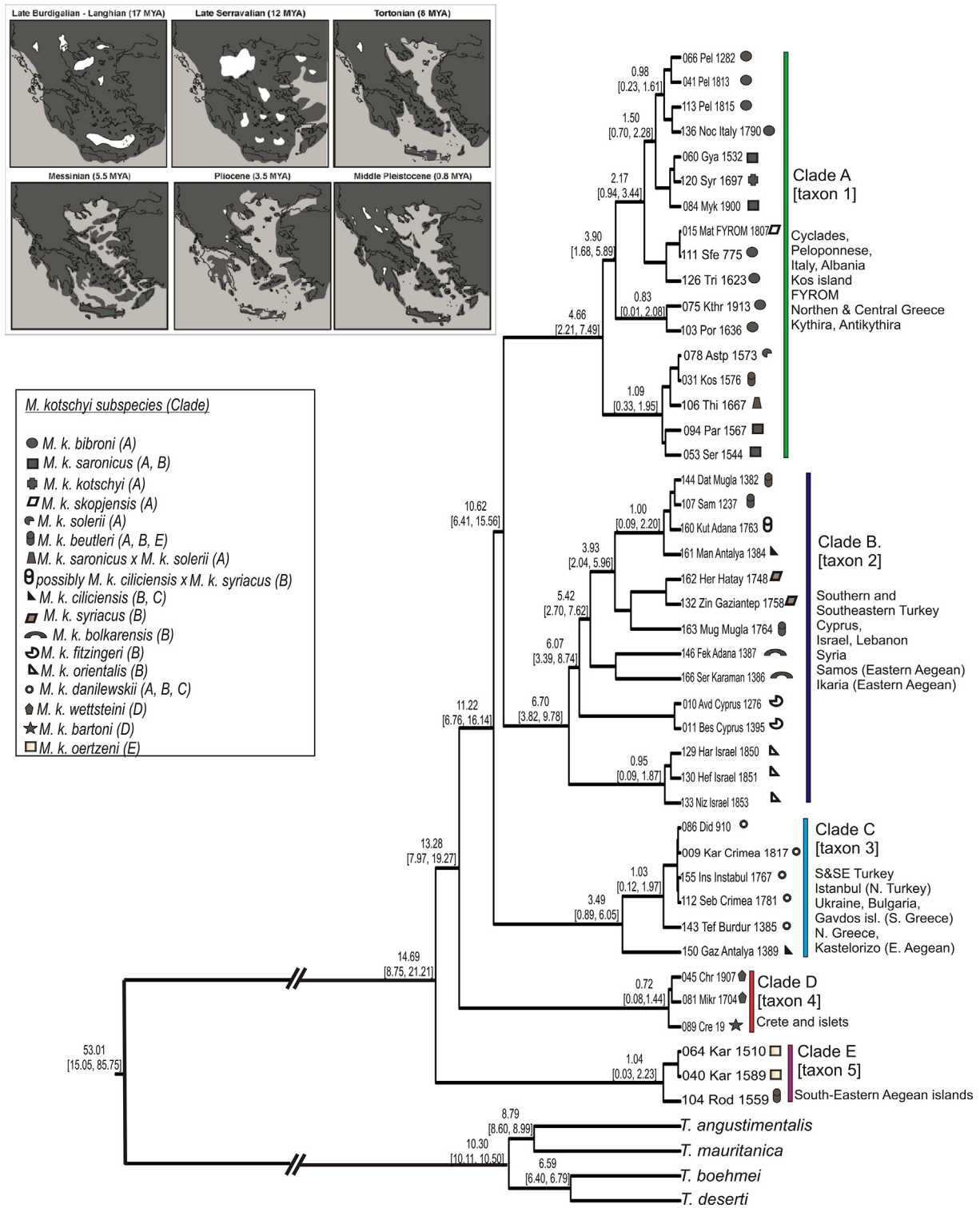
Fig. 3. The ultrametric species tree with the estimated times of divergence between the main lineages of *M. kotschyi* as reconstructed by the *BEAST based on the concatenated (mtDNA and nuDNA) dataset. The distinct taxa identified by Stacey analysis are consistent with the major clades and are indicated by bar of different color. Individual codes and clades' symbols are consistent with those in Table S1 and Fig. 2 respectively. Different symbols on the Clades' tips indicate the different described subspecies within each clade. In the embedded map the paleogeographic history of the Aegean area is presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

territory.

Within the Western region, there are two biogeographically important results; the first concerns the split of Cyclades islands to northern and southern part showing moderate/high differentiation (Table 1) in terms of genetic distance and the second one regards the

clustering of the island of Kos with the Cyclades in subclade A2 (Fig. 2) instead of the eastern Aegean islands (within clade B). The majority of the populations distributed in the northern (subclade A1) and southern Cyclades (subclade A2) do not show great morphological differentiation (most of them are assigned to a single subspecies *M. k. saronicus*;

Supplementary File 2) and the time frame of this differentiation (Fig. 3; ~3.9 Mya) seems to be slightly older than the known geological separation in this area (see map in Fig. 3; ~3.5 Mya). Interestingly, the same geological splitting between northern and southern Cyclades at ~3.6 Mya is evident in *Podarcis* lizards (Poulakakis et al., 2005) as well as in scorpions (Parmakelis et al., 2006) but the time estimates in this case was much older (~10 Mya). Given the broad confidence intervals in the time divergence estimation we cannot safely conclude if this split reflects the known geological separation of Cyclades (~3.5 Mya) or if it is older than that. In the latter case, vicariance events before the known geological separation of Cyclades may have created this divergence, although the exact geological or biological processes that contributed to the observed pattern remain unclear. The case of the island of Kos, which geographically belongs to eastern Aegean islands but phylogeographically is clustered with the Cyclades, is probably either one more case of 'naughty' animals (Poulakakis et al., 2015), namely animals that passed the Mid Aegean Trench (MAT) by dispersal, or a human-aided dispersal. Three representatives of the Aegean vertebrate fauna (*Ablepharus kitaibelii, Podarcis erhardii,* and *Pelophylax bedriagae*) and several invertebrates (*Dolichopoda* spp., *Trachelipus aegaeus, Albinaria brevicollis Dichomma dardanum, Zonites rhodius*) also have disjunct distributions and may also be a member of the 'naughty' animals (Lymberakis and Poulakakis, 2010; Poulakakis et al., 2015).

The Eastern region comprises two clades (B and C). Clade B hosts specimens from Anatolia, eastern Aegean islands, Syria, Lebanon, Israel, and Cyprus. Within this clade, specimens from the eastern Aegean islands cluster with those from Turkey, a pattern consistent with the biogeography of several animal species, such as *Ablepharus, Ophisops, Anatololacerta, Pelophylax* (Bellati et al., 2015; Kyriazi et al., 2008; Lymberakis et al., 2007; Skourtanioti et al., 2016), and is likely due to land connections among the majority of the eastern Aegean islands to Anatolia until the Upper Pleistocene (Perissoratis and Conispoliatis, 2003). Moreover, the specimens from Cyprus form a separate clade that is sister to *M. kotschyi* from southern Turkey and Samos/Arkoi islands in the eastern Aegean. The geotectonic evolution of Cyprus has not been clarified yet to a detailed enough level for biogeographic reconstructions [for details see (Poulakakis et al. 2013) and (Tamar et al., 2014)], as it is not clear if the island has ever been joined by a land bridge to the neighboring mainland (Anatolia, Syria). Thus, the colonization of Cyprus could have occurred either by a land bridge that connected Cyprus with the mainland (perhaps during the Messinian Salinity Crisis) as it has been suggested for the Eurasian blindsnake in Cyprus (Kornilios, 2017) or by transmarine dispersal. More specifically, species considered as old colonizers (Late Miocene, Early Pliocene) could have arrived to Cyprus either by geodispersal or transmarine dispersal, while the species considered as younger colonizers could have inhabited the island either through transmarine dispersal from the Middle East or by human translocations (Poulakakis et al., 2013). If we consider that the age of the split of Cyprus subclade at ~6 Mya is accurate (Fig. 3), then *M. kotschyi* seems to belong to the first group of colonizers that reached the island during the Messinian period. Our time estimates for the *M. kotchyi* lineage from Cyprus, considerably coincide with the time divergence estimated (~6 Mya) between Cyprus and Turkey lineages of *Acanthodactylus schreiberi* (Tamar et al., 2014) and close to that of *Xerotyphlops socotranus* (Kornilios, 2017).

Clade C hosts specimens from a very broad geographic region (from Crimea through Bulgaria and Turkey to an isolated population on Gavdos island) and can be divided into four distinct highly supported subclades, each one including specimens from a specific geographic region (Figs. 1 and 2). One of the most striking findings within this clade was the grouping of the lineage from Gavdos island (south-west of Crete) with some southern Anatolian populations and population from Kastelorizo island, which was also observed in the previous phylogeographic study of *M. kotschyi* (Kasapidis et al., 2005). The close phylogenetic relationship of the lineage from Gavdos island with the lineages

from Turkey, Crimea and Bulgaria, and not with the geographically closer island of Crete remains a challenging issue. Gavdos is expected to have been an isolated island since the Tortonian at ~8 Mya (Drinia et al., 2010; Pomoni et al., 2013; Tsaparas, 2004) suggesting overwater dispersal to Gavdos as the most likely colonization (Kasapidis et al., 2005), though it is not obvious if the dispersal was human-aided or not as the limited sampling from the Eastern region may not contain the source population. However, the fact that specimens from Gavdos Island are morphologically differentiated from the remaining Anatolian samples (see Supplementary File 2; Distinct sub-species) supports the idea that it is possibly an old dispersal event.

Finally, the last two clades (D and E) that are the most differentiated clades of *M. kotschyi,* are well located at the south and southeastern part of the Aegean (Southern region). The differentiation of Crete from the southeastern Aegean islands is a well-defined biogeographic pattern observed in other taxa as well [e.g. land snails (Parmakelis et al., 2005) and frogs (Lymberakis et al., 2007)]. Although the partially resolved relationships of these two lineages in *M. kotschyi* do not allow precise estimates, our time estimates between 11 and 13 Mya (Fig. 3), are consistent with the observed pattern in other taxa and the palaeogeographic separation of this region from the mainland (see map in Fig. 3).

4.2. Species delimitation and taxonomy

Several subspecies and intermediate forms have been described, and the subspecific taxa are distributed across all of the five STACEY-defined species. More specifically, the populations of *M. kotschyi* of the Western region have been assigned to eight morphological subspecies and two transitional forms (Valakos et al., 2008). Two of them (*M. k. bibroni* and *M. k. saronicus*) cover the majority of the geographical distribution within the western group (see Supplementary File 2; Fig. S5). Six more subspecies (*M. k. tinensis, M. k. kotschyi, M. k. beutleri, M. k. skopjensis, M. k. solerii* and possibly *M. k. danilewskii*) and two transitional forms *(M. k. saronicus × M. k. schultzewestrumi* and *M. k. saronicus × M. k. solerii)* clustered within clade A but they show (see Supplementary File 2) very restricted geographical distributions (Valakos et al., 2008). In other words, the subspecific taxonomy of *M. kotschyi* is uncertain as several subspecies are polyphyletic.

Similarly considering the Eastern region, eight subspecies together with a transitional form have been described within clade B and three subspecies together with a transitional form have been described within clade C (Fig. 3 and Supplementary File 2; Figs. S6 and S7). Interestingly, the subspecies delimitation within clade B largely corresponds to the defined sub-clades [B2; *M. k. danilewski,* B3; *M. k. fitzingeri,* B4; *M. k. bolkarensis,* B5; *M. k. orientalis* and *M. k. syriacus*]. However, STACEY supported that all clade B samples form a single species (Fig. 3; taxon 2).

Samples from clade C originate from a broad geographic distribution, however, the majority of the specimens of this clade have been assigned to a single subspecies (*M. k. danilewskii*) that corresponds to the subclade C1. The remaining specimens of clade C are subdivided into three distinct subclades, two of which correspond to different subspecies [C3; *M. k. kalypsae* in Gavdos island, C4; *M. k. ciliciensis* in Antalya]. However, we should note that the taxonomic position of *M. kotschyi* populations in southwestern Turkey is very doubtful since there are populations showing features of three subspecies (*M. k. beutleri, M. k. danilewskii* and *M. k. ciliciensis*) and there is a wide inter-breeding zone between *M. k. beutleri* and *M. k. ciliciensis* (Baran and Gruber, 1982) or there is exclusively *M. k. ciliciensis* (Kumlutaş et al., 2004). Nonetheless, the species delimitation analysis showed, as in the case of clade C, that all of these populations belong to one single species (Fig. 3; taxon 3).

Last, considering the Southern region, most of the specimens that originate from the islands of southeastern Aegean (clade E) belong to *M. k. oertzeni* with the exception of those from Sofrano, Symi and maybe Rhodos (see Supplementary File 2). Two subspecies (see Supplementary
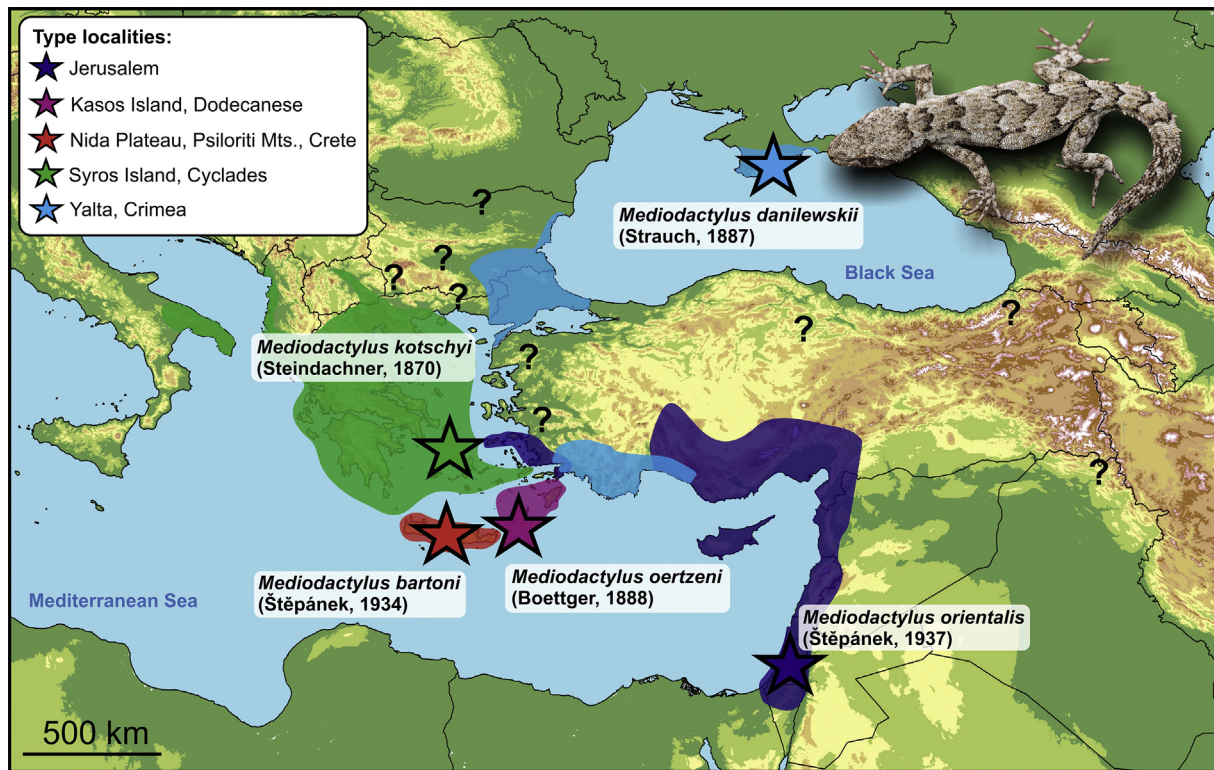
Fig. 4. The newly proposed species-level taxonomy of the *Mediodactylus kotschyi* species complex with type localities. Question marks indicate populations, which are not represented in the present study and may be important for taxonomy.

File 2) were recognized in clade D; *M. k. bartoni* and *M. k. wettsteini* (Valakos et al., 2008), which are not monophyletic, indicating that in this clade, as in the previous ones, the subspecific taxonomy is uncertain. Both clades of this region (D and E) are again recognized as distinct species in the species delimitation analysis (Fig. 3; taxa 4 and 5).

According to our estimates for the divergence time among *M. kotschyi* clades (Fig. 3), members of the species complex has been diversifying over the last ~15 Mya. There are several examples of genera that diversified into many species in a similar time frame [in ~16 My nine *Acanthodactylus* species (Tamar et al., 2016), in < 16 My three *Stenodactylus* species (Metallinou et al., 2012), in ~16 My six *Ablepharus* species (Skourtanioti et al., 2016), in ~9 My five *Podarcis* species, in ~15 My seven *Pelophylax* species (Lymberakis and Poulakakis, 2010)].

In summary, the species delimitation analysis recognized five species (Fig. 3 and Supplementary File 2). In each one of which at least two subspecies of *M. kotschyi* are assigned (Ajtić, 2014; Baran and Gruber, 1981, 1982; Rösler et al., 2012; Valakos et al., 2008). Interestingly, these taxa are very close to the *M. kotschyi* groups defined by Beutler (1981). More specifically, clade A (taxon 1) to the kotschyi-group (with possible exceptions of Kos and Lemnos islands), clades B and C (taxa 2 and 3) coincide with the danilewskii-group (with the exception of Gavdos isl.), clade D (taxon 4) to the bartoni-group, and clade E (taxon 5) largely corresponds to the *oertzeni*-group (with possible exceptions of Symi and Rhodos islands).

The genetic diversity within the *M. kotschyi* complex in mtDNA is high, and specifically the genetic distances among the major phylogenetic clades (Table 1), which correspond to the STACEY-defined species, are similar or even higher than the divergence found between some other lizard species (Bellati et al., 2015; Edwards et al., 2012; Sampaio et al., 2015). For example, in COI (Table 1; 17–18%) the genetic distance between *Tenuidactylus* species range between ~7.0% and 18.6% (Nazarov and Poyarkov, 2013) and between species of the

Gekkonidae family from 11.9% to 35.7% (Hawlitschek et al., 2013). The same issue was observed in cyt *b* (Table 1; 16–20%) and 16S (Table 1; ~11–17%) which are equal or higher than the corresponding distances in *Hemidactylus* (Smid et al., 2013) and *Stenodactylus* (Metallinou et al., 2012). The high genetic divergence of *M. kotschyi* combined with its great morphological diversification (Ajtić, 2014), and with the large number of recognized subspecies (Baran and Atatur, 1998; Franzen et al., 2008; Valakos et al., 2008), are in congruence with the hypothesis that *M. kotschyi* represents a species complex. Considering the deeply divergent clades (A-E; Fig. 2), their estimated ages (Fig. 3), distributions (Figs. 1 and 4), and the oldest available names, taxa 1 to 5 could be recognized as full species. Nevertheless, we stress the need of a complex approach with combination of genetics, morphology, ecology, and based on type or topotypic material to fully resolve the taxonomy. If we consider taxa 1–5 as full species we propose the names below while our detailed rationale on names proposed is explained in detail in Supplementary File 2:

Clade A; *Mediodactylus kotschyi* (Steindachner, 1870), type locality: Syros Island, northern Cyclades. Distribution: mainland Balkans, most of Aegean islands, Italy (Fig. S5).

Clade B; *Mediodactylus orientalis* (Štěpánek, 1937) (new status), type locality: Jerusalem. Distribution: Levant, Cyprus, southern Anatolia, south-eastern Aegean islands (Samos, Ikaria and neighboring islets) (Fig. S6).

Clade C; *Mediodactylus danilewskii* (Strauch, 1887) (new status), type locality: Yalta, Crimea. Distribution: Black Sea region, south-western Anatolia, and Gavdos Island (south of Crete) (Fig. S7).

Clade D; *Mediodactylus bartoni* (Štěpánek, 1934) (new status), type locality: Nida Plateau, Psiloriti Mts., Crete. Distribution: Crete, including nearby islets (Fig. S8).

Clade E; *Mediodactylus oertzeni* (Boettger, 1888) (new status), type locality: Kasos Island, Dodecanese. Distribution: southern Dodecanese Islands (Fig. S9).

For synonymy see Supplementary File 2.

5. Conclusions

We provide new information about the phylogenetic relationships of populations of *M. kotschyi* covering almost its entire distribution, indicating that it is one more case where phylogenetic analyses revealed hidden (unrecognized) species diversity (Ahmadzadeh et al., 2012; Garcia-Porta et al., 2017; Simó-Riudalbas et al., 2017). The levels of genetic divergence between the major phylogenetic clades of *M. kotschyi* are extremely high, reaching or even overcoming the levels of interspecific genetic divergences between species within other genera of gekkonid lizards. Thus, *Mediodactylus kotschyi* is actually a species complex, in which the applied species delimitation approach supported the five-species hypothesis. Phylogenetic information can now be added to the morphology-based subspecific taxonomy and the knowledge of distribution in order to perform detailed subspecific taxonomic revisions within the newly recognized species, and to assess conservation priorities since some of the defined subspecific taxa may be vulnerable or endangered.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.03.022.

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