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Cryptic diversity within the *Anatololacerta* species complex (Squamata: Lacertidae) in the Anatolian Peninsula: Evidence from a multi-locus approach

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ABSTRACT

The rapid development of innovative molecular tools for characterizing biodiversity is leading to an extensive and sometimes unexpected renovation of taxonomic classifications. Particularly, for species having allopatric or parapatric distributions or resulting from recent speciation processes, the absence of clear phenotypic differentiation may hinder the recognition of closely related taxa, while intraspecific polymorphism may be confused with the presence of more than one single species. In the present work, we apply different phylogenetic methods in order to infer relationships within the genus *Anatololacerta*, and to assess the taxonomy of this morphologically diversified group of lizards endemic to western and southern Anatolia and some neighboring Aegean islands. According to morphology, three species have been recognized (*Anatololacerta anatolica*, *A. oertzeni* and *A. danfordi*) as well as several subspecies, but small variation at immunological markers led some authors to join all the populations into one single taxon, *A. danfordi*. By selecting both mitochondrial and nuclear informative markers, we tested the effectiveness of classical "gene tree" (i.e. Bayesian Inference) vs. innovative (i.e. coalescent-based) "species tree" methods in resolving the *Anatololacerta* taxonomic enigma, as a case in point for similar studies on species complexes resulting from non-obvious and cryptic diversification patterns. According to our results, the gene tree method failed in resolving phylogenetic relationships among clades, whereas the multi-locus species tree approach, coupled with species delimitation methods, allowed the identification of four well distinct species. These species probably diversified in different allopatric refugia located in southern and western Anatolia, where isolated populations may have persisted during Pleistocene glacial cycles.

1. Introduction

The recent emergence of modern molecular techniques for studying biodiversity is revealing that traditional taxonomy and systematics today require an extensive and sometimes unexpected renovation (e.g. Mallet and Willmott, 2003; Wilson, 2003; Wiens, 2007; de Carvalho et al., 2008). One of the most important outcomes of molecular studies is indeed the awareness that morphological approaches to species identification often fail to resolve the specific identity of closely related taxa, which generally look very similar. Particularly, while the study of morphological variation seems to be informative in delimiting boundaries among syntopic species, the distinction of allopatric or parapatric sibling taxa generally appears to be problematic (e.g. Bruna et al., 1996; Fernandez et al., 2006; Ibáñez et al., 2006). Most cryptic species also result from recent speciation processes, so that morphological or other diagnosable traits have not yet evolved or become evident (Saez and Lozano, 2005; Kaliontzopoulou et al., 2012). Moreover, intraspecific polymorphism, sometimes resulting from random processes or local adaptation of populations, has been historically confused with the existence of distinct species (Darwin, 1859; Mayr, 1963). Finally, in addition to resolving the true phylogenetic relationships among taxa and to shedding light on their taxonomy, ecology, biogeography and evolution, assessing the real extent of species diversity is essential for conservation purposes.

In this framework, and with the use of ever-increasing multi-locus genetic data, coalescent-based methods for species delimitation are being developed and applied to the recognition and delimitation of unknown diversity (Pons et al., 2006; O'Meara, 2010;

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Ence and Carstens, 2011; Yang and Rannala, 2010; Fujita et al., 2012). Indeed, a growing number of studies is revealing that gene tree approaches, such as Maximum Likelihood (ML) and Bayesian Inference (BI), may infer the genealogical pathway of individuals rather than the true evolutionary relationships among species (e.g. Nichols, 2001; Rannala and Yang, 2008). In contrast, multilocus coalescent methods (i.e. species tree, Edwards, 2009) have been proven to be more effective in delimiting species (Knowles and Carstens, 2007; Carstens and Dewey, 2010), allowing for reliable estimation of species divergence, while taking into account the uncertainties associated with gene tree inference, such as incomplete lineage sorting (ILS, Heled and Drummond, 2010). For this reason, the multi-species coalescent method has now become the default option for phylogenetic and phylogeographic investigations, especially when dealing with non-obvious and cryptic diversification patterns such as those characterizing species complexes.

The lizard family Lacertidae includes about 44 genera and 318 species widely distributed in Eurasia and Africa (Arnold et al., 2007; Uetz, 2014), divided into two subfamilies, Gallotinae and Lacertinae; the latter comprises two main tribes, the Eremiadini and the Lacertini. As a result of the high level of morphological similarity and convergence among different groups, the systematics of Lacertini has been particularly controversial, with most of the taxa being lumped for decades under the paraphyletic genus "*Lacerta*" (see Arnold et al., 2007 for a review). This systematic confusion was resolved with the description of eight new genera of Lacertini using a combination of molecular and morphological data, thus reconciling phylogeny and taxonomy and highlighting the diversity of this group (Arnold et al., 2007). Although molecular data have not been able to resolve the phylogenetic relationships between the different genera of Lacertini (Arnold et al., 2007; Pavlicev and Mayer, 2009; Kapli et al., 2011), DNA sequences have been very useful in resolving the phylogenetic relationships, and especially in uncovering high levels of cryptic diversity within some genera (e.g. Carranza et al., 2004; Pinho et al., 2007; Ahmadzadeh et al., 2013a,b). However, with the only exception of Ahmadzadeh et al. (2013b), which used a species tree approach, all the phylogenetic analyses of the Lacertini carried out to date have been done with gene trees.

The genus *Anatololacerta* was erected by Arnold et al. (2007) and refers to a small group of lizards endemic to western and southern Anatolia and some neighboring Aegean islands, including Samos, Ikaria, Rhodes and a few other small islets (Fig. 1). Although this genus originated approximately 12 Ma (Arnold et al., 2007), phylogenetic relationships with other Lacertini are still poorly resolved, and only sister relationships with *Parvilacerta* have been suggested based on mitochondrial (Carranza et al., 2004) and nuclear data (Mayer and Pavlicev, 2007). According to previous studies based on morphological characters (Eiselt and Schmidtler, 1986), the genus consists of three species with parapatric distributions: *Anatololacerta danfordi* (Günther, 1876), *A. anatolica* (Werner, 1900), and *A. oertzeni* (Werner, 1904). Populations of *A. anatolica* occur in northwestern Anatolia (north of Büyük Menderes river; nominal form) and in Samos Island (ssp. *aegaea* Eiselt and Schmidtler, 1986). *A. oertzeni* is distributed in southwestern Turkey, from Büyük Menderes river to Anamur area (ssp. *budaki, finikensis, ibrahimi*, all of them described by Eiselt and Schmidtler, 1986), with insular populations inhabiting Ikaria (nominal form), Rhodes (ssp. *pelasgiana* Mertenz, 1959) and the surrounding islets of Symi (ssp. *quandttaylori* Börner, 1974) and Pentanisos (ssp. *pentanisiensis* Wettstein, 1964). The remaining populations belong to *A. danfordi*, which is spread across the central and eastern portion of the Taurus Mountains from Acıgöl (in Burdur province) to Mersin (ssp. *bileki* Eiselt and Schmidtler, 1986) and in the Bolkar Dağları (nominal form) (Fig. 1). However, the existence of some cases of partial range overlap between taxa, e.g. between *A. oertzeni*

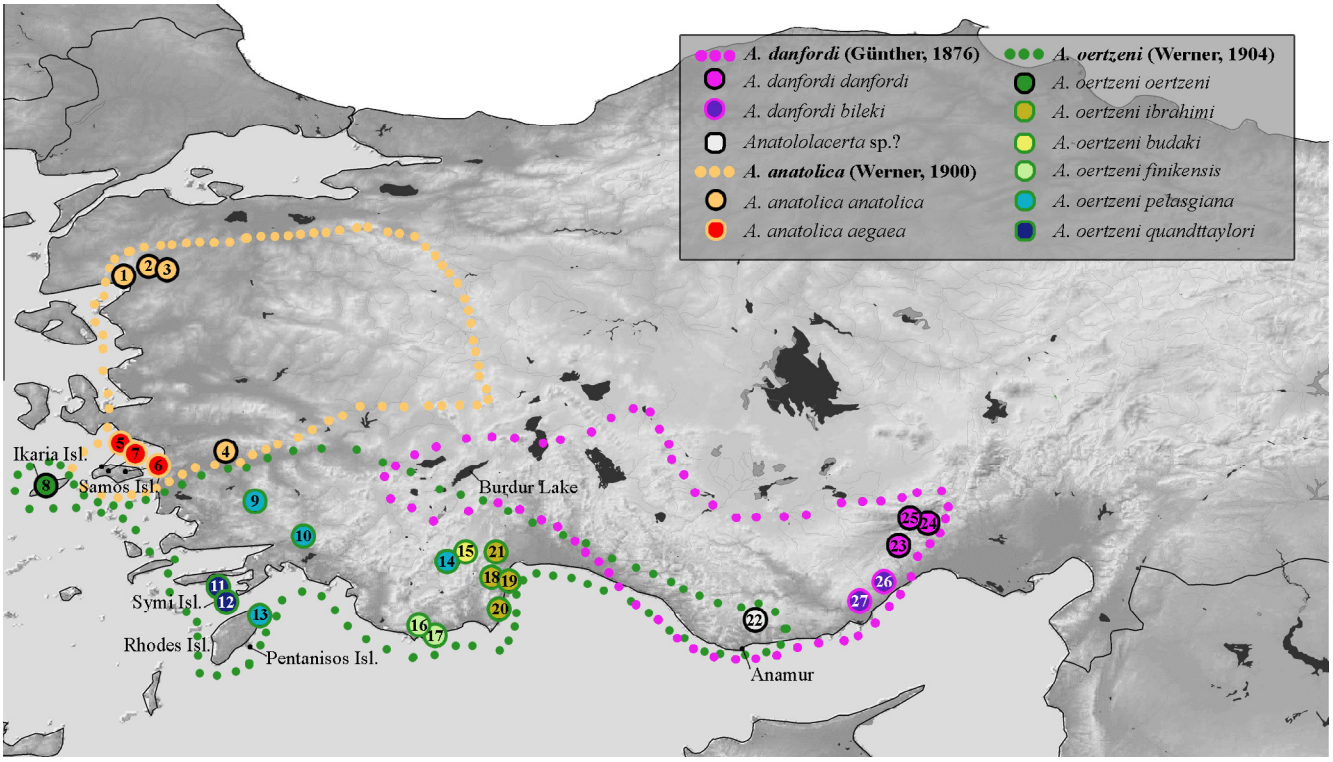

Fig. 1. Sampling localities considered in this study (circles), and approximate range (dotted lines) of the *Anatololacerta* morphospecies according to Eiselt and Schmidtler (1986): orange, *A. anatolica*; green, *A. oertzeni*; purple, *A. danfordi*. Alternative colors identify distinct morphological subspecies, whose correspondence are detailed in the box at right. Within circles, numbers refer to locality codes listed in Supplementary Table S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ibrahimi and *A. danfordi bileki* east of Antalya and around Anamur, and between *A. d. bileki* and *A. o. budaki* southwest of the Burdur Lake (Fig. 1), poses difficult taxonomic scenarios, raising uncertainty about the current taxonomy of the group. Moreover, in a chemosystematic study based on albumins, Mayer and Lutz (1989) stated: "*the biochemical differences between* Lacerta danfordi, L. oertzeni *and* L. anatolica *are too small to confirm their taxonomic revalorisation*". Following these authors, Sindaco and Jeremčenko (2008) considered all the populations as belonging to a single species, *Anatololacerta danfordi*, waiting for the taxonomy to be addressed with modern molecular techniques.

Recent evidence from the investigation of genetic variation in amphibians and reptiles acknowledges the potential role of the Anatolia region as a major refugium and a source of re-expansion for several amphibians and reptiles taxa during the Pliocene and Pleistocene (e.g. Veith et al., 2003; Kutrup et al., 2006; Joger et al., 2010; Kornilios et al., 2011; Bilgin, 2011; Ahmadzadeh et al., 2013a; Sindaco et al., 2014). Particularly in the south, the Anatolian mountains played an important role in speciation and definition of biogeographical subregions, promoting endemisms and great intraspecific genetic diversity in this area (Çıplak, 2003, 2004 and references therein). Accordingly, several cryptic genetic lineages of amphibians and reptiles showing continuous geographic distributions in Anatolia have been revealed (Kyriazi et al., 2008; Plötner et al., 2001; Akın et al., 2010; Fritz et al., 2007).

With the primary aim of assessing the real extent of genetic divergence lying within the genus *Anatololacerta*, in this study we selected informative molecular markers (both mitochondrial, mtDNA, and nuclear, nuDNA) and compared the reliability of "gene tree" vs. "species tree" approaches in solving the true relationships among closely related taxa. Our aims were to: (i) use a multi-locus coalescent-based approach to revise the current taxonomy of *Anatololacerta*; (ii) test the reliability of different phylogenetic methods to infer true relationships among taxa identified by species delimitation approaches; (iii) clarify the evolutionary history of each taxon by estimating the time of divergence of cladogenetic events and the biogeographic scenario that allowed the diversification of distinct lineages.

2. Materials and methods

2.1. Taxon sampling and laboratory procedures

We sampled 45 *Anatololacerta* museum specimens from 27 localities (hereafter locs.) distributed across the entire genus range (Fig. 1). Specimens were representative of all previously described taxa, with the exception of *A. oertzeni pentanisiensis*, from the Pentanisos islet close to Rhodes. Our data set included 11 out of the 15 "population groups" defined by Eiselt and Schmidtler (1986) (specimens from groups "g", "l", "m" and "k" were missed in our analysis). According to geographic information, 11 individuals were ascribed to *A. anatolica*, 23 to *A. oertzeni* and 8 to *A. danfordi*. The last three specimens, sampled in the only area of partial range overlap surveyed in our study (locality 22 around Anamur; see Fig. 1) were not ascribed to any taxon due to uncertain assignment. A list of all the specimens with their taxonomic assignments, sample codes, voucher codes, locality codes, and GenBank accession numbers is presented in Supplementary Table S1, including relevant information for network and species delimitation analyses.

Genomic DNA was extracted from ethanol-preserved museum specimens (approximately 2 mm^2 of muscle) using the Archive Pure DNA Tissue kit (5 PRIME Hamburg, Germany) and following manufacturer's protocol. For phylogenetic inference and genetic distance estimations, museum specimens of *Parvilacerta parva* ($N = 1$) and *Hellenolacerta graeca* ($N = 2$) were selected as outgroups based on published evidence (Carranza et al., 2004; Arnold et al., 2007). Since our aim was to identify the distinct evolutionary units within the complex which deserve species status, both mitochondrial and nuclear markers were selected, to assess the reciprocal monophyly at mtDNA markers and a significant allele frequencies divergence at nuclear loci (Moritz, 1994). In particular, 350 bp (base pairs) of the 12S ribosomal DNA (*12S*) and 420 bp of the protein-encoding cytochrome b (*cytb*) mitochondrial genes were PCR amplified with available primer pairs 12SA-12SB (Kocher et al., 1989) and GludG (Kocher et al., 1989)-Cytb2 (Palumbi et al., 1991), respectively. Two nuclear markers were also amplified for all samples, corresponding to 525 bp of the oocyte maturation factor (*c-mos*) gene and 642 bp of the melanocortin 1 receptor (*MC1R*) gene, by selecting already published primer pairs Lsc1F-Lsc2R (Godinho et al., 2005) and MC1RF-MC1RR (Pinho et al., 2010), respectively. All the amplifications were performed in 20 μl-volume reactions containing 10X PCR Buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, each primer 0.2 μM, 0.5 U Master*Taq* enzyme polymerase (5PRIME, Hamburg, Germany) and approximately 1 μl of genomic DNA (see Supplementary Table S2 for details on primers' sequences and marker-specific thermal profiles). Amplicons were sequenced by Macrogen Europe Inc. (Amsterdam, The Netherlands) using the same amplification primers.

2.2. Sequences analysis

Chromatograms were imported in Geneious 5.3.6 (Biomatters Ldt.) and checked manually for insertions or deletions (indels) and ambiguous positions in protein-encoding mitochondrial gene fragments. Nuclear sequences were assembled and edited with the same software. All protein-encoding fragments (i.e. *cytb*, *c-mos* and *MC1R*) were translated into amino acidic sequences to exclude the presence of non-functional copies of target markers (i.e. pseudogenes) in the dataset, which can be detected by premature stop codons or non-sense codons occurring in the coding frame. Sequences were aligned for each gene independently with the online version of MAFFT (Katoh and Toh, 2008) applying parameters by default (Auto strategy, Gap opening penalty: 1.53, Offset value: 0.0). Nuclear sequences (*c-mos* and *MC1R*) with multiple heterozygous single-nucleotide polymorphisms (detected in the presence of two peaks of approximately equal height at a single nucleotide site) were resolved using the coalescent-based Bayesian method implemented by the software PHASE 2.1 (Stephens et al., 2001; Stephens and Scheet, 2005). The on-line web tool SeqPHASE (available at http://www.mnhn.fr/jfflot/seqphase/, Flot, 2010) was used to generate input files, then 3 runs with different seeds for the random-number of generator were performed, checking for consistent gametic phase estimation through runs according to the goodness-of-fit values. All the alternative alleles were estimated with high probability (>0.9), coding polymorphic sites with a probability of <0.9 with the appropriate IUPAC ambiguity code in both alleles. Finally, individual sequences were merged into single gene haplotypes using the on-line web tool DnaCollapser 1.0 available at FaBox site (http://www.birc.au.dk/fabox) to calculate the number of variable sites in each gene alignment, including both parsimony informative (*Pi*) and singletons. Mitochondrial haplotypes were used to estimate pairwise genetic distances (*p*-distance) between clades and species subsequently identified using MEGA 5.2 (Tamura et al., 2011).

2.3. Phylogenetic gene tree and haplotype network reconstructions

Phylogenetic relationships among individuals were initially inferred by standard Bayesian Inference (BI), using the concatenated mtDNA + nuDNA (unphased) alignment with *Parvilacerta* and *Hellenolacerta* as outgroups, and partitioning sequences

(nearly 1940 bp long) in order to estimate appropriate model parameters separately for each gene subset. Best-fit models of nucleotide substitution were assessed by means of Bayesian Information Criterion (BIC) as implemented in jModeltest 2.1 (Darriba et al., 2012), estimating also Γ-distributed rates among sites (Uzzell and Corbin, 1971; Yang, 1994) or a proportion of invariant sites, or a combination of the two (Gu et al., 1995; Waddell and Steel, 1997) to describe rate heterogeneity among sites (see Supplementary Table S3 for models' specifications).

Bayesian analyses were performed using the Markov chain Monte Carlo (MCMC) method in MrBayes 3.2 (Ronquist et al., 2012), running two independent analyses consisting in four MCMC chains each one. Each analysis was run 20×10^6 generations sampling each 10^3 generation. Convergence of chains upon a stationary distribution and appropriate sampling were assessed by monitoring the standard deviation of split frequencies between the two simultaneous runs (<0.002) and the potential scale reduction factor (PSRF) diagnostic (= 1.000). Distributions of log-likelihoods and parameter estimates were examined in TRACER 1.5 (available at http://beast.bio.ed.ac.uk/Tracer, Rambaut and Drummond, 2003) to determine the burn-in threshold after which MCMC runs converged (corresponding to the first 20% of generations). After discarding the burn-in, a majority-rule consensus tree was generated and visualized in FigTree 1.3.1 (available at http://tree.bio.ed.ac.uk/software/figtree, Rambaut, 2009). Nodes were considered strongly supported if posterior probability support values were $\geqslant 0.95$ (Huelsenbeck and Rannala, 2004).

Relationships among haplotypes were visualized by reconstructing statistical parsimony haplotype networks for the concatenated mtDNA dataset and for each nuDNA marker, selecting the 95% connection limit as a reliable parsimony threshold as implemented in TCS 1.21 (Clement et al., 2000). This procedure allowed examining the extent of haplotype sharing among taxa previously described only on morphological basis as well as among evolutionary divergent units identified by our species delimitation approaches, under the statistical parsimony method (Templeton et al., 1992). Accordingly, the maximum numbers of mutational steps (pairwise nucleotide differences) constituting a parsimonious connection between two haplotypes were calculated (Posada and Crandall, 2001; Templeton, 2001). In the mitochondrial analysis, gaps were treated as a fifth state. Although the 95% threshold does not necessarily correspond to species boundaries, also because distinct species may still share the same mtDNA as a result of (past) hybridization events, it is often assumed to separate groups that roughly coincide with named species or species groups (Hart and Sunday, 2007). Therefore, when distinct networks were identified in the analyses, we also assessed the minimum number of mutational steps required to join them together, although in a non-parsimonious (i.e. non-significant) way.

2.4. Coalescent-based species tree and species delimitation approaches

We also applied the coalescent-based approach (i.e. species tree, Edwards, 2009) coupled with species delimitation methods, in order to resolve the taxonomy of the genus *Anatololacerta*. Interestingly, the multi-locus coalescent-based method implemented in *BEAST (Heled and Drummond, 2010) is considered to outperform concatenated data sets in the reconstruction of phylogenetic relationships (Liu et al., 2009) as it takes into account the gene tree variation in the phylogenetic inference (Degnan and Rosenberg, 2009; Knowles, 2009; Heled and Drummond, 2010). Since *BEAST analysis requires the *a priori* definition of putative species to infer phylogenetic relationships among them, we adopted a three-stage procedure for delimiting *Anatololacerta* species: briefly, (i) individuals were firstly assigned to putative groups by using the Generalized Mixed Yule-coalescent analysis (GMYC; Pons et al., 2006); (ii) secondly, phylogenetic relationships among GMYC units were assessed by inferring a multi-locus Bayesian species tree (using *BEAST); (iii) finally, a nuDNA-based Bayesian species delimitation analysis as implemented in Bayesian Phylogenetics & Phylogeography (BP&P; Rannala and Yang, 2003; Yang and Rannala, 2010) was performed to validate species identified by previous methods (see Supplementary Table S3 for details on single gene substitution models, priors and parameter specifications). Specifically, the General Mixed Yule-coalescent (GMYC) approach allowed us to identify putative species boundaries, assessed as a shift in branching rates from a Yule (interspecific) to a coalescent (intraspecific) model on an ultrametric tree that contains multiple populations (Pons et al., 2006; Monaghan et al., 2009). The likelihood peaks of such transitions between cladogenesis (i.e. interspecific diversification) and allele intraspecific coalescence were also estimated along the branches. As this method relies on single-locus phylogenies, and given the lower sequence variability of nuclear data, we conducted the GMYC analysis onto the mtDNA concatenated alignment considering only unique haplotypes. The ultrametric mtDNA tree (excluding outgroups) was generated with BEAST 1.6.2 (Drummond and Rambaut, 2007). Model and prior specifications applied were as in Supplementary Table S3. The substitution rate was fixed to one, that is, no calibrations were used as our aim was to estimate the branching rates only. Three BEAST runs of 5×10^7 generations were performed, sampling every 10^4 steps. Convergence was evaluated reading the log files with TRACER 1.5 to verify that the effective sampling size (ESS) values were adequate (>1000), then trees were combined with Logcombiner and summarized in a maximum credibility tree with TreeAnnotator (available in BEAST package at http://beast.bio.ed.ac.uk/). Species delimitation analyses were conducted in R Version 3.0.1 (R Core Team, 2013) using the 'SPLITS' (Species Limits by Threshold Statistics, Ezard et al., 2009) package available at http://r-forge.r-project.org/projects/splits. The single threshold algorithm was applied and compared to the null model (i.e. all individuals belong to a single species cluster) using a log-likelihood ratio test as implemented in the GMYC package. A lineage-through-time plot as produced by the software was visually evaluated for changes in branching rate. We then used the hierarchical Bayesian model implemented in *BEAST to estimate a multi-locus species tree for the putative groups identified by GMYC (i.e. considering the complete data set of two mitochondrial and two nuclear genes excluding outgroups). Model and prior specifications applied to the *BEAST analysis were as in Supplementary Table S3. Each run of 5×10^7 generations was repeated three times, sampling every 10^4 steps, setting unlinked clock models across loci. Convergence and adequate effective sample size (ESS) were checked using TRACER 1.5, then independent analyses were combined using Logcombiner, discarding the first 10% of each run as burn-in. The species tree was summarized using TreeAnnotator and visualized with FigTree 1.3.1.

Finally, we performed a multi-locus coalescent species delimitation analysis with the phased dataset for the two nuclear loci using the Bayesian specie tree-based method implemented in BP&P 2.2, which uses a reversible jump Markov chain Monte Carlo (rjMCMC) approach to calculate the posterior probabilities of competing models that contain greater or fewer lineages, accommodating confounding processes like ILS because of ancestral polymorphism, as well as uncertainties due to unknown gene trees (Yang and Rannala, 2010). Following this method, distinct species entities were identified by the software according to the biological species concept (BSC, i.e. as members of populations that actually or potentially interbreed in nature) using a species phylogeny represented by a user-specified guide tree (Yang and Rannala, 2010). The guide tree, which specifies the relationships among the species included in the analysis and guides the Markov chain, plays a crit-

ical role in the outcome of the species delimitation model, even moderate changes sometimes impacting the support for models (Leaché and Fujita, 2010). To test the reliability of putative species identified by previous approaches (GMYC, *BEAST), we therefore selected the guide tree generated from species tree analyses, but we also repeated the analysis using an alternative topology where clade III and V were treated as sister clades.

We ran rjMCMC analyses for 250,000 generations, sampling interval of five. Both algorithms 0 and 1 implemented in BP&P were used, assigning each species delimitation model equal prior probability. Since the prior distributions on the ancestral population size (θ) and root age (τ_0) can affect the posterior probabilities for models (with large values for θ and small values for τ_0 favoring conservative models containing fewer species, Yang and Rannala, 2010) and since no empirical data were available for the studied species, we ran the species delimitation analyses by considering different combinations of priors (Leaché and Fujita, 2010) (see Supplementary Table S3 for specific of priors and burn in values). Marginal posterior probabilities associated with each bifurcation in the guide tree were estimated by summing the probabilities for all models that support a particular speciation event in the guide tree. A probability of 1 on a node indicates that every species delimitation model visited by the rjMCMC algorithm supports the two lineages descending from that node as species. Conversely, a speciation probability of 0 reflects the situation where all of the species delimitation models in the posterior distribution collapsed that particular node to one species. We then considered speciation probability values $\geqslant 0.95$ as strong support for a speciation event.

2.5. Estimation of divergence times

The lack of internal calibration points in *Anatololacerta* precluded the direct estimation of the timing of cladogenetic events in our phylogeny. We used for this purpose the mean substitution rates and standard errors for the same *12S* and *cytb* gene regions used in the present study, that have been calculated for the Canary Islands radiation of lacertid lizards of the endemic genus *Gallotia*, applied under an uncorrelated lognormal clock model (see Appendix II in Carranza and Arnold, 2012). Specifically, we set a normal distribution prior for the ucld.mean parameter of the *12S* and *cytb* partitions based on the meanRate posterior (mean and standard error) of the calibration analysis of *Gallotia* (0.00553 ± 0.00128 for the *12S* and 0.0164 ± 0.00317 for the *cytb*) (see Carranza and Arnold, 2012). Estimation of divergence times was carried out with *BEAST (see Section 2.4) with models, priors and parameter specifications as in Supplementary Table S3.

3. Results

3.1. Patterns of sequence variation within the Anatololacerta species complex

In the final concatenated alignment of our 45 *Anatololacerta* specimens (i.e. 2 mitochondrial and 2 nuclear gene fragments; 1937 bp), 116 sites were variable and 98 parsimony informative (*Pi*), while considering the outgroups (1939 bp) 253 variable sites were identified (*Pi* = 186). The resulting mitochondrial data set, excluding the outgroups, contained 770 bp of which 96 were variable and 83 parsimony informative. Nor gaps neither premature stop codons were found in protein-encoding gene fragments. Concerning nuclear data, the number of polymorphic sites, excluding the outgroups, were 8 and 12 variable sites (out of which 7 and 8 *Pi*) for *c-mos* and *MC1R*, respectively. The newly sequenced samples returned 24 unique haplotypes, when the concatenated *12S* + *cytb* alignment was considered. Altogether 18 gametic-phased haplotypes were found for *MC1R* when the high-probability phasing threshold was adopted (>0.9). The *c-mos* fragment was less variable, with 16 distinct haplotypes identified in the phased alignment (see Supplementary Table S1 for all haplotypes codes).

3.2. Phylogenetic gene tree and genetic differentiation between Anatololacerta clades

The Bayesian phylogenetic tree, obtained by considering the concatenated alignment (i.e. 1939 bp) reveals five clades that mainly reflect variation according to the spatial distribution of the specimens rather than their taxonomic designation (Fig. 2). Clade I includes northwestern *Anatololacerta* populations (locs. 1–8, Fig. 1) morphologically ascribed to *A. anatolica* (including *A. a. aegaea* from Samos Island, locs. 5–7) and specimens sampled in loc. 8 from Ikaria Island, which is the type locality of *A. oertzeni*. In contrast, southwestern populations are assigned to two well distinct clades: one (clade II) includes specimens classified as *A. o. pelasgiana* (locs. 9–14, Fig. 1) both from the mainland (locs. 9, 10 and 14) and Rhodes Island (loc. 13), together with *A. o. quandttaylori* specimens sampled from Symi Island (locs. 11–12); the other (clade III) includes only mainland specimens (locs. 15–21, Fig. 1) classified as *A. o. budaki* (loc. 15), *A. o. finikensis* (locs. 16–17) and *A. o. ibrahimi* (locs. 18–21). Finally, the easternmost specimens morphologically ascribed to *A. danfordi* (locs. 23–27, Fig. 1), including *A. d. danfordi* (locs. 23–25) and *A. d. bileki* (locs. 26–27) belong to clade IV. Interestingly, specimens sampled at locality 22, where the ranges of *A. oertzeni* and *A. danfordi* partially overlap, are assigned with high support to a small but well-differentiated distinct clade V, together with one specimen of *A. danfordi* (1501) from locality 25 (Figs. 1 and 2). Although distinct clades show high support according to Bayesian posterior probability values, only the sister taxa relationships between clades III and V is recovered, while the others appear overall poorly resolved by the standard gene tree phylogenetic approach.

Average uncorrected divergences (*p*-distance) at mtDNA loci among the five clades identified by our gene tree approach are 7.0% for *cytb* (from 3.1%, between clades III and V to 8.2% between clades II and IV) and 2.1% for *12S* (from 0.6% between clades III and V and 3.3% between clades II and IV) (Table 1). Considering each clade separately, different degrees of intra-clade structuring can be recognized, generally consistent with the geographic distribution of haplotypes. The most striking observations are the clear genetic affinity of specimens morphologically ascribed to *A. oertzeni* (nominal form, locality 8 from Ikaria Island) with those morphologically classified as *A. anatolica* within clade I, as well as the deep divergence of specimens previously assigned to *A. o. pelasgiana* and *A. o. quandttaylori* (clade II) from those ascribed to the other *A. oertzeni* subspecies from clade III (Fig. 2).

3.3. Haplotype network reconstruction

Statistical parsimony produces five independent mtDNA (*12S* + *cytb*, 770 bp) networks (named 1–5; Fig. 3a) when applying a 95% connection limit (12 mutational steps), which correspond to clades I–V identified by Bayesian gene tree phylogenetic inference (see Fig. 2). Within network 1, private haplotypes are observed for the three morphological taxa *A. a. anatolica* (A01), *A. a. aegaea* (A02, A03) and *A. oertzeni oertzeni* (A04, A05). Interestingly, the latter taxon from Ikaria Island appears to be well differentiated from haplotypes found in "*anatolica*" populations from northwestern Anatolia, including those from the very close Samos Island. Network 2 includes private haplotypes from specimens ascribed to *A. oertzeni pelasgiana* (A06 to A08, A10, A11) and *A. o. quandttaylori* (A09). Morphological representatives of *A. o. budaki*, *A. o. ibrahimi*

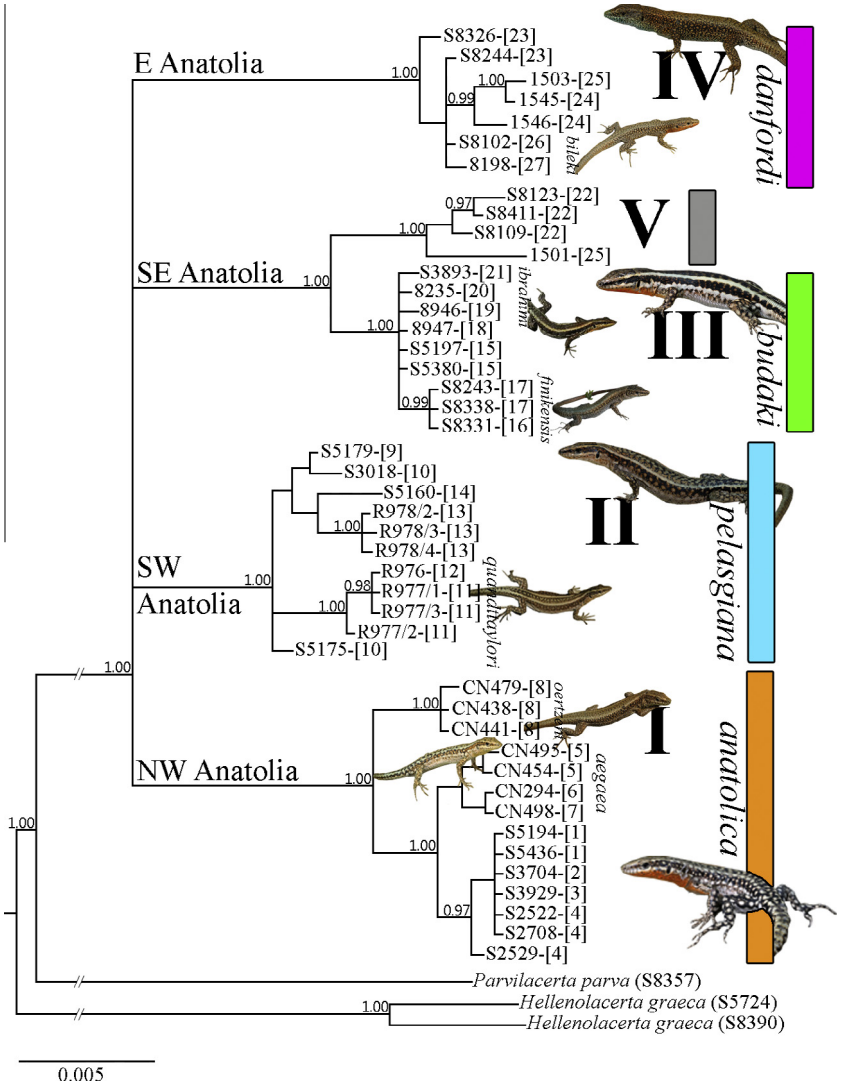

Fig. 2. Bayesian phylogenetic gene tree of the *Anatololacerta* species complex inferred from 1939 bp of two mtDNA (*cytb*, *12S*) and two nuDNA (*MC1R*, *c-mos*) loci, revealing five evolutionary lineages (i.e. clades I–V). Posterior probabilities are shown above nodes only if >0.95. Numbers in square parenthesis behind taxa refer to sampling localities shown in Fig. 1 and listed in Supplementary Table S1.

Table 1
Uncorrected *cytb* (below the diagonal) and *12S* (above the diagonal) pairwise (*p*) genetic distances between the five clades (I–V, see Fig. 2) identified by our gene tree phylogenetic approach. Intra-clade variation is given (in italic) along the diagonal for both markers (*cytb*, left side; *12S*, right side). n/c = not calculated.

ID	[I]	[II]	[III]	[IV]	[V]	[Ppa]	[Hgr]
Clade I [I]	*1.1/0.6*	2.0	2.1	1.7	2.5	8.0	8.9
Clade II [II]	7.3	*0.9/0.6*	3.0	2.6	3.3	8.0	8.9
Clade III [III]	6.7	6.7	*0.5/0.3*	1.6	0.6	8.7	10.2
Clade IV [IV]	8.1	8.2	7.6	*0.6/0.0*	1.9	8.3	9.7
Clade V [V]	7.2	6.9	3.1	8.1	*0.8/0.7*	8.8	10.2
Parvilacerta parva [Ppa]	20.0	18.3	18.4	18.8	18.8	*n/c*	9.7
Hellenolacerta graeca [Hgr]	18.3	18.3	19.1	19.1	18.4	20.5	*3.8/1.1*

and *A. o. finikensis* belong to network 3 (A12 to A15), whereas network 4 joins together *A. danfordi bileki* with the nominal subspecies *A. d. danfordi* (A18 to A21, A23, A24). Unclassified samples from locality 22 (A16, A17) join together with haplotype A22 from locality 25 (morphologically *A. danfordi*) in network 5, which was disconnected from network 3 by only one mutational step more than the threshold limit (i.e. they join together at 94% parsimony threshold). In contrast, the highest number of mutational steps required to connect two distinct networks is 36 steps (between networks 2 and 3). Interestingly, these two networks should be classified as a single species (*A. oertzeni*) according to morphological taxonomy.

Considering only nuclear variation, most of the observed polymorphism contributes to the differentiation of specimens assigned to phylogenetic clades I and II, since relative private haplotypes were found in both nuDNA markers considered in our study (mainly in *MC1R* for clade I and in *c-mos* for clade II, Fig. 4b and c). Higher levels of allele sharing occur among the other three clades

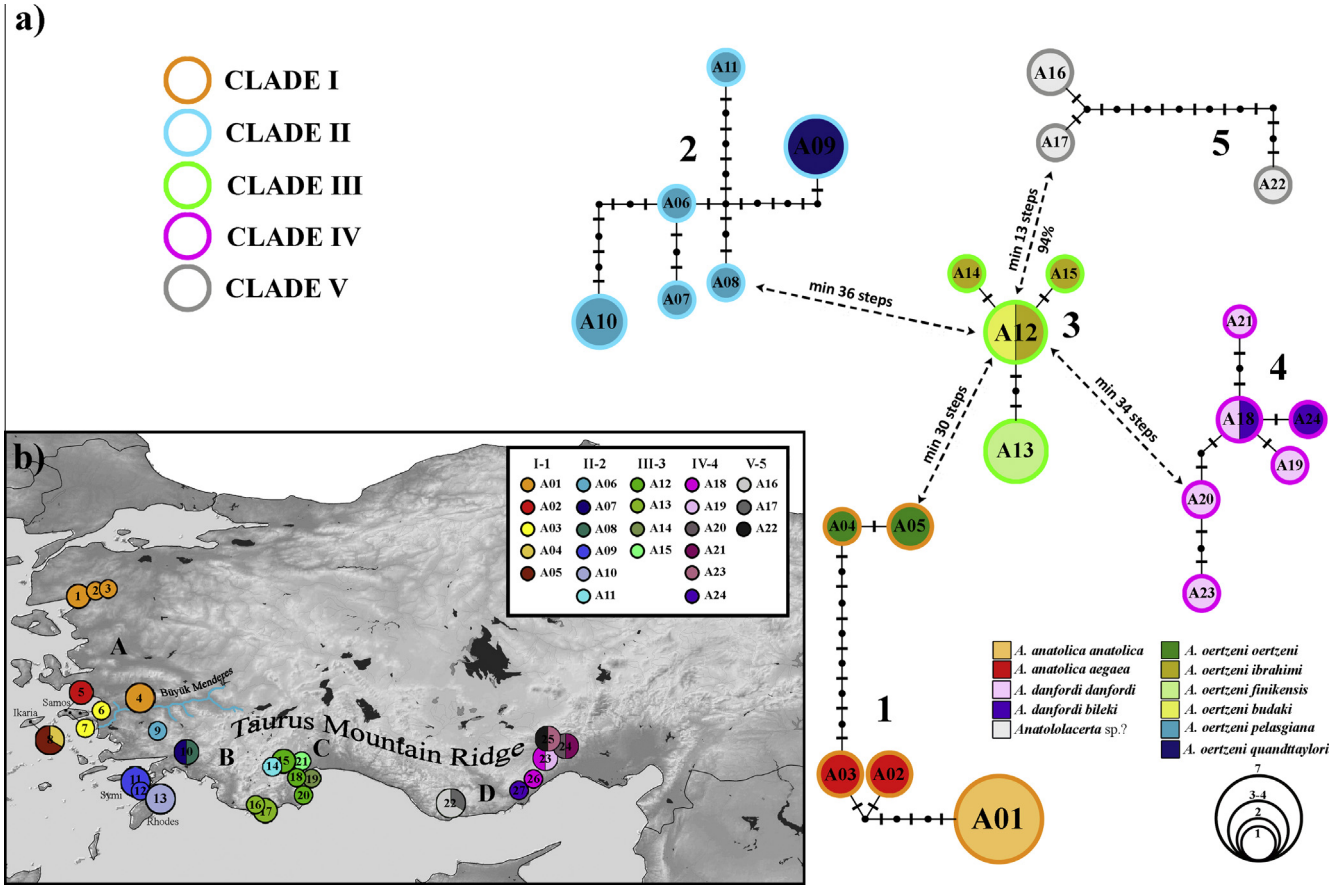

Fig. 3. (a) Unrooted mtDNA haplotype network of concatenated *12S + cytb* sequences (770 bp). Circle size is proportional to haplotype frequency (i.e. the number of samples sharing the same haplotype). Bars correspond to one single point mutation, while dots are missing (i.e. extinct or unsampled) haplotypes. The minimum number of connections required to join single networks (95% cut-off) is also given using double-arrowed dashed lines. In distinct networks, circles have been colored according to the morphological assignment of specimens, while alternative colors of the outlines indicate different clades identified by our gene tree phylogenetic reconstruction (BI) (see Fig. 2). (b) Distribution of mtDNA haplotypes (concatenated *12S + cytb*) across our sampling area. Each haplotype is represented by a distinct color, while numbers within circles identify sampling sites listed in Supplementary Table S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

according to parsimony network analyses, particularly between clades IV and V, which should belong to the same species according to subsequent *BEAST and BP&P species delimitation analyses (see Section 3.4.). Overall, the observed patterns of mtDNA and nuDNA variation agree only partially, the latter showing allele sharing particularly between some clades (III, IV and V), thus suggesting possible incomplete lineage sorting or gene flow processes especially among southern and eastern *Anatololacerta* populations.

3.4. Multi-locus species tree and species delimitation within *Anatololacerta*

The ultrametric tree resulting from the BEAST analysis of the mtDNA data set (excluding the outgroups) provides strong support for the persistence of clades I–V (all posterior probabilities = 1.00), although sister relationships among them remain unresolved (not shown). The GMYC analysis (i.e. single threshold model, only mtDNA-based) recovers clades I–V as effective putative species, according to the lineage-through-time plot and the likelihood function estimated by the software (LR = 6.051984, $P < 0.05$; Fig. 5). Interestingly, the *BEAST analysis performed by treating clades I–V as five separate putative species supports the presence of only 4 distinct taxa (Fig. 4a). Indeed, previously identified clades I, II and III correspond to three distinct species (hereafter named A, B and C), while clades IV and V are sister lineages belonging to the same species (hereafter named D). The posterior probability (pp) of this relationship exceeding 0.99 implies that virtually all species trees in the posterior distribution had clade IV and V monophyletic. Moreover, in the maximum clade credibility species tree, species A (previous clade I) is sister to all the other *Anatololacerta* lineages (pp = 1.00). Therefore, the species tree provides a substantial difference compared to the previous gene tree (Fig. 2), since the taxonomically ambiguous clade V, previously recovered as a sister lineage of clade III by Bayesian analysis and as a distinct putative species by the mtDNA-based GMYC approach (Figs. 2–5c), here shows a highly supported sister relationships with clade IV (Fig. 4a).

Finally, the BP&P analysis (nuDNA-based only) supports the guide tree topology of the multi-locus coalescent-based species tree (i.e. species A–D, Fig. 6b). Again, species A, B and C are recovered as distinct entities with speciation probabilities of 1.00 on all nodes, whereas the split between clades IV and V is no longer supported (= 0.30). Noteworthy, different algorithms or prior distributions for θ and τ_0 did not affect this outcome (results not shown). Similarly, the alternative tree topology did not support monophyly for clades III and V, which should be regarded as distinct species according to the nuclear-based Bayesian species delimitation approach (results not shown).

Uncorrected genetic variation (*p*-distance) at mitochondrial loci appear highly comparable when calculated across the four distinct species (A–D) identified by multi-locus species tree and species delimitation approaches (Table 2). Particularly, species diverge from 6.1% (species C vs. D) to 7.8% (species A vs. D and species B

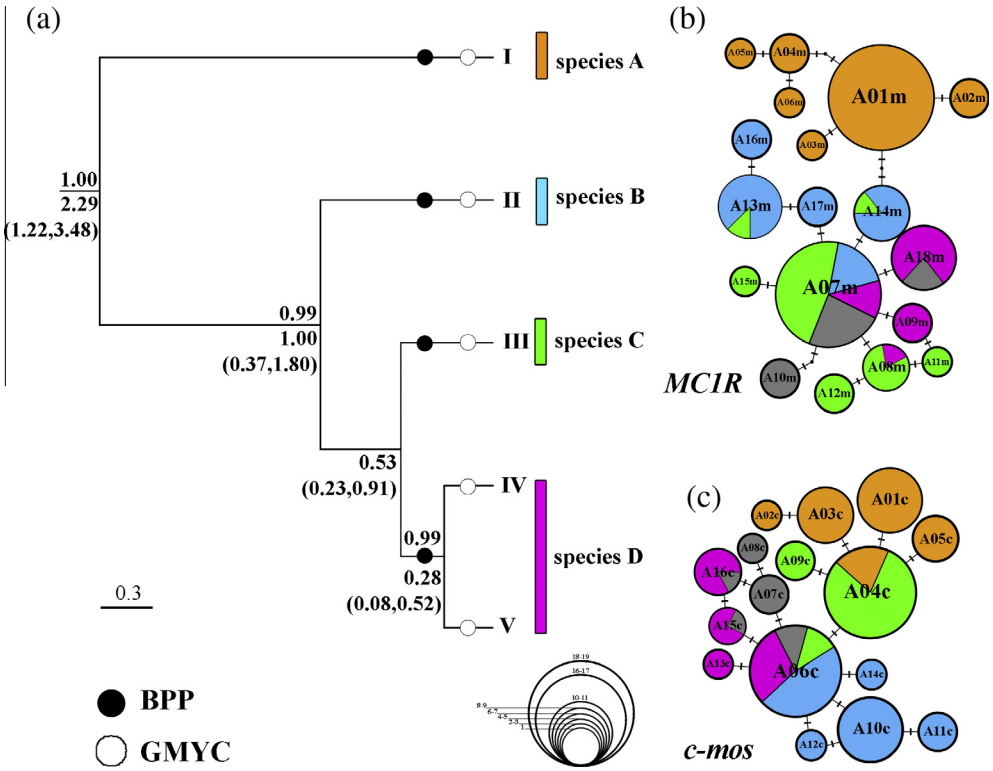

Fig. 4. (a) Dated species tree inferred in *BEAST considering two mtDNA (*12S*, *cytb*) and two nuDNA (*MC1R*, *c-mos*) loci, with posterior probabilities reported above each node. For the analysis, specimens have been assigned to putative species based on the GMYC species delimitation result (see Fig. 5c). Circles on branches indicate taxa recognized by the GMYC (white) and the BP&P (black) species delimitation analyses. Mean node ages (My) are reported below nodes with 95% highest posterior density (HPD) interval (in brackets). (b and c) Unrooted haplotype networks of both nuclear markers used in this study. Circle size is proportional to haplotype frequency (i.e. the number of individuals sharing the same haplotype). Bars correspond to one single point mutation, while dots are missing (extinct or unsampled) haplotypes. The minimum number of connections required to join independent networks (95% cut-off) is also given using double-arrowed dashed lines. Colors reflect the phylogenetic clade in which each haplotype occurs (i.e. I–V, see Fig. 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vs. D) for *cytb*, and from 1.0% (species C vs. D) to 3.0% (species B vs. C and species B vs. D) for *12S*. Considering each species separately, it is worth noticing that some, particularly species D, are characterized by high intraspecific variation (*cytb*: 4.3%; *12S*: 1.3%), according to the presence of well-differentiated genetic lineages (i.e. previously identified mitochondrial clades IV and V), while others, as for instance species C, appear highly homogeneous (*cytb*: 0.4%; *12S*: 0.3%). Only two species (A and B) show comparable intraspecific values (*cytb*: 1.1% and 0.9%, respectively; *12S*: 0.6% for both).

3.5. Molecular dating of speciation events

The results of the dating analysis indicate that diversification in *Anatololacerta* started approximately 2.29 Ma (95% HPD 1.22–3.48 Ma) and that species B–D originated between 1 and 0.53 Ma (see Fig. 4a). According to the clear differentiation recovered by both mtDNA and nuDNA data, species A split first within the genus, definitely predating the speciation of other taxa. Similarly, the high differentiation of species B agrees with our dating estimation of speciation events (1 Ma, 95% HPD 0.37–1.80 Ma), while separations of species C and D appear definitely more recent (less than 1 Ma, see Fig. 4a).

4. Discussion

4.1. Phylogenetic resolution of the Anatololacerta species complex

In the present study, we provide the first comprehensive and robust assessment of the phylogenetic relationships within the *Anatololacerta* species complex, according to both mitochondrial and nuclear data. In order to clarify the intra-specific taxonomy of the numerous taxa described on morphological ground, we applied both the traditional single-locus and the modern multi-locus coalescent-based methods for phylogenetic inference and species delimitation. Our results depict a very high genetic variability which lies within the genus, despite its relatively small geographic range, limited to western and southern Anatolia and some Aegean islands. Actually, *Anatololacerta* is a very diversified taxon with a complex evolutionary history.

The traditional phylogenetic reconstruction (BI) highlights the presence of five major monophyletic clades (I–V), showing high statistical support for each group (posterior probabilities >0.95) and perfectly matching the main mitochondrial lineages occurring within our data set (Figs. 2 and 3a and b). According to mtDNA variation, genetic divergence within each clade (and species) is very low, especially compared to variation between them (Tables 1 and 2). The lowest genetic distance occurs between clades V and III, while both clades I and II (species A and B, respectively) appear well-differentiated from all the others, suggesting restricted gene flow among populations due to physical or ecological barriers. Particularly, the occurrence of different mtDNA networks suggests that the five lineages have been genetically isolated for a long time in multiple distinct refugia probably located on warm mountainous areas near the coast. At least in one case (clade I/species A), the genetic divergence of northwestern populations could be further explained by the presence of a physical barrier isolating them from southern populations: the valley of the Büyük Menderes River, rising in west-central Turkey near Dinar and then flowing 560 kilometers west through the "Büyük Menderes graben" down to the Aegean Sea near the ancient Ionian city Miletus (Figs. 3b and 6a).

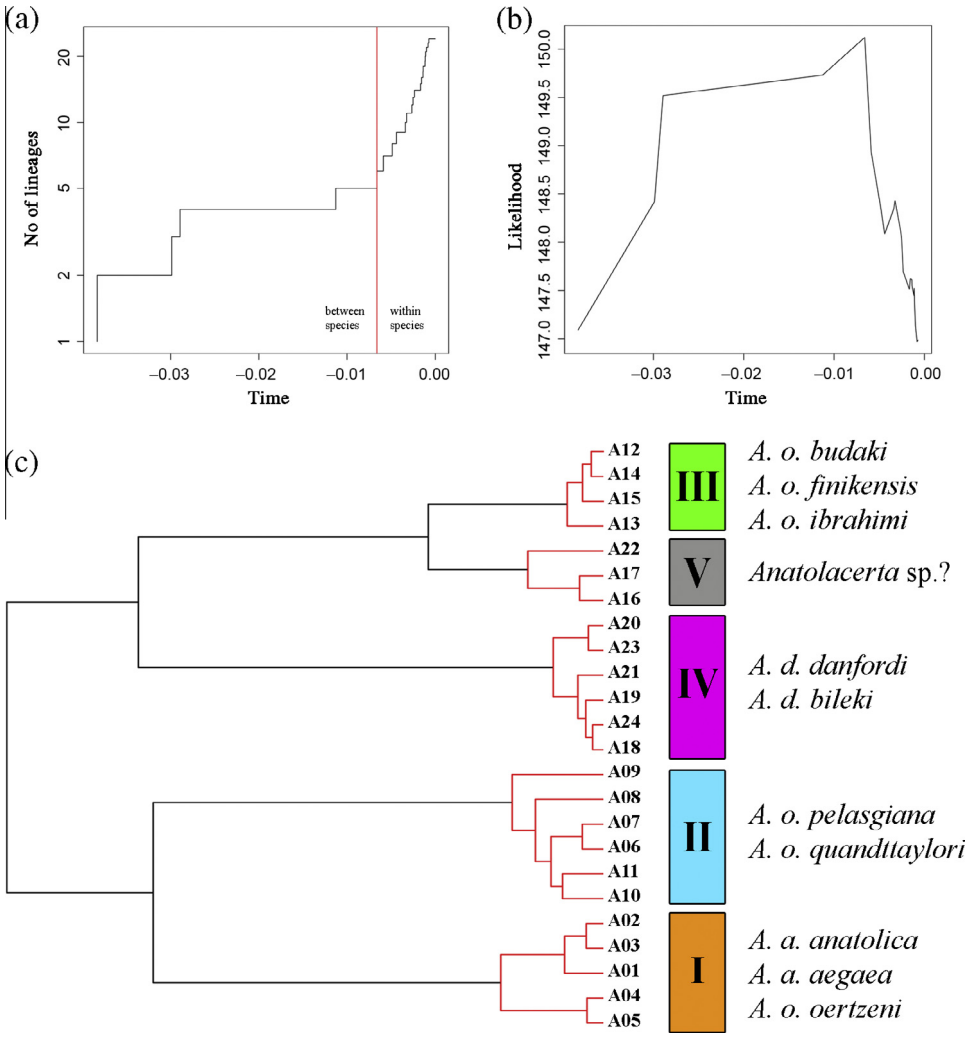

Fig. 5. Results of the species delimitation analysis according to the GMYC single-threshold model (mtDNA-based only). (a) Lineage-through-time plot based on the ultrametric tree obtained from all mtDNA haplotypes. The sharp increase in branching rate, corresponding to the transition from interspecific to intraspecific branching events, is indicated by a red vertical line; (b) likelihood function produced by GMYC to estimate the peak of transition between cladogenesis (interspecific diversification) and allele intraspecific coalescence along the branches and (c) ultrametric tree obtained in BEAST setting coalescent prior and relaxed log-normal clock model for concatenated mtDNA. Putative species are colored in red on the tree. Information concerning haplotype codes are listed in Supplementary Table S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The use of two mitochondrial sequences, even of relatively short length, is generally considered to provide adequate information to point out the occurrence of intra- vs. inter-specific relationships in reptile phylogenies (e.g. Beukema et al., 2010; Carranza et al., 2006; Lymberakis et al., 2007; Poulakakis et al., 2005; Rato et al., 2010; Vasconcelos et al., 2010). Nevertheless, phylogenetic relationships between the five clades appear unresolved according to the standard mtDNA + nuDNA gene tree approach (BI), suggesting either a possible loss of information in nuclear gene variation, which only partially corroborated the subdivisions observed according to mtDNA only, or a lack in our data set of unsampled phylogenetic lineages, some of which could even be extinct, together with the possibility of multiple and simultaneous divergence events. Interestingly, our species delimitation within the *Anatololacerta* complex using multi-locus data reveals the potential for recognizing at least four genetically distinct species. Their non-overlapping geographic distributions, and the strong association of both nuclear and mitochondrial genetic diversity with the geographic pattern suggest a history of allopatric divergence within the species complex. Even the present geographic scenario, in which distinct species partially overlap their ranges, may be the outcome of secondary contacts following post-glacial recolonization of previously unsuitable regions by populations that spread from their allopatric refugia in recent times. Therefore, we conclude that new cryptic lineages could have become isolated from each other as a result of habitat fragmentation, which drove allopatric speciation. Cryptic genetic lineages occur in other reptilian or amphibian species with continuous geographic distribution in Anatolia. For instance, *Ophisops elegans* shows four distinct genetic lineages in Anatolia (Kyriazi et al., 2008). The Anatolian water frogs exhibit a similar pattern with various lineages having been identified (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 specific level, and were also confirmed in some cases by a thorough morphological approach (Türkozan et al., 2010). In Anatolia, four well-supported mtDNA lineages of *Typhlops vermicularis*, corresponding to respective refugia, have been recently identified (Kornilios et al., 2011), and four main lineages, corresponding to three species and one subspecies, have been found in Turkish *Blanus* (Sindaco et al., 2014).

Controversies may arise with the delimitation of allopatric species, owing to the difficulties associated with assessing properties

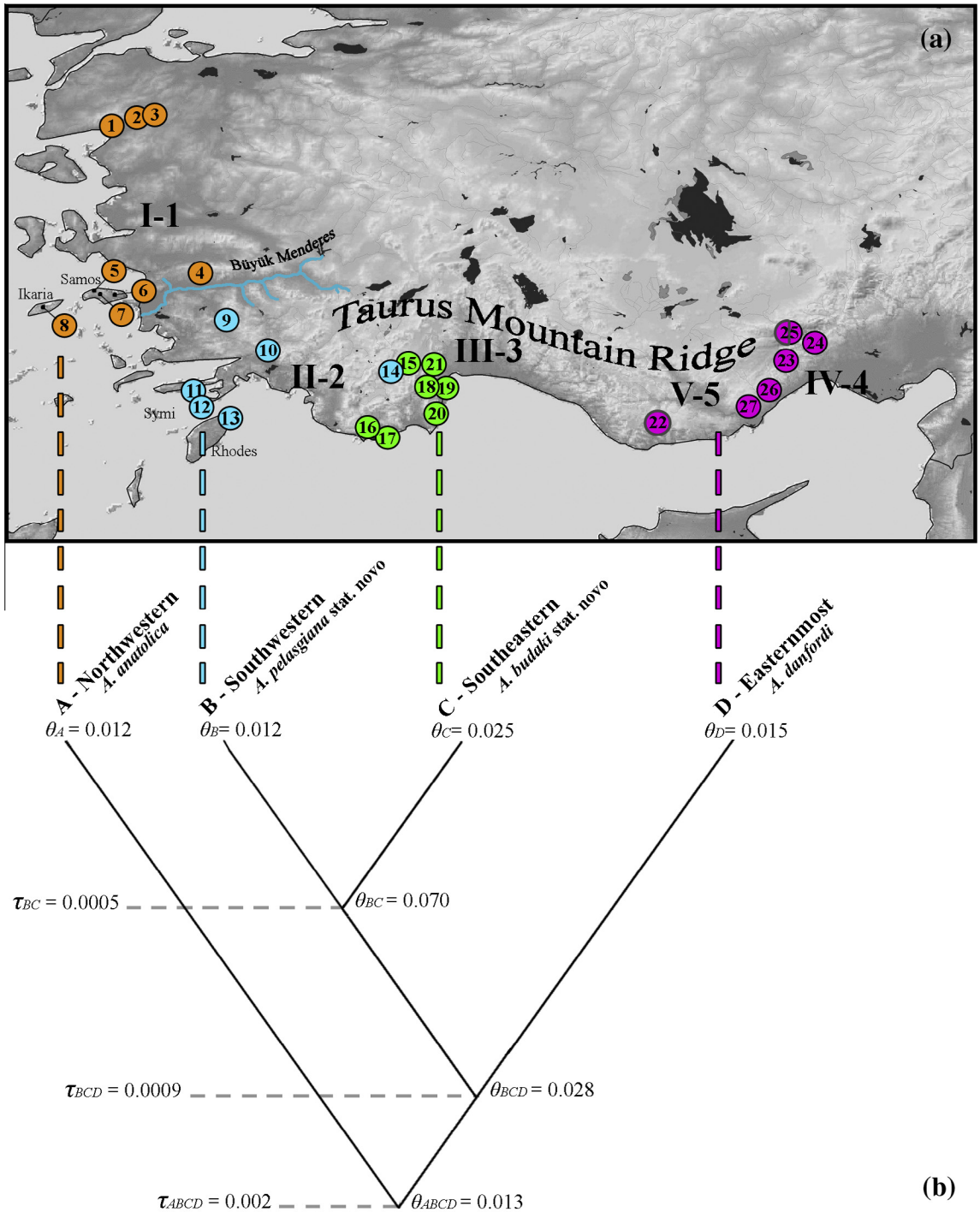

Fig. 6. (a) Distribution of the four distinct *Anatololacerta* species identified by multi-locus coalescent species tree approach and supported by Bayesian species delimitation analysis. Indication of the 5 distinct phylogenetic clades identified by gene tree approach (i.e. I–V, Fig. 2) corresponding to the 5 main mtDNA lineages (i.e. 1–5, Fig. 3a) is also given for comparison; gray outlines indicate the geographic locations where specimens assigned to the doubtful clade V have been sampled and (b) results of the species delimitation analysis according to the Bayesian species delimitation approach of BP&P (nuDNA-based only). The posterior estimates (mean of the distribution) for θ and τ are provided on the tree.

inherent to the biological species concept (BSC), such as natural reproduction resulting in viable and fertile offspring and intrinsic reproductive isolation (Mayr, 1942). Nevertheless, this is not a major concern from the perspective of a lineage-based species concept (de Queiroz, 1998), since reproductive isolation represents just one of the many criteria available to delimit species in nature (de Queiroz, 2007). In any case, we acknowledge that ideally a combination of genetic, morphological and ecological criteria should be used in species delimitation (e.g. Leaché et al., 2009; Ross et al., 2009). Our present study did not include morphological, ecological and physiological characteristics that may differentiate these lineages, and we recognize that such data would strongly be required in order to confirm our conclusions.

Unfortunately, to date information concerning eco-physiological aspects of the studied species are scarce and would require an improvement in the sampling effort and the acquisition of a

Table 2
Uncorrected *cytb* (below the diagonal) and *12S* (above the diagonal) pairwise (*p*) genetic distances between the four *Anatololacerta* species identified by our species tree reconstruction and BP&P species delimitation approach (see Figs. 4a and 6b). Intra-clade variation is given (in italic) along the diagonal for both markers (*cytb*, left side; *12S*, right side). n/c = not calculated.

ID	[spA]	[spB]	[spC]	[spD]	[Ppa]	[Hgr]
A. anatolica [spA]	*1.1/0.6*	2.0	2.1	2.2	8.0	8.9
A. pelasgiana stat. novo [spB]	7.3	*0.9/0.6*	3.0	3.0	8.0	9.8
A. budaki stat. novo [spC]	6.7	6.7	*0.4/0.3*	1.0	8.7	10.2
A. danfordi [spD]	7.8	7.8	6.1	*4.3/1.3*	8.6	10.0
Parvilacerta parva [Ppa]	19.9	18.3	18.4	18.8	*n/c*	9.7
Hellenolacerta graeca [Hgr]	18.3	18.3	19.2	18.9	20.4	*3.8/1.1*

deeper knowledge on the ecology of the different populations in the future. Similarly, a quantitative multivariate analysis of diagnostic morphological characters would be desirable in order to validate taxa, although again, larger sampling sizes than those considered in our research should be obtained. However, given the fairly strict conditions that the Bayesian species delimitation (BP&P) method assumes to designate species, we feel that recognizing at least four species is a conservative estimate.

4.2. Taxonomic conclusions

Our genetic results only partially confirm the taxonomic arrangement proposed by Eiselt and Schmidtler (1986) for the genus *Anatololacerta*. The most surprising outcome from a taxonomical point of view is that *A. o. oertzeni* is closely related to *A. anatolica* and not to other taxa previously included in the "*oertzeni*" group (Figs. 2, 3a and b and 5c). Moreover, other southern Anatolian taxa previously considered subspecies of *A. oertzeni* group into two distinct and well supported clades, which can be recognized at species rank: a southwestern one (*A. pelasgiana*), and a southeastern one, for which the name *budaki* has priority. Lastly, it is confirmed that the easternmost populations from southern Anatolia belong to a fourth species, *A. danfordi*.

Overall, it becomes obvious that current taxonomy does not properly reflect phylogenetic relationships and the genetic diversity of this species complex. According to our findings and in order to reflect the evolutionary relationships, the taxonomy of the genus *Anatololacerta* should be changed as follows:

***Anatololacerta anatolica* (Werner, 1900)**

Taxa included: *Lacerta anatolica* Werner, 1900; *Lacerta o. oertzeni* Werner, 1904; *Lacerta anatolica aegaea* Eiselt and Schmidtler, 1986.

Distribution. All localities from western Anatolia north of the Büyük Menderes River, including insular populations from Samos and Ikaria.

Remarks. Specimens from Ikaria Island form a well-differentiated lineage within species A (*A. anatolica*), having private haplotypes both at mtDNA and nuDNA loci. Therefore, subspecific status for this insular population could be proposed: *Anatololacerta anatolica oertzeni* **comb. nova** (Werner, 1904).

***Anatololacerta pelasgiana* (Mertens, 1959) stat. novo**

Taxa included: *Lacerta oertzeni pelasgiana* (Mertens, 1959); *Lacerta danfordi quandttaylori* Börner, 1974.

Distribution. Southwestern Anatolian Peninsula (south to the Büyük Menderes River, east to Çobanisa/Isparta), including insular populations from Rhodes and Symi.

Anatololacerta budaki (Eiselt and Schmidtler, 1986) **stat. novo**

Taxa included: *Lacerta oertzeni budaki* Eiselt and Schmidtler, 1986; *Lacerta oertzeni finikensis* Eiselt and Schmidtler, 1986.

Distribution. Southwestern Anatolia, from east-north-east of Kaş and Karaman Pass (north of Elmalı) to Antalya.

***Anatololacerta danfordi* (Günther, 1876)**

Taxa included: *Lacerta d. danfordi* Günther, 1876; *Lacerta danfordi bileki* Eiselt and Schmidtler, 1986.

Distribution. All populations that cluster with those from the eastern portion of the southern Anatolian peninsula, from Abanoz (north of Bozyazı/Içel) to the Bolkar Dağları (near Çamlıyayla).

Although the examined specimens cover almost all the described taxa and 11 out of 15 "populations groups" identified on morphological basis by Eiselt and Schmidtler (1986), more intensive sampling is needed to draw more precisely the boundaries of species' geographic ranges, as well as the intraspecific taxonomy. For instance in our data set, samples of the very doubtful *A. oertzeni pentanisiensis* Wettstein, 1964 from Pentanisos Islet, which very probably belong to *A. pelasgiana*, are missing. This study is also unable to allocate the taxon *A. oertzeni ibrahimi*, which is supposed to range from the Antalya to the Karaman provinces, due to missing samples from the type-locality ("ca. 20 km NW Anamur, vilayet Mersin"). At present, only samples from the close locality of Çukurabanoz, in the Mersin province (locality 22, Fig. 1) have been analyzed, resulting in a divergent lineage that should be included in species D, together with the eastern populations ascribed to *A. danfordi*. However, since the type-locality of *A. o. ibrahimi* is situated in the contact zone between species C and D, we could hypothesize at least two different scenarios: (1) topotypic specimens of *A. o. ibrahimi* belong to species D, and therefore *A. o. ibrahimi* should be included in *Anatololacerta danfordi* (Günther, 1876); (2) they cluster within species C, thus the older name *A. o. ibrahimi* (Eiselt and Schmidtler, 1986) would become available instead of *A. o. budaki*. In this second scenario, small-scale parapatry rather than sympatry could be invoked to explain spatial distribution of distinct species along this contact zone. Further analyses considering topotypic samples of ssp. *ibrahimi* are therefore particularly needed to solve this issue.

4.3. Estimation of divergence times and biogeographic considerations

Anatolia, and 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 plate was an island-archipelago almost totally submerged under the Tethys. At the Eocene-Oligocene boundary (*ca.* 34 Ma) the formation of the Paratethys began and Anatolia was only connected to central Asia, while no connection occurred with Europe and the Middle East. Beside these major geological events, severe climatic changes also played a key role on the evolutionary and biogeographical history of the taxa inhabiting this area, since climatic oscillations between significantly wetter and drier conditions have produced repeated changes in habitat, and periodic modifications of the major biota (Rognon, 1993; Anhuf, 2000; Prentice and Jolly, 2000; Douady et al., 2003; Schuster et al., 2006). Particularly, the formation of the Anatolian mountain chains (e.g. the Anatolian Diagonal, the Taurus and the Black Sea Mountains) can be tracked back to the Tertiary, when the northward movement of Europe resulted in the formation of the Alps. At the Plio-Pleistocene boundary, wide sea-level changes and extensive tectonic uplifts of landmasses, particularly occurring in western and southern Anatolian regions, were the main geological factors affecting taxa distributions (e.g.

Glover and Robertson, 1998). Later on, Anatolian populations persisted in several microrefugia similarly to those surviving in the three main southern European peninsulas (i.e. Iberia, Italy and the Balkans, Hewitt, 2004) during the Quaternary climatic oscillations. During glacial phases, high mountains provided barriers to species dispersion, while during inter-glacial periods, individuals radiating from their refugia often met and promoted secondary contacts and hybridization among their partially distinct lineages. Such orographic and climatic barriers to gene flow have been invoked to explain the great cryptic genetic differentiation recently revealed by molecular studies in several Anatolian taxa (e.g. *Lyciasalamandra* spp., Veith and Steinfartz, 2004; *Pelophylax* spp., Akın et al., 2010; *Typhlops vermicularis*, Kornilios et al., 2011; *Blanus* spp., Sindaco et al., 2014). At the same time, local variation in environmental factors may also have determined striking morphological diversification among populations of the same species (e.g. *Hyla* spp., Gvoždik et al., 2008).

Although formerly considered to be a single refuge, today the idea of "refugia within refugia" (Gómez and Lunt, 2007) has also been proposed for Anatolia, suggesting that multiple smaller refugial areas existed within this region resulting in high levels of cryptic genetic diversity, particularly after glacial retreats, when populations radiated from refugia in the same area. Our study confirms that several distinct vicariant events occurred in Anatolia, when formerly larger geographic ranges of *Anatololacerta* populations were reduced across this region, and genetic lineages were isolated in disjunct areas. Particularly, according to our calibration results (Fig. 4a), the first disruption leading to allopatric fragmentation of northernmost *A. anatolica* populations (species A) possibly resulted from major tectonic events (i.e. a regional uplift of about 400 m along the Büyük Menderes river valley, Westaway et al., 2003) that occurred at the Pliocene-Pleistocene boundary (i.e. 2.3 Ma). Later on, the predominant Pleistocenic climate fluctuations should have strongly affected the distribution of the other lineages and significantly shaped their genetic structure in the past 2 My. During that time, divergent evolutionary lineages could have originated within populations in southern refugia, already identified as suitable for reptiles and amphibians persistence during glacial phases (e.g. Kornilios et al., 2011; Plötner et al., 2001; Weisrock et al., 2001; Fritz et al., 2007; Kyriazi et al., 2008; Akın et al., 2010; Gvoždik et al., 2010a,b; Kornilios et al., 2010; Wielstra et al., 2010). Anatolia is predominantly mountainous, and its varied geomorphology produces many different climatic regions and vegetation types (see Sindaco et al., 2000 for detailed description of these characteristics). Particularly, southwestern Anatolia has acted as a "biodiversity pocket" for several amphibian and reptile taxa, such as *Lyciasalamandra*, *Vipera anatolica*, and *Blanus strauchi* due to its geomorphological and ecological features. Indeed, this is a mountainous and densely forested area that reaches altitudes of 3000 m a.s.l., and represents the western edge of the Taurus Mountain ridge. Therefore, repetitive shifts between intense aridifications (glacial phases) and wetter Mediterranean conditions probably were the major climatic changes that affected the distribution of species during Quaternary glacial cycles (Fauquette et al., 1999; Cavazza and Wezel, 2003; Jiménez-Moreno et al., 2010). Accordingly, extreme and sudden aridification during glaciations led to shrinking ranges of *Anatololacerta*, with genetic lineages surviving in refugia and producing the differentiation of *A. pelasgiana* (species B), *A. budaki* (species C) and *A. danfordi* (species D).

Molecular data also support the non-existence (or maximal rarity) of hybridization between *A. anatolica* and *A. pelasgiana* along the Büyük river valley barrier, as well as between *A. pelasgiana* and *A. budaki*. Nevertheless, more detailed data, particularly from the zone of parapatry between *A. budaki* and *A. danfordi*, are required to clarify the sympatric vs. parapatric relationships of those populations. The low intra-specific genetic divergence observed within some taxa (i.e. *A. budaki*) suggests a recent recolonization of the species range, probably from one single refuge located in the Lycian mountains (Schmidtler, 1998). By contrast, *A. anatolica* and *A. pelasgiana* exhibit a considerable intra-clade genetic variability, possibly related to the persistence of isolated populations on islands that were disconnected from the mainland during the Pleistocene (particularly Ikaria for *A. anatolica* and Symi for *A. pelasgiana*). Finally, the highest intraspecific variation recovered within *A. danfordi* suggests that its current range might have been recolonized from distinct refugia, probably located in the Eastern Taurus Mountains (clade IV) and in the Bolkar Mountains (clade V).

5. Conclusions

Our study suggests that strong geomorphological and environmental changes including tectonic uplifts (Davis, 1971; Kosswig, 1955; Schmidtler, 1998) and Quaternary climatic oscillations resulting in southwards progression of ice sheets and intense aridification during phases of cooling (Avise, 2000; Hewitt, 2001, 2004), might have affected the distribution of the *Anatololacerta* ancestor, triggering the evolution and allopatric divergences of various lineages within Anatolia. Other studies have suggested the existence of cryptic genetic lineages within some amphibian and reptile taxa with apparently continuous geographic distribution in Anatolia (Kyriazi et al., 2008; Plötner et al., 2001; Akın et al., 2010; Fritz et al., 2007). Recent results very similar to the ones here described for *Anatololacerta* have been recovered for the Eurasian blindsnake, *Typhlops vermicularis* (Kornilios et al., 2011, 2012) and the *Blanus strauchi* species complex (Sindaco et al., 2014). Interestingly, the areas that seem to have played a key role in the diversification of these taxa are roughly the same as for *Anatololacerta*.

Our approach allowed the identification of four distinct species, which have important implications for conservation and natural resource management. Integrated assessments of other faunal elements from southern Anatolia are clearly needed in order to fully understand the biodiversity of this geologically and climatically complex region, whose central role for producing and sustaining biodiversity has been further confirmed by our study.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.10.003.

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