

The story of a rock-star: multilocus phylogeny and species delimitation in the starred or rougtail rock agama, *Laudakia stellio* (Reptilia: Agamidae)

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Situated at the junction of three continents, Europe, Asia and Africa, the Eastern Mediterranean is an ideal region to study the effects of palaeogeography, ecology and long human presence on animal evolution. *Laudakia stellio* (Squamata: Agamidae) is found across this region and offers an excellent opportunity for such studies. The high morphological variation across their range suggests that these lizards might represent a species complex. This is the first study exploring their evolutionary history, using molecular markers and individuals from all described subspecies. We employed the latest phylogenetic and species-delimitation methods to identify all distinct evolutionary lineages, their genetic variation and divergence times. The phenotypical diversity of *L. stellio* matches its genetic differentiation: almost all subspecies correspond to well-supported retrieved subclades and additional distinct lineages representing intermediate morphs have been retrieved. '*Laudakia stellio*' represents three distinct evolutionary entities that diverged during the Plio-Pleistocene transition, which we propose as distinct species. One includes Greek and Turkish populations, as well as cryptic Anatolian lineages. The second comprises all other Near East populations and the third is endemic to Cyprus. Our results indicate a role of humans in shaping present distribution patterns, and highlight the importance of the Aegean, Anatolia and the Levant as glacial refugia and diversity hotspots.

ADDITIONAL KEYWORDS: molecular phylogeny – molecular systematics – phylogeography – phylogenetic systematics – species delineation – subspecies – taxonomy.

INTRODUCTION

Lizards of the family Agamidae are among the most diverse and widespread squamates, belonging to 551 species and 67 genera, and are distributed across the Old World, from Africa and West Asia, to South-East

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Asia and Australia (Uetz *et al.*, 2021). *Laudakia stellio* (Linnaeus, 1758) is one of the few agamids present in Europe (Speybroeck *et al.*, 2020) and, like most of its African and Asian counterparts, it is a robust, rock-dwelling, diurnal lizard. The taxonomic history of this species is complex. Along with other Palaearctic species, it was initially placed within the genus *Agama* Daudin, 1802 that was later split into six distinct genera (Moody, 1980). One of them was named ‘*Stellio*’ after the focal species, albeit this name had already been made unavailable (Henle, 1995). ‘*Stellio*’ was later divided into *Acanthocercus* Fitzinger, 1843 and *Laudakia* Gray, 1845 by Baig and Böhme (1997).

The phylogenetic position of *L. stellio* in the genus *Laudakia* has historically been considered controversial. The first phylogenetic studies, using mitochondrial markers, rendered this genus paraphyletic (Macey *et al.*, 2000, 2006), whereas subsequent studies, including both mitochondrial and nuclear markers, supported its monophyly (Melville *et al.*, 2009; Edwards & Mellville, 2011). This controversy led to a morphology-based reassessment of the genus (Baig *et al.*, 2012), which was then split into three genera: *Paralaudakia* Baig *et al.*, 2012, *Laudakia* and the monotypic *Stellagama* Baig *et al.*, 2012. However, an extensive phylogenetic study using five mitochondrial and seven nuclear markers strongly supported the monophyly of *Laudakia*, rendering the aforementioned division unjustified (Pyron *et al.*, 2013). Even though this study was lacking a lot of data from particular squamate families, and in view of additional new data that support the split of *Laudakia* into three different genera (Wagner *et al.*, unpubl. data), we will use the name *Laudakia stellio* in compliance with the Taxonomic Committee of Societas Europaea Herpetologica (SEH), which proposes the inclusion of the ‘genera’ *Stellagama* and *Laudakia* / *Paralaudakia* within *Laudakia*, until new published studies suggest otherwise (Speybroeck *et al.*, 2020).

The entire distribution of *L. stellio* falls within an important biodiversity hotspot, the East Mediterranean (Myers *et al.*, 2000; Blondel, 2010; Mittermeier *et al.*, 2011), which stretches from northern Africa and the Levant to Turkey and Greece. Throughout this range, *L. stellio* occupies a variety of habitats, from Aegean island coasts to the mountain tops in inner Anatolia, and from the Negev desert to the humid forests along the Black Sea coast. This habitat variability goes hand in hand with phenotypic variation that has led to the description of seven subspecies since the beginning of the 19th century (Crochet *et al.*, 2006 and references therein; Baig *et al.*, 2012 and references therein).

In North Africa, the species is mainly represented by *L. s. vulgaris* (Sonnini & Latreille, 1802), which occurs in north-eastern Egypt. In addition to *L. s. vulgaris*,

described from Alexandria (and introduced to Giza in 1896 and Cairo in 1901), Flower (1933) noted the existence of two more morphs. The first corresponds to *Laudakia s. brachydactyla* (Haas, 1951), which is found in the northern Sinai, southern Israel, Jordan and Saudi Arabia. As its name suggests, *brachydactyla* has shorter toes in comparison to the other subspecies. It is mainly associated with the Negev desert, whereas another intermediate morph, namely the ‘Near-East *L. s. ssp.*’, inhabits the northern Mediterranean parts of Israel (Werner, 1988; Panov & Zykova, 1997, 2016; Federman & Werner 2007). The other morph observed by Flower (1933) refers to *Laudakia s. salehi* (Werner in Lachman *et al.*, 2006), which is restricted to the granite mountains of the southern Sinai. This population was initially classified as *L. s. brachydactyla*, but later was identified as a distinct subspecies, joining the other endemic reptiles found in this high-elevation and relatively cold area, such as *Mesalina bahaeldini* Segoli Cohen & Werner, 2002 (Kapli *et al.*, 2008, 2014), *Eirenis coronella ibrahimi* Sivan & Werner, 2003 and *Hemidactylus mindiae* Baha El Din, 2005. However, a recent study has suggested that *L. s. salehi* should be synonymized with *L. s. vulgaris* (Kadry *et al.*, 2020).

The smallest of all subspecies, *L. s. picea* (Parker, 1935), is present in Syria, Jordan and Saudi Arabia. Having a distinctive dark coloration with orange spots (Parker, 1935; Werner, 1992), it is characteristic of the Black Lava desert of Syria, its type locality. The Lebanon and the Golan heights’ populations had been erroneously attributed to *L. s. picea*, but today are assigned to the ‘Near-East *L. s. ssp.*’ that broadly encompasses all populations from eastern Anatolia to the Negev and Jordan (excluding *L. s. picea* and *L. s. brachydactyla*) (Werner, 1988, 1992; Panov & Zykova, 1997; Federman & Werner, 2007).

Further north in Anatolia, distribution of subspecies remains a puzzle. Initially, all Anatolian populations had been classified as *L. s. stellio* (Bodenheimer, 1944; Daan, 1967), with some intermediate morphs being present in south-eastern Anatolia (Bird, 1936; Clark & Clark, 1973). Later, *L. s. daani* was described by Beutler & Frör (1980) from the East Aegean island of Ikaria. It differs from the nominate subspecies mostly in colour. However, a subsequent extensive morphological review by Baran & Öz (1985) assigned the western and southern Anatolia populations to *daani* and the south-eastern Anatolian populations to *stellio*, and highlighted the differentiation of the Hatay population. In contrast, more recent studies have suggested that all Anatolian populations were similar to *L. s. daani*, again excluding the Hatay population (Almog *et al.*, 2005; Gül *et al.*, 2010 and references therein; Kumlutaş *et al.*, 2004, 2015; Gül & Tosunoglu, 2011). The high phenotypical variation of the latter, as well as of other

south-east Anatolian populations, has been supported by additional morphological examination (Kete & Yılmaz, 2006) and by a molecular study that revealed that south-eastern Anatolian populations correspond to one of the two distinct mitochondrial lineages found in Turkey (Özdemir *et al.*, 2011). Thus, the boundaries of *L. s. stellio* and *L. s. daani* in Anatolia, as well as the taxonomic status of all reported intermediate morphs, still pose questions that are waiting to be answered.

Both these subspecies are present in Greece, where they have a mainly insular distribution: *L. s. daani* is found in most East Aegean islands, in some of the Cyclades (Paros and Naxos) and has been introduced to Thessaloniki and Crete (Klaptocz, 1910; Spaneli & Lymberakis, 2014), whereas *L. s. stellio* is found exclusively in the Cyclades (Mykonos, Delos, Mikros Rematiaris and Rineia) and has been introduced to Corfu (Ionian Sea) (Koch, 1932). In the past, *L. s. stellio* from Mykonos and the nearby islets was described as a different subspecies '*Laudakia stellio mykonensis*' (Xyda, 1983, 1986). The presence of *L. s. stellio* and *L. s. daani* in separate but nearby Cycladic islands raises the question about their origin. Crossing the Aegean Sea barrier and reaching the Cyclades would be possible for *L. s. daani*, but not for *L. s. stellio*, whose nearest population is found in south-eastern Anatolia. A study by Brammah *et al.* (2010), focusing on the genetic divergence among these populations using microsatellite data, yielded contrasting results, leaving the question on their origin and their non-overlapping current distribution unanswered.

The only exclusively insular subspecies is *L. s. cypriaca* (Daan, 1967). Apart from its larger body size, it also has a distinctive tail segmentation pattern (Osenegg, 1989). Along with *Phoenicolacerta troodica* (Werner, 1936), *Hierophis cypriensis* (Schätti, 1985) and *Telescopus fallax cyprianus* Barbour & Amaral, 1927, it is one of the most prominent endemic reptiles of Cyprus. Although the morphological distinctiveness of *L. s. cypriaca* has not been challenged by past research, its origin and genetic affinity to nearby mainland populations has not yet been investigated.

The phenotypical variance exhibited by *L. stellio* has yet to be examined in view of the complex palaeogeographic and palaeoclimatic history of the eastern Mediterranean. Anatolia is recognized as an important evolutionary hotspot and a major glacial refugium that hosts species originating from, or expanding to, Eurasia, Africa and the Arabian Peninsula (Bilgin, 2011 and references therein; Gür, 2013, 2016; Korkmaz *et al.*, 2014; Ali *et al.*, 2019). Its complex geomorphology consists of imposing mountain ranges and massifs, such as the Pontides, Caucasus, Taurus and Amanos mountains (Yılmaz, 1993; Popov *et al.*, 2006; Seyrek *et al.*, 2008) surrounding the Central Anatolian plateau and lake system. Acting

as a physical and/or ecological barrier to dispersal, mountain ranges like the Anatolian Diagonal (Davis, 1971; Ekim & Güner, 1986) have promoted endemism and high intraspecific diversity in various reptiles and other animals and plants (Çıplak *et al.*, 1993; Rokas *et al.*, 2003; Gündüz *et al.*, 2007; Mutun, 2010; Bilgin, 2011; Gür, 2016; Allegrucci *et al.*, 2017; Arslan *et al.*, 2020). The adjacent Aegean archipelago consists of numerous islands forged by tectonic plate movements leading to volcanism and orogenesis, sea introgression and subsequent land fragmentation. These islands vary in landscape heterogeneity and environmental conditions (Fassoulas, 2018 and references therein), enabling animal dispersal, especially during the Messinian Salinity Crisis (MSC = 5.96–5.33 Mya) when extensive land-bridge connections existed between islands (Krijgsman *et al.*, 1999, 2010; Duggen *et al.*, 2003). Moreover, Pleistocene climatic fluctuations contributed to repeated events of island isolation/reconnection due to eustatic sea-level changes (Webb & Bartlein, 1992; Raymo *et al.*, 1996). The combined effect of these processes gave rise to complex biogeographic patterns and a high degree of endemism, rendering the eastern Mediterranean an ideal place to study evolution and species diversification (Lymberakis & Poulakakis, 2010; Sfenthourakis & Triantis, 2017; Poulakakis & Parmakelis, 2018).

Considering the high intraspecific polymorphism exhibited by *L. stellio* along a range of ecologically different habitats, one might presume that it is a species-complex (Panov & Zykova, 1997, 2016). Nevertheless, all the aforementioned studies on the taxonomy of *L. stellio* are mainly comparisons among subspecies, based exclusively on colour features and other morphological and osteological characters. In addition, numerous intermediate morphs have an unknown taxonomic status because they do not fit with the description of any described subspecies. This volume of research has provided detailed information about phenotypical variation, but has not addressed the evolutionary history of this taxon, nor how it was shaped by palaeogeography and palaeoclimate. Only three studies so far have attempted to resolve the phylogenetic relationships among different subspecies using molecular data, but included few populations and just one or two genetic markers (Brammah *et al.*, 2010; Özdemir *et al.*, 2011; Kadry *et al.*, 2020). Thus, the phylogenetic relationships in this putative species complex still remain unresolved.

Here we present the first comprehensive study of the evolutionary history of *L. stellio* based on genetic data extracted from all known subspecies and populations throughout its distribution. We follow a multilocus approach and apply the latest phylogenetic and coalescence-based species-delimitation methods in order to: (1) reconstruct the phylogenetic history of

L. stellio; (2) estimate the degree of genetic variation and the extent to which subspecies represent distinct evolutionary lineages; (3) explore the pattern of divergence and distribution of the encompassed evolutionary lineages; (4) shed new light on their origin and possible effects of major palaeogeographic and palaeoclimatic events; and (5) revise the taxonomy accordingly.

MATERIAL AND METHODS

SAMPLES AND LABORATORY PROCEDURES

Tissue samples for DNA extraction were collected from a total of 140 individuals, representing all nominal subspecies of *L. stellio* and covering its entire range, from north-western Greece to eastern Turkey and south to the Sinai Peninsula (Fig. 1). Specimens from type localities were available for *L. s. daani* (Ikaria) and *L. s. stellio* (Mykonos and Delos). For the other subspecies, we examined specimens collected more recently from areas near the type localities. Additional information on specimen localities, voucher codes and

GenBank accession numbers for all amplified genetic loci is presented in [Supporting Information, File S1](#).

Three mitochondrial DNA (mtDNA) markers (*ND4*, the adjacent histidine, serine, leucine tRNAs and 16S rRNA) were sequenced for all 140 individuals. Of those, 75 samples were additionally sequenced for six nuclear DNA (nuDNA) markers (*CMOS*, *MC1R*, *NKTR*, *PNN*, *RAG1* and *R35*) representing all well-supported mitochondrial lineages produced by the phylogenetic analyses, and all 'independently evolving entities' delimited by the Bayesian implementation of the Poisson tree processes (PTP) model for species delimitation (Zhang *et al.*, 2013) ([Supporting Information, File S2](#)). Moreover, 13 sequences of other agamids were used as outgroups and for calibrating the species tree: *Agama agama* (Linnaeus, 1758), *A. boensis* Monard, 1940, *A. bottegi* Boulenger, 1897, *A. boueti* Chabanaud, 1917, *A. boulengeri* Lataste, 1886, *A. impalearis* Boettger, 1874, *A. planiceps* Peters, 1862, *A. sankaranica* Chabanaud, 1918, *A. spinosa* Gray, 1831, *Paralaudakia* (= *Laudakia*) *caucasica* (Eichwald, 1831), *Phrynocephalus mystaceus* (Pallas, 1776), *Pseudotrapelus sinaitus* (Heyden, 1827) and *Xenagama*

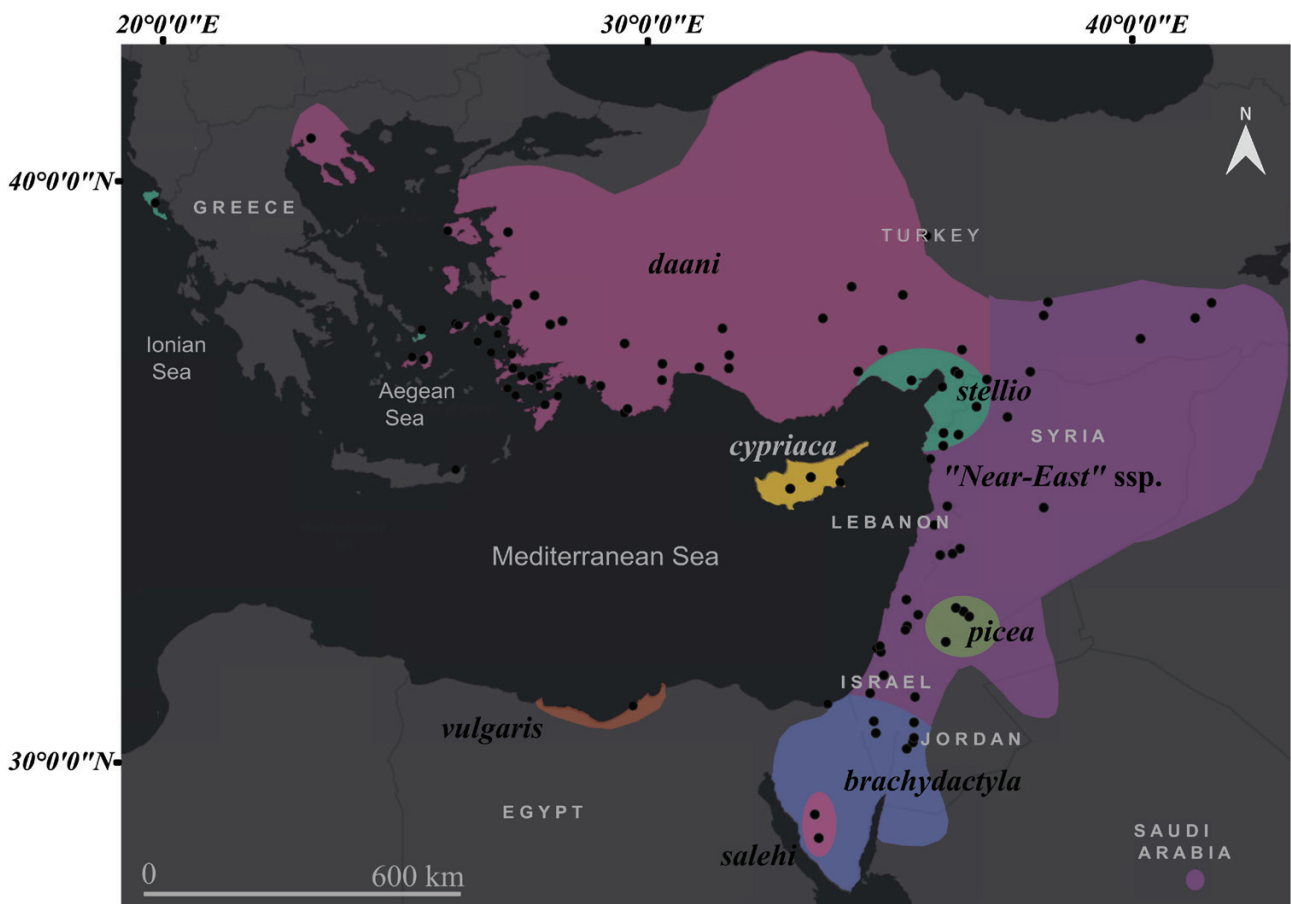


Figure 1. Map showing the distribution of all known morphological subspecies of *Laudakia stellio* in the East Mediterranean.

taylori (Parker, 1935) (Supporting Information, File S1; Leaché *et al.*, 2014). Thus, two datasets were created: one with mitochondrial markers (mtDNA, $N = 153$) and one with concatenated sequences (mtDNA and nuDNA, $N = 88$).

Total genomic DNA was extracted using different protocols depending on condition, age and mass of each sample. Older samples were initially washed in 0.01 mol/L Tris-HCl (changed daily) for a seven-day period and extracted using commercially available DNA extraction kits (DNeasy Blood & Tissue Kit – Qiagen, Germantown, MD, USA, NucleoSpin Tissue kit – Macherey-Nagel, Düren, Germany). DNA from relatively fresh samples was extracted following the Ammonium Acetate based method (Bruford *et al.*, 1998). Primer sequences and polymerase chain reaction (PCR) amplification conditions for each marker are given in Supporting Information, File S1. When PCR amplification failed for a certain marker using external primers, nested primer pairs were designed using Primer3 (Rozen & Skaletsky, 2000) on the GENEIOUS 8.1.2 bioinformatics software platform (Kearse *et al.*, 2012, Biomatters Ltd, San Diego, CA, USA) (Supporting Information, File S1). Sanger sequencing data were collected using BigDye Terminator cycle sequencing chemistry (v.3.1) on an ABI3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA).

SEQUENCES ALIGNMENT, GENETIC DISTANCES AND HAPLOTYPE NETWORKS

Sequences of each locus were aligned using MAFFT v.7.017 (Katoh *et al.*, 2002) with default parameters and FFT-NS-1 algorithm in GENEIOUS 8.1.2. All coding sequences were translated into amino acids to check for the presence of premature stop codons. Genetic divergence among all main evolutionary lineages produced by the phylogenetic analyses were estimated using the uncorrected pairwise genetic distance (p -distance) in MEGA v.10.0.5 (Kumar *et al.*, 2018), based on pairwise deletion of gaps or missing data and including both transitions and transversions.

Sequences of nuclear genes containing heterozygous positions were resolved using the coalescent-based Bayesian method implemented by the PHASE algorithm (Stephens *et al.*, 2001; Stephens & Donnelly, 2003) in DNASP v.6.12.03 (Rozas *et al.*, 2017), using the default settings (100 burn-in iterations and 100 additional iterations with thinning interval equal to 1). Then, they were used to produce a haplotype network reconstruction using the default settings of the statistical parsimony algorithm TCS (Templeton *et al.*, 1992) as implemented in PopART (<http://popart.otago.ac.nz>) (Leigh & Bryant, 2015). For visualization

purposes, haplotypes were assigned to different clades (in agreement with the phylogenetic analyses results) using a trait file, according to the PopART manual.

PHYLOGENETIC ANALYSES

Phylogenetic reconstruction was performed separately on the mtDNA and the concatenated datasets, using both Bayesian inference (BI) (Yang & Rannala, 1997; Huelsenbeck *et al.*, 2001) and maximum likelihood (ML) methods (Felsenstein, 1973, 1981). Data-partitioning schemes (by codon position for each protein-coding fragment) and the best-fit models of DNA substitution (excluding +G+I models; Yang, 2006) were estimated under the Bayesian information criterion (BIC) implemented in PartitionFinder2 (PF) (Lanfear *et al.*, 2016). PartitionFinder2 analyses were done separately for BI (setting models = mrbayes) and ML (setting models = all; and specifying the option - -raxml in the command line), using the greedy algorithm and linked branch lengths. Model selection for each phylogenetic method (BI and ML) was performed in three separate runs: first, for the mtDNA dataset, second for the mitochondrial sequences included in the concatenated dataset and third for the nuclear sequences of the concatenated dataset. The final concatenated alignment was partitioned into 25 blocks (18 corresponding to the six nuDNA genes, three to *ND4*, three to the tRNAs and one to the non-coding 16S rRNA). The resulting partition schemes and best-fit nucleotide substitution models were subsequently used in the phylogenetic analyses (Supporting Information, File S3).

Bayesian inference was performed using MrBayes v.3.2.7a (Ronquist *et al.*, 2012) with four independent runs consisting of five Markov chain Monte Carlo (MCMC) chains each, for 2×10^7 generations and recording samples every 10^3 generations. Partitions were allowed to have their own nucleotide substitution model parameters [unlink statefreq = (all) revmat = (all) shape = (all) pinvar = (all)] and evolve at different rates [prset applyto = (all) ratepr = variable]. The first 25% of samples per run were discarded as burn-in (relburnin = yes burninfrac = 0.25). Convergence of independent runs was assessed by the standard deviation of split frequencies (< 0.01) as well as the potential scale reduction factor (PSRF) diagnostic (= 1.0). Results were further inspected in TRACER v.1.7.1 (Rambaut *et al.*, 2018) by examining the traceplots of multiple runs and the effective sample sizes (ESS) of all parameters. The produced majority-rule consensus tree (contype = halfcompat) was visualized in TreeGraph v.2.14.0 (Stöver & Müller, 2010) and nodes were considered to be strongly supported when posterior probability values were

> 0.95 (Huelsenbeck & Rannala, 2004). Maximum likelihood analyses were performed using RaxmlHPC v.8.2.12 (Stamatakis, 2014). The RAxML rapid hill-climbing algorithm was run under the GTRGAMMA model and rapid bootstrapping was halted using the autoMRE criterion. Both phylogenetic reconstruction analyses were conducted on CIPRES Science Gateway platform v.3.3. (Miller *et al.*, 2010).

SPECIES TREE AND DIVERGENCE TIME ESTIMATION

Species tree and divergence time estimation was based on all loci, but using two individuals per well-supported subclade, produced by the BI analysis on full mitochondrial data. *Laudakia s. salehi* was omitted from this analysis because we did not obtain sequences for its nuclear genes. The final species tree dataset contained a total of 37 samples (two ingroup sequences per 12 populations, plus 13 outgroup species). Analyses were conducted on CIPRES Science Gateway platform, using the StarBEAST2 v.0.15.5 package (Ogilvie *et al.*, 2017) on Beast2 v.2.6.1 (Bouckaert *et al.*, 2014). The input file was formatted in BEAUTi2 v.2.6.2 (Bouckaert *et al.*, 2019; Supporting Information, File S4).

Data partitions and DNA substitution models were set according to PF analyses as described above (setting models = beast), only this time each protein-coding fragment was set as one block (Supporting Information, File S3). The dataset was not phased; therefore, heterozygous positions were treated as unknown (N). Site and clock models were 'unlinked' across loci. Gene trees were also 'unlinked' for the nuclear loci and 'linked' for the mitochondrial ones. Moreover, gene ploidy was equal to 2 for the first and 0.5 for the latter. Population model was set to 'linear with constant root' and the 'uncorrelated lognormal' clock was used for all partitions. The species tree was estimated under a 'birth-death' model with all priors set to default, apart from the 'lwcPopScale.species' that was set to lognormal. Seven dates that represent splits among other Agamidae species (Leaché *et al.*, 2014) were used as calibration points. We should note that this calibration scheme could lead to compounded inaccuracy of estimated divergence times, as it is based on secondary dates, but it was a necessary option due to the lack of available fossil calibration points for the genus *Laudakia*. In order to account for uncertainty in these dates, divergence times were set as means of a normal prior distribution with S values adjusted so that the 5% and 95% quantiles contained the 95% highest posterior density (HPD) interval of each node (Supporting Information, File S5).

Four runs with random starting seeds were performed, with chain lengths of 500 million generations and sampling every 5000 steps. The first 25% of samples were discarded as burn-in. Results

were inspected in TRACER (Rambaut *et al.*, 2018) to ensure that the posterior distributions of parameters were congruent among runs. Log files were subsequently combined and resampled (every 20 000) in LogCombiner v.2.6.2 (Beast2 package) and the outcome was re-inspected in TRACER to ensure that effective sample sizes (ESS) were high enough (> 200) (Rambaut & Drummond, 2009) for all parameters. Species trees from both runs were also combined and resampled so that a final dataset of 75 000 trees was used as input in TreeAnnotator v.2.6.2 (Beast2 package) to produce a maximum clade credibility tree and to calculate mean node ages (mean heights) and their 95% highest posterior densities (HPD). The calibrated species tree produced was visualized in TreeGraph2 v.2.14.0.

SPECIES DELIMITATION: BAYESIAN PHYLOGENETICS AND PHYLOGEOGRAPHY (BPP) AND HEURISTIC SPECIES DELIMITATION USING THE GENEALOGICAL DIVERGENCE INDEX (GDI)

Species delimitation was conducted on the concatenated dataset, following a multispecies coalescence-based approach, the Bayesian phylogenetics and phylogeography (BPP; Rannala & Yang, 2003; Yang & Rannala, 2010). Individuals were assigned to different populations (putative species) as described in the calibrated species tree analysis and outgroups were excluded. Nuclear sequences were phased as previously described.

The BPP analyses A10 (species delimitation using a fixed guide tree) and A11 (joint species delimitation and species tree estimation) were performed in BPP3.4a (Flouri *et al.*, 2018). Because the retrieved phylogenetic position of the *cyprica* phylogenetic lineage was found to be ambiguous (see Results), all analyses were run under three different scenarios: *cyprica* as sister to Clade 1 (scenario 1), as sister to Clade 2 (scenario 2) and as sister to both major sister-clades (1 and 2) (scenario 3). The rest of the guide-tree topology agrees with the species-tree produced by the StarBEAST analysis. MtDNA was considered as one locus (heredity scalar = 0.25) in contrast to the nuclear loci that were set as separate (heredity scalar = 1 in each case). Individual assignment to putative species was done in the Imap file provided as input to BPP. Analyses were run with the rjMCMC algorithm = 1 and under ten different prior settings: we investigated the consistency of results under a more diffuse ($\alpha = 3$) and a more informative ($\alpha = 21$) inverse-gamma prior, with β values varying by orders of magnitude following the example of Flouri *et al.* (2018) (Supporting Information, File S6). MCMC was set to 100 000 samples with sample frequency equal to 5 and 10 000 samples set as burn-in.

Moreover, both A10 and A01 analyses were repeated under all three scenarios, this time using empirical priors, calculated from the dataset: $\alpha = 3$ was set as a diffuse prior for both theta (θ) and tau (τ), and β was, respectively, estimated from the equation: $m = \beta / (\alpha - 1)$, for $\alpha > 1$, with 'm' being equal to the mean nucleotide diversity in the case of (θ) and the mean node height (according to the StarBEAST analysis results) in the case of (τ) (Supporting Information, File S6). MCMC was set to 200 000 samples with sample frequency equal to 5 and 10 000 samples set as burn-in.

Given that the species-delimitation method based on the MSC model implemented in BPP may actually reflect population splits rather than species divergence events (Jackson *et al.*, 2017; Sukumaran & Knowles 2017), we also employed the genealogical divergence index (gdi) (Jackson *et al.*, 2017) coupled with a thorough likelihood analysis (Leaché *et al.*, 2019) under all three scenarios. First, empirical priors were used to perform the A00 analysis in BPP in order to estimate the parameters of species divergence times and population sizes (τ s and θ s) under the MSC model. Three separate runs were performed, inspected for convergence and then combined in order to create the posterior distribution of all sampled MSC parameters (200 000 samples, sample frequency = 5, burn-in = 10 000 in each run). Then, gdi was calculated following the equation $\text{gdi} = 1 - e^{-2\tau/\theta}$ (Jackson *et al.*, 2017; Leaché *et al.*, 2019). Given two populations, A and B, $2\tau_{AB}/\theta_A$ is used to decide whether population A is distinguished from population B. When gdi is lower than 0.2, the two populations are considered as belonging to the same species, while the opposite is true when gdi is greater than 0.7. Intermediate values ($0.2 > \text{gdi} < 0.7$) represent ambiguous species status results. The analysis was performed multiple times, by progressively collapsing sister-clades and thus allowing the comparison of internal branches and of all phylogenetic subclades.

RESULTS

SEQUENCES ALIGNMENT AND PHYLOGENETIC RECONSTRUCTION

The mitochondrial dataset contains a total of 1311 bp (ND4: 681 bp, tRNAs: 163 bp, 16S: 467 bp) from 153 (140 ingroup and 13 outgroup) individuals and the concatenated dataset has a total length of 5547 bp from 88 (75 ingroup and 13 outgroup) individuals (mtDNA: 1312 bp, MC1R: 669 bp, NKTR: 638 bp, RAG1: 1009 bp, PNN: 854 bp, R35: 705 bp, CMOS: 360 bp). The best-fit nucleotide substitution models and data-partitioning schemes for the phylogenetic analyses (BI, ML), as well as for the calibrated species-tree reconstruction

(StarBEAST2), are presented in Supporting Information, File S3.

Bayesian inference (LnL = -12,762.22) and ML (LnL = -12,759.57) analyses on the mtDNA dataset yielded trees with similar topologies (Fig. 2). In ML, rapid bootstrap was halted after 456 replicates according to the MRE based-criterion. The focal taxon, *L. stellio*, consists of 13 different evolutionary subclades grouped into two major clades (1 and 2), along with a distinct phylogenetic lineage from Cyprus. All known morphological subspecies correspond to distinct subclades, with the exception of *L. s. vulgaris* that clusters with the *brachydactyla* subclade from Egypt and Israel.

Clade 1 consists of four subclades with unresolved phylogenetic relationships: (1) the *brachydactyla* subclade that includes *L. s. brachydactyla* from Israel and Egypt and the unique specimen of *L. s. vulgaris* (1/100); (2) the *picea* subclade that contains *L. s. picea*; (3) the *Jordan* subclade that appears to have a sister-group relationship with the *picea* lineage, although with low statistical support; and (4) the *salehi* subclade from Sinai. The well-supported (1/97) lineage of *cypriaca*, which corresponds to *L. s. cypriaca* from Cyprus, appears as the sister to Clade 1, although support for this relationship was low in BI (PP = 0.80) but not in ML (bs = 85).

Clade 2 contains populations from north and north-west Syria, Lebanon, Greece and Turkey (Fig. 2). Within this clade, two insular populations from the east Aegean (Symi and Kastellorizo), along with individuals from the neighbouring region of south-western Turkey (Kaş), form a well-supported subclade (1/90), hereafter named *Symi-Kaş* (Figs 2, 3), which is sister to all the rest. After that, two well-supported subclades diverge: the *Hatay* subclade (1/100), which extends along the Syrian-Turkish borders, from Aleppo (Syria) in the south towards Adıyaman (Turkey) in the north and the Amanos (Nur) mountains in the west, and the *Lebanon* subclade (1/99), which is found in a narrow strip parallel to the Mediterranean coast, from north-western Lebanon towards the western side of the Syrian Coastal Mountain Range (Fig. 3). These two subclades have a sister-group relationship (0.95/79) forming the 'Near East' group (Fig. 2).

The 'Near East' group has a sister-group relationship with the rest of Clade 2, which comprises the Greek and Turkish populations, broadly corresponding to the subspecies *L. s. stellio* and the *L. s. daani*. Three subclades are recognized in *L. s. stellio*: the first from the Greek insular populations (Delos, Mykonos and Corfu) and populations from Adana and Osmaniye (*stellio* GR-TR), the second from south and central Anatolia populations (*stellio* TR) and the third from eastern Anatolia (1/97) (*Anatolia*). Together, they cluster with the last two subclades: the *daani* subclade,

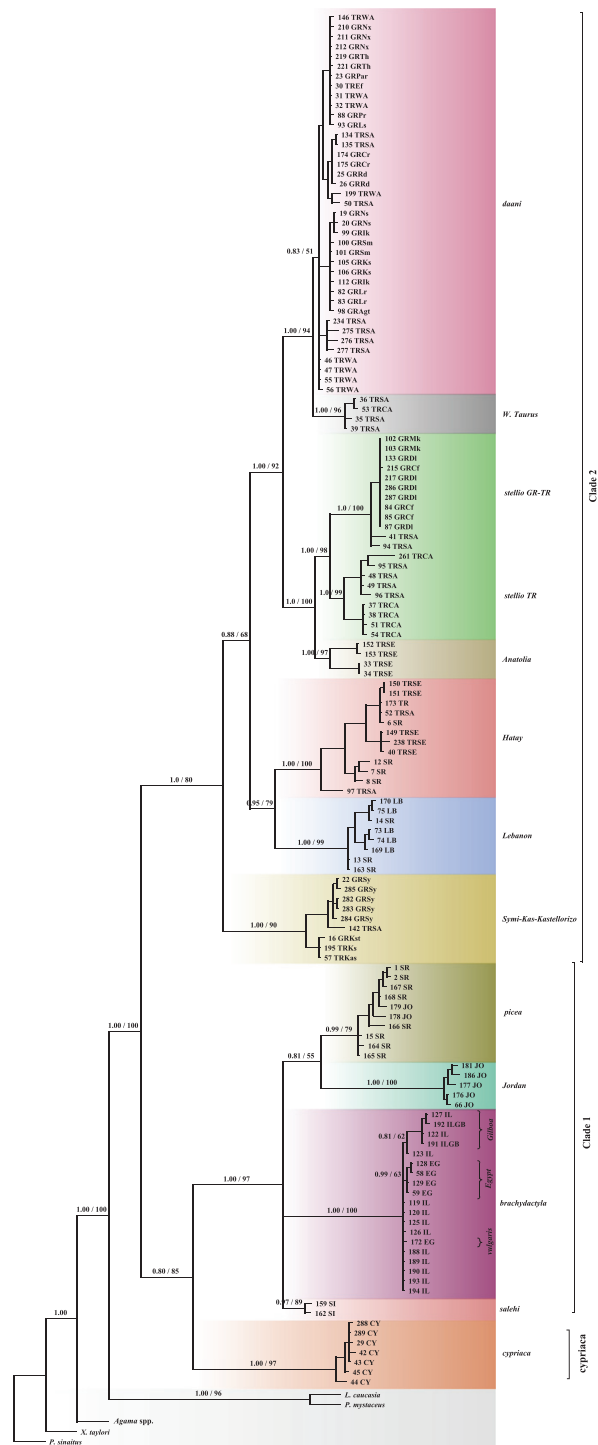


Figure 2. Phylogenetic tree based on mtDNA (ND4-tRNAs and 16S rRNA). Bayesian posterior probabilities (PP) and maximum likelihood bootstrap support (bs) values are represented in the form PP/bs above or beside nodes. (*Agama* spp. contains *Agama agama*, *A. boensis*, *A. bottega*, *A. boueti*, *A. boulengeri*, *A. impalearis*, *A. planices*, *A. sankaranica* and *A. spinosa*).

which includes all the *L. s. daani* specimens from east Aegean islands and Turkey, and its sister-subclade of West Taurus (1/96) (*W. Taurus*). According to its name, the latter subclade is limited to the western Taurus mountain range (south of Lake Beyşehir), whereas the mainland population of Thessaloniki (N. Greece) and the recently discovered population from the island of Crete (Spaneli & Lymberakis, 2014), which clusters with specimens from Rhodes (East Aegean, Greece), cluster with the *daani* subclade (Figs 2, 3).

On the concatenated multilocus dataset, BI (LnL = -23,373.67) and ML (LnL = -24,078.21) analyses produce similar topologies (Fig. 4). In ML, rapid bootstrap was halted after 252 replicates according to the MRE based-criterion. Again, the position of *cyprica* is unresolved, forming a polytomy with the other two major clades (in BI) or being sister to Clade 1 (in ML), but with low support (bootstrap value = 57). Comparing the mitochondrial and the concatenated analyses, apart from small changes in support, the greatest difference lies in the phylogenetic position of the *Symi-Kas* subclade. In the former, it appears to be sister to all other subclades within Clade 2, whereas in the latter, it has sister-group relationship to subclades *daani* and *W. Taurus* (1.0/81).

GENETIC DISTANCES AND HAPLOTYPE NETWORKS

The pairwise uncorrected *p*-distances for the mitochondrial and nuclear markers are presented in Tables 1 and 2 and Supporting Information, File S7. In the ingroup, they reach 13.8% in ND4-tRNAs and 4.6% in 16S rRNA. In ND4-tRNAs the highest distance is observed between *Anatolia* and *brachydauctyla* (13.8%) and the lowest between *daani* and *W. Taurus* (1.8%). Divergence reaches 5% in Clade 1, 4.9% in Clade 2 and 13% between these two major clades. Moreover, the *cyprica* lineage differs by approximately 10% from all subclades.

The *p*-distances in 16S rRNA follow the same pattern as in ND4-tRNAs. The greatest distance is recorded between *Jordan* and *cyprica* (4.6%) and the lowest between *daani* and *stellio TR*, as well as *daani* and *Lebanon* (0.6%). Divergence within the two major clades (1 and 2) is roughly 1% in each case and reaches 2.8% between the two (Table 2). Again, *cyprica* is highly divergent with a 4% distance from Clade 1 and a 3.8% distance from Clade 2.

In nuclear loci, maximum *p*-distances measured are 0.3% in *CMOS*, 0.9% in *R35*, 1.0% in *NKTR*, 1.7% in *PNN*, 1.8% in *RAG1* and 2.1% in *MC1R* (Supporting Information, File S7). As in the mtDNA, the largest values are recorded between any subclades belonging to the two distinct major clades (1 vs. 2), followed by the ones between *cyprica* and the rest of the subclades.

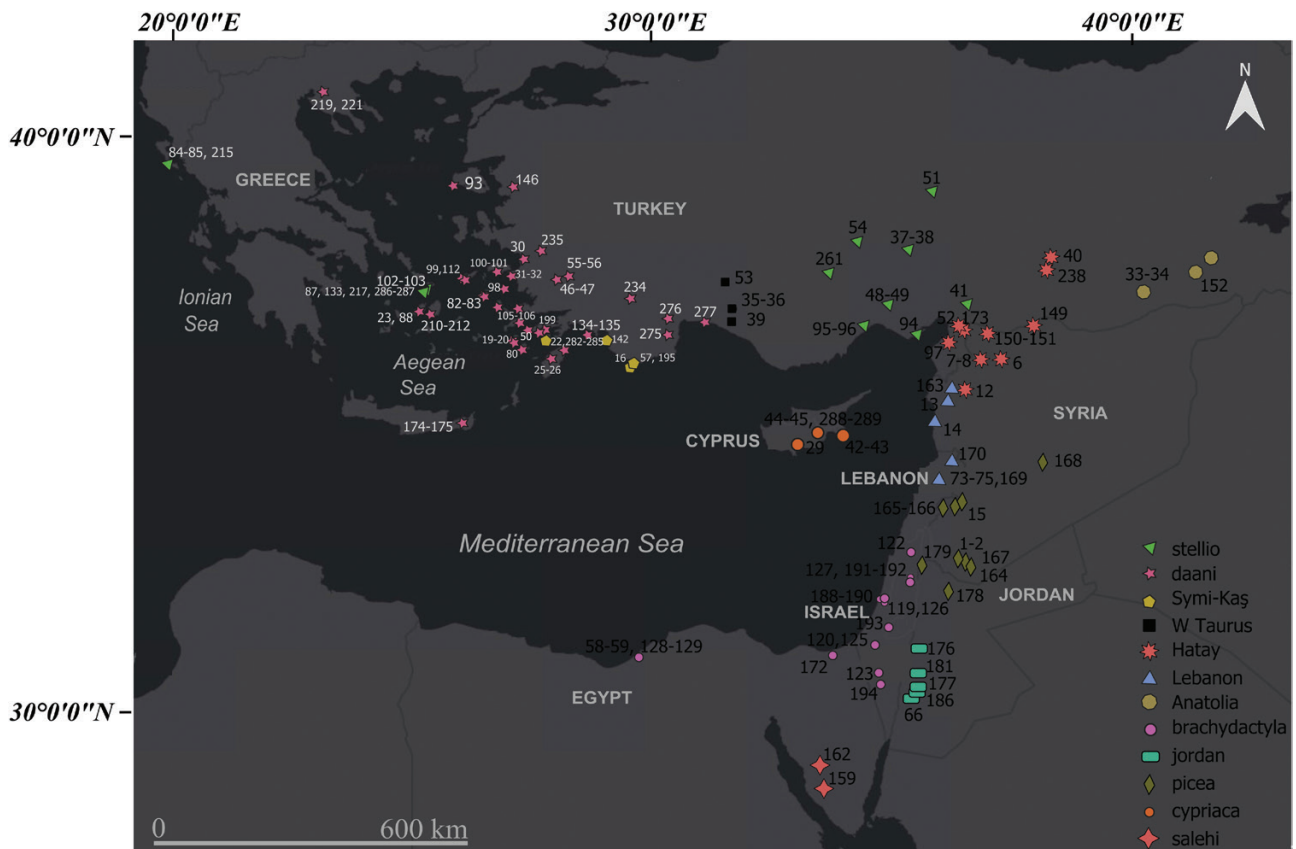


Figure 3. Map showing the sampling localities of specimens used in the present study. Different colours represent the phylogenetic subclades indicated in Figures 2 and 4.

Haplotype networks inferred from all nuclear markers are given in [Supporting Information, Files S8 and S9](#). The highest level of variation, expressed as the highest number of haplotypes, is observed in *NKTR*, followed by *MC1R* and *R35*, whereas the lowest is in *CMOS*. Patterns of nuclear haplotypes generally agree with the results of phylogenetic analyses, with higher levels of allele sharing within each of the two major clades (1 and 2). In contrast, *cypriaca* alleles are mostly private and grouped with those of Clade 2.

SPECIES TREE AND DIVERGENCE TIME ESTIMATION

The multilocus calibrated species tree with the mean, and the posterior probability support of estimated divergence times are presented in [Figure 5](#) and [Supporting Information, File S1](#). The effective sample size (ESS) values are high (> 716). The topology is similar to the one produced by the concatenated dataset, placing *cypriaca* as sister to Clade 1, and the *Symi-Kaş* subclade, together with the ones of *daani* and *W Taurus*, but with low support in both cases.

Laudakia stellio diverged during the Middle Miocene (13.7 Mya, 95% HPD: 8.7–19.0). The first split, which

took place during the Early Pleistocene (2.7 Mya, 95% HPD: 1.8–3.7), separated Clade 2 from Clade 1 and *cypriaca*. The differentiation of the latter from Clade 1 followed shortly, around 2.4 Mya (95% HPD: 1.5–3.3). All other splits are relatively recent. The temporal differentiation of Clade 1 started 1.2 Mya (95% HPD: 0.7–1.8) and coincides with the split of the Near-East subclades (*Hatay* and *Lebanon*) from the rest of Clade 2 (1.00 Mya, 95% HPD: 0.6–1.4). The differentiation of *stellio* and *daani* (including their sister subclades) occurred around 0.8 Mya (95% HPD: 0.5–1.2).

SPECIES DELIMITATION

The BPP analyses A10 (species delimitation using a fixed guide tree) and A11 (joint species delimitation and species tree estimation) produced overall similar results ([Supporting Information, File S 6](#)). Results are not sensitive to different prior combinations (including empirical ones), nor to topological differences of starting-trees (A11) and fixed guide-trees (A10) among the three scenarios. In almost all analyses, 12 evolutionary entities are delimited, each corresponding to a well-supported phylogenetic subclade. In A10, run

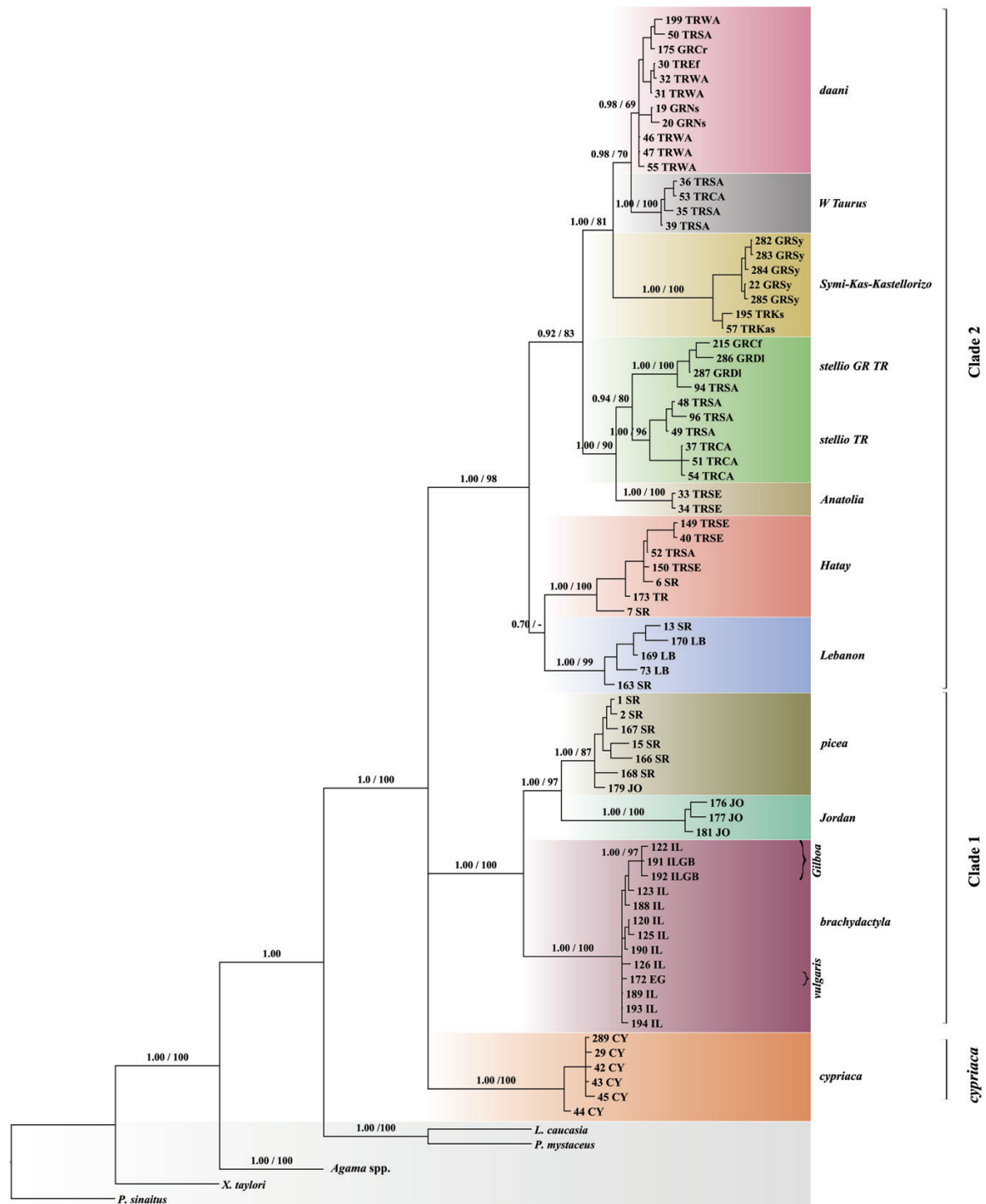


Figure 4. Phylogenetic tree based on the concatenated dataset (mtDNA & nuDNA). Bayesian posterior probabilities (PP) and maximum likelihood bootstrap support (bs) values are represented in the form PP/bs above or beside nodes. (*Agama* spp. contains *Agama agama*, *A. boensis*, *A. bottega*, *A. boueti*, *A. boulengeri*, *A. impalearis*, *A. planices*, *A. sankaranica* and *A. spinosa*).

8 resulted in 11 and ten evolutionary entities, with the merging of the *Hatay* and *Lebanon* subclades in all scenarios, and the additional merging of *daani*–*W Taurus*–*Symi-Kas* in scenario 2. In A11 analyses,

run 8 produced similar results, with the additional lumping of *brachydactyla* with *Jordan*, which lowers the number of specified evolutionary entities to eight (in scenario 1).

Table 1. Sequence divergence (p -distance, %) among the main subclades of *Laudakia stellio* and two outgroups (*Laudakia caucasia* and *Phrynocephalus mystaceus*). Values below the diagonal represent distances of mtDNA fragments (ND4-tRNAs), whereas values above the diagonal represent the mtDNA fragment 16S rRNA. Values in diagonal are the within lineages sequence divergences (ND4-tRNAs and 16S rRNA). Dashes refer to non-estimated values. All values are rounded to two decimal places

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. daani	0.64 & 0.08	0.88	1.93	1.16	0.61	1.88	0.78	0.61	2.74	1.59	2.49	3.67	1.69	15.69	14.74
2. WTaurus	1.78	0.28 & 0.26	2.03	1.77	1.15	2.03	0.88	1.17	2.66	2.41	3.09	4.28	2.56	15.05	13.67
3. Symi	6.57	6.83	1.23 & 0.16	2.18	1.48	2.15	1.74	2.07	4.20	3.05	4.00	3.98	3.43	16.24	14.95
4. stellio GR-TR	5.20	5.61	8.07	0.18 & 0.15	0.85	2.10	1.46	1.72	3.60	2.45	2.55	4.10	2.60	16.49	15.37
5. stellio TR	4.71	5.32	7.64	3.31	1.37 & 0.18	1.50	0.83	1.14	3.11	1.93	2.68	3.56	2.04	15.30	14.43
6. Anatolia	3.79	4.27	7.16	3.44	3.03	1.18 & 0.9	1.31	2.23	4.33	3.18	3.38	4.39	3.35	16.34	15.10
7. Hatay	6.54	6.59	8.44	7.42	7.89	6.84	1.73 & 0.25	1.13	3.47	2.29	2.83	3.60	2.49	15.78	15.16
8. Lebanon	6.44	6.82	7.39	7.79	7.42	6.55	6.62	0.67 & 0.13	2.81	1.70	3.07	3.83	2.28	15.27	14.53
9. brachydactyla	13.72	13.53	12.99	13.29	13.64	13.81	13.36	12.37	0.38 & 0.1	1.55	2.63	4.02	1.59	15.61	14.96
10. picea	12.47	12.10	12.30	12.35	12.67	12.33	12.38	11.67	7.64	0.97 & 0.09	1.87	3.86	0.94	16.21	15.11
11. Jordan	13.01	12.62	12.11	12.20	12.98	12.54	13.59	13.12	9.74	6.58	0.51 & 0.13	4.57	1.79	17.50	15.84
12. cypriaca	10.27	10.22	11.13	10.66	10.93	10.66	9.79	10.60	10.76	10.13	11.19	0.44 & 0	3.82	14.87	14.30
13. salehi	-	-	-	-	-	-	-	-	-	-	-	-	- & 0	16.08	15.43
14. L. caucasia	22.01	21.71	21.46	22.03	22.45	22.61	22.71	22.44	22.77	22.98	23.85	21.42	-	- & -	14.38
15. P. mystaceus	21.97	22.19	22.05	22.43	23.21	22.01	22.29	21.54	22.70	23.16	23.53	21.68	-	21.34	- & -

Table 2. Sequence divergence (*p*-distance, %) among the main clades of *Laudakia stellio* and two outgroups (*Laudakia caucasia* and *Phrynocephalus mystaceus*). Values below the diagonal represent distances of mtDNA fragments (*ND4*-tRNAs), whereas values above the diagonal represent the mtDNA fragment 16S rRNA. Values in diagonal are the within lineages sequence divergences (*ND4*-tRNAs and 16S rRNA). Dashes refer to non-estimated values. All values are rounded to two decimal places

	<i>Clade II</i>	<i>Clade I</i>	<i>cypriaca</i>	<i>L. caucasia</i>	<i>P. mystaceus</i>
<i>Clade II</i>	4.85 & 1.01	2.75	3.80	15.78	14.81
<i>Clade I</i>	13.03	5.02 & 1.22	4.04	16.08	15.16
<i>cypriaca</i>	10.44	10.64	0.44 & 0	14.87	14.30
<i>L. caucasia</i>	22.13	22.99	21.42	-	14.38
<i>P. mystaceus</i>	22.17	22.96	21.68	21.34	-

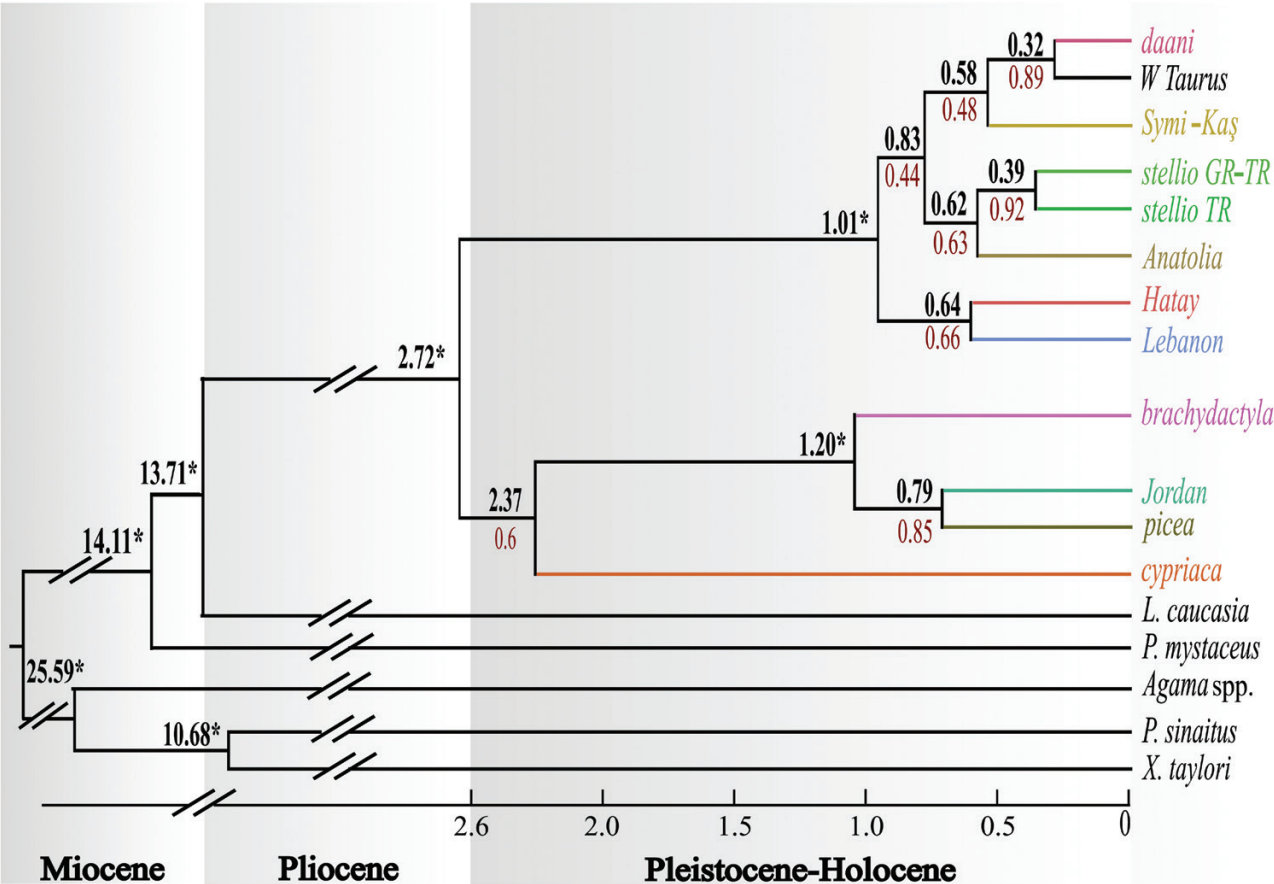


Figure 5. Multilocus calibrated species-tree produced by StarBEAST2. Numbers above branches represent mean divergence times (Myr), while numbers below represent posterior probabilities. Asterisks represent posterior probabilities equal to 1. (*Agama* spp. contains *Agama agama*, *A. boensis*, *A. bottega*, *A. boueti*, *A. boulengeri*, *A. impalearis*, *A. planices*, *A. sankaranica* and *A. spinosa*).

The BPP approach strongly supports the splitting of all phylogenetic subclades in the majority of the analyses. However, the heuristic calculation of the gdi shows a different picture. The results of four

combined runs, after a multiple-step progressive hierarchical lumping of sister-taxa, are shown in Figure 6. In all scenarios (Fig. 6A1–3), *stellio* GR-TR, *stellio* TR, *WTaurus*, *Symi-Kaş* and *Jordan* subclades

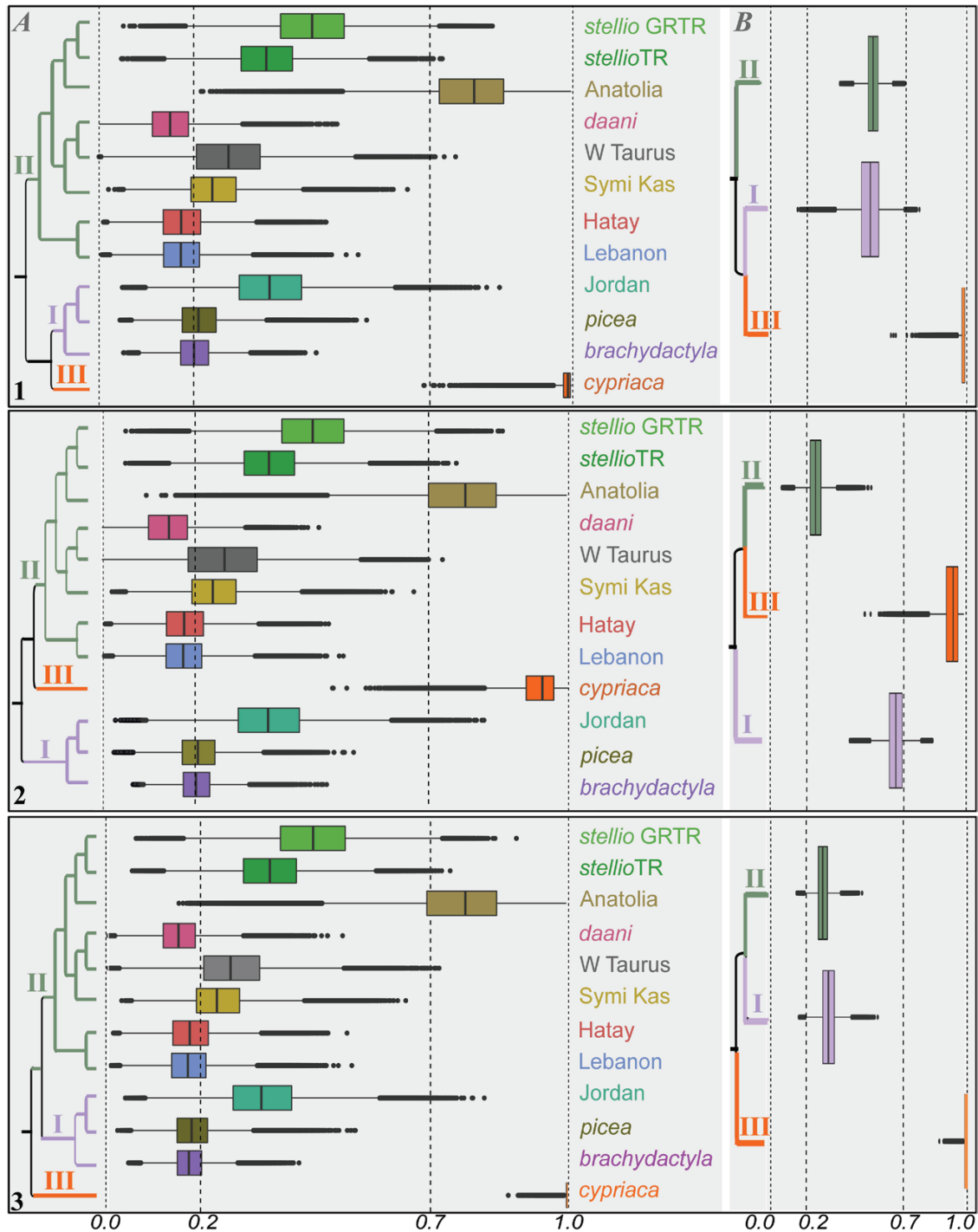


Figure 6. Summary of heuristic BPP delimitation based on the gdi under three topological scenarios (1, 2 and 3). Four BPP runs were combined in every step of a multiple analysis of progressive hierarchical lumping of sister taxa. Boxplots in the left (A) refer to the gdi of each well-supported phylogenetic subclade while those on the right (B) correspond to the three distinct evolutionary entities: Clade 1 (I), Clade 2 (II) and *cypriaca* (III).

fall within the ambiguous zone ($0.2 < \text{gdi} < 0.7$), whereas *Hatay*, *Lebanon* and *daani* have a $\text{gdi} \leq 0.2$. The *picea* and *brachydactyla* subclades exhibit

marginal gdi values (Fig. 6A2) that are slightly greater (Fig. 6A1) or smaller (Fig. 6A3) than 0.2. Last, the highest gdi values (> 0.7) are exhibited

by *Anatolia* and *cypriaca*, with the latter exhibiting values close to 1.

Progressive merging of subclades allowed the estimation of gdi values for the two major clades (1 and 2) under the three scenarios explained above (Fig. 6B1–3). Irrespective of its topological placement, *cypriaca* exhibits gdi values close or equal to 1. However, the two major clades (1 and 2) exhibit values that shift along the ambiguity zone, depending on the position of *cypriaca*: when *cypriaca* is sister to Clade 1, the two major clades exhibit higher values (Fig. 6B1), while the opposite is true if the two clades have a sister-relationship (Fig. 6B3). Finally, when Clade 2 is sister to *cypriaca*, it shows a gdi value close to 0.2, and Clade 1 shows a gdi value close to 0.7 (Fig. 6B2).

DISCUSSION

This is the first multilocus molecular study that explores the phylogenetic history of the '*L. stellio*' complex, including samples from its entire distribution and of all currently accepted morphological subspecies. Phylogenetic analyses (BI and ML) and species delimitation analysis, combined with the latest heuristic estimation of the genealogical divergence index (BPP and gdi), complement the existing, thorough morphological assessment of this taxon. Our results reveal that the striking phenotypic differentiation within '*L. stellio*' is paralleled by high levels of genetic diversity. All but one (*L. s. vulgaris*) of the subspecies described on morphological grounds correspond to well-supported evolutionary subclades, which, together with a few new ones, form two major clades (1 and 2). The Cypriot population corresponds to a distinct genealogical lineage, with uncertain phylogenetic position regarding the two major clades.

SPECIES DELIMITATION

The current taxonomy of '*L. stellio*' does not precisely reflect its genetic diversity. Several populations do not fall clearly within the morphological subspecies described so far, leaving many open questions about their status and distribution. Species delimitation analysis mostly supports a scheme of 12 distinct evolutionary entities, each one corresponding to a well-supported phylogenetic subclade. Nevertheless, these delimitations may actually reflect population splits rather than speciation events (Jackson *et al.*, 2017; Sukumaran & Knowles, 2017). This issue becomes more evident when comparing these results with the ones produced by the coupling of Bayesian parameter estimation with the genealogical divergence index (gdi) assessment (Leaché *et al.*, 2019). The majority of

the 12 evolutionary entities produced by BPP actually exhibit small gdi values, placed on the lower end of the 'speciation gradient' (Fig. 6A1–3). The only exception is the *cypriaca* phylogenetic lineage, which is clearly distinct under all possible topological scenarios, with gdi values ranging from 0.99 to 1. Moreover, gdi estimation for the two major clades through progressive merging of sister subclades, reveals greater values (Fig. 6B1–3), towards the higher end of the 'speciation spectrum'. Given the discrepancy of species delimitation results and taking into account the phylogenetic analyses and the divergence times estimated in the present study, it appears that '*L. stellio*' actually comprises three distinct evolutionary species: Clade 1, Clade 2 and *cypriaca*, with high intraspecific divergence.

DIVERGENCE TIMES AND GENETIC DISTANCES

Laudakia stellio originated in the Middle Miocene (13.7 Mya) and further diversified in the Late Pliocene (2.7 Mya) with the split of Clade 2 from Clade 1 and *cypriaca*. *Cypriaca* subsequently branched off from Clade 1, in the Early Pleistocene (2.4 Mya). This relatively short period of time between these cladogenetic events, could be the reason behind the uncertainty of the phylogenetic position of *cypriaca*. This trifold diversification is also reflected by the mtDNA genetic distances among Clade 1, Clade 2 and *cypriaca*. Distances between Clades 1 and 2 reach 13.0% in *ND4* and 2.7% in 16S rRNA, with *cypriaca* exhibiting equally high distances from both (c. 10% in *ND4* and 4% in 16S rRNA). These results are comparable to the *p*-distances observed among species of the lacertid genus *Podarcis* Wagler, 1830 (16S: 2.2–7.3%; Psonis *et al.*, 2017), the agamid genera *Phrynocephalus* Kaup, 1825 (*ND4*: 6.3%, 16S: 4.5%; Pang *et al.*, 2003) and *Agama* Daudin, 1802 (*ND4*: 13.1%; Gonçalves *et al.*, 2012). They are also similar to the intraspecific distances among candidate *L. nupta* species (De Filippi, 1843) (*ND4*: 1.3–9.7%; Sanchooli *et al.*, 2015). The two major clades (1 and 2) diversified further and almost simultaneously during the Pleistocene, in a relatively short period of time (from 1.2 to 0.3 Mya).

PHYLOGENETIC RELATIONSHIPS

Clade 1 extends from North Africa to the Levant, more specifically from Egypt and Israel to Jordan and south Syria (Fig. 3). It is divided into the *brachydactyla* subclade that corresponds to *L. s. brachydactyla*, and the *picea*–Jordan subclades, comprising *L. s. picea* and the Jordan populations of Wadi Araba, respectively. All specimens from Egypt form a small cluster in the first subclade, but without support (Fig. 2). This is also the case for the specimen representing *L. s. vulgaris*

(no. 172), as it shows no genetic differentiation from the rest of *brachydactyla*. On the other hand, *L. s. salehi* represents a distinct subclade that has an uncertain position in Clade 1 (Fig. 2). These results contradict the findings of Kadry *et al.* (2020) who suggested the synonymy of *L. s. brachydactyla* and *L. s. salehi*. However, that study was limited in both sample size and loci sequenced. Moreover, the southern part of the Sinai Peninsula is considered to be a biodiversity hotspot for reptiles, hosting a number of endemic taxa (Milito, 2017). Taking into account the disagreement over the extent of morphological differentiation between *L. s. vulgaris* and *L. s. brachydactyla* [Lachman *et al.* (2006) vs. Panov & Zykova (2016)], and the incongruence of molecular findings regarding *L. s. vulgaris*, the question regarding the validity of both *L. s. salehi* and *L. s. vulgaris* still remains open.

Subclade *brachydactyla* also encompasses a small group of lizards originating from the Gilboa mountains that is weakly supported (Figs 2, 4). This small population corresponds to part of what has been previously termed as the 'Near-East *L. s. ssp.*' [the colour morph 'F' according to Daan (1967)], named after individuals originating from Mount Hermon, the Golan Heights and those distributed along the Lebanon -Israeli borders (Werner, 1988; Sivan & Werner, 1992; Panov & Zykova, 1997). It has also been used to describe morphs of '*L. stellio*' that inhabit the Mediterranean landscapes of northern Israel, in contrast to *L. s. brachydactyla*, which is mainly found in the Negev desert (Panov & Zykova, 2016). Toe-length differences between the two have been attributed to adaptation to these different habitats (Izhaki & Haim, 1996). Apart from ecological and morphological differences, an allozyme analysis showed that the 'Near-East *L. s. ssp.*' and *L. s. brachydactyla* exhibit a genetic distance comparable to the one between the latter and *L. s. salehi* (Nevo, 1981; Panov & Zykova, 1997; Federman & Werner, 2007). Nevertheless, our results do not reveal two distinct phylogenetic lineages in Israel. According to the study of Federman & Werner (2007), the southward development of irrigation, road network expansion and human-mediated transportation has led to the mingling of the two morphs, and thus the reduction of their morphological differences, especially in the contact zone. Indeed, our results reflect this morphological continuum, because all samples form one well-supported subclade (1/100) (Figs 2, 4) and a clear division in central or southern Israel is lacking. Whether the small, weakly supported cluster of the Gilboa population represents a particular subspecies awaits further investigation considering the morphological, behavioural and ecological differences reported between the latter and *L. s. brachydactyla* (Panov & Zykova, 1997, 2016), and the fact that this wider area in northern Israel and Jordan is home to

other endemic taxa, such as *Lacerta media israelica* Peters, 1964 and *Ablepharus rueppellii festae* Peracca, 1894 (Disi, 2011; Loos *et al.*, 2011).

The rest of Clade 1 consists of *L. s. picea* (*picea* subclade) and its sister-taxon, in Jordan (*Jordan* subclade). *Laudakia s. picea*, the smallest and darkest subspecies, forms a well-supported phylogenetic lineage extending from Palmyra in central Syria, to the eastern side of the Anti-Lebanon mountains in the west, and from the Sea of Galilee to the Black lava desert in the south (Fig. 3), where a great number of endemic reptiles can be found (Disi & Böhme, 1996; Disi, 2011). The Jordan subclade refers to a population limited on the mountainous strip extending from the Dead Sea to Petra (Fig. 3). Restricted to the Mediterranean ecotone between Jordan and Israel, this group has also been characterized as '*L. s. ssp.*' by Disi (2011). The morphological differentiation of this population is reflected in the results of the present study, as it represents a well-supported (1/100) subclade (Figs 2, 4).

Clade 2 contains the rest of the Levant and European populations from Lebanon, north-west Syria, Turkey and Greece (Fig. 3), which are allocated to seven subclades that can be clustered into three major groups (Figs 2, 4). The first one is formed by the *Lebanon* and the *Hatay* subclades. Mitochondrial genetic distances between the two are comparable to the ones they have with other members of Clade 2. The *Lebanon* subclade occupies a narrow area, from the high elevations of Mount Lebanon to the western side of the Syrian coastal mountain range (An-Nusayriyah) and the neighbouring coast (Fig. 3). The phylogenetic differentiation of this subclade agrees with the observations by Zinner (1967), who was the first to note the striking differences in colour and behaviour of *Laudakia* lizards, as he moved from the coast of northern Lebanon towards higher elevations, finally reaching the Beqaa valley.

The *Hatay* subclade is found in the north-western part of Syria and extends towards the Nur (Amanos) mountains in Hatay and the south-eastern Taurus mountain range, both in Turkey (Fig. 3). In the past, numerous researchers have reported the great phenotypic variation exhibited by the eastern Anatolian populations (Bird, 1936; Clark & Clark, 1973), which has caused much taxonomic confusion on whether they belong to *L. s. stellio* or *L. s. daani* (Baran & Öz, 1985; Göçmen *et al.*, 2003; Almog *et al.*, 2005 and references therein; Kumlutaş *et al.*, 2015). The Hatay population has certain morphological (neck and dorsal coloration and tail pholidosis) and osteological differences from *L. s. daani* and *L. s. stellio* (Göçmen *et al.*, 2003; Gül & Tosunoğlu, 2011) and has been characterized as a hybrid between the two (Kete & Yılmaz, 2006). The distinctiveness of *Hatay* partly agrees with a previous study (Özdemir *et al.*, 2011), which showed the presence of two mitochondrial

lineages in Anatolia (although with low support), one of which matches the *Hatay* subclade described above.

The second major group of subclades of Clade 2 consists of *stellio* and *daani* subclades, corresponding to the homonymous subspecies, along with three geographically restricted subclades, namely *Anatolia*, *W Taurus* and *Symi–Kaş*. The phylogenetic position of the latter is not clear. It appears as sister to all other subclades within Clade 2 in the mtDNA analyses, in contrast to the concatenated gene tree and the calibrated species tree, where it appears to have sister-group relationship to its neighbouring, *daani* and *W Taurus* subclades. Considering its geographic position, genetic distances and divergence times, the last scenario seems more plausible. The differentiation of the *W Taurus* subclade is also supported by its coloration and meristic features (Kumlutaş et al., 2004). The only mainland European population, located in the broader area of Thessaloniki (Greece), belongs to *L. s. daani* and so does the newly discovered insular population from Crete (Spaneli & Lymberakis, 2014), as it clusters with specimens from the island of Rhodes.

These results help to clarify the limits of *L. s. daani* in Anatolia. From the E. Aegean islands and the Turkish coast, *L. s. daani* reaches the west side of Taurus mountains and is replaced by *L. s. stellio* on the east side of the mountain range. This Turkish population of *L. s. stellio* (*stellio* TR subclade) corresponds to the second mitochondrial lineage found by Özdemir et al. (2011) that was misallocated to *L. s. daani* and has a sister-relationship with the Greek populations of *L. s. stellio*, which form a well-supported (1/100) subclade (*stellio* GR-TR) (Figs 2, 4). Finally, the easternmost group of lizards, the *Anatolia* subclade, is sister to these two *stellio* subclades.

The Cypriot population is a well-defined, distinct evolutionary entity, according to phylogenetic and species delimitation analyses. Individuals of this insular lineage are also the largest (Osenegg, 1989; Baig, 1992) and have a unique tail pattern (Baig et al., 2012). The phylogenetic relationship of *cyprica* with the other two major clades still remains ambiguous because they all diversified in a relatively short period of time. According to the mtDNA and the multilocus StarBEAST analysis, *cyprica* is sister to Clade 1, but with low statistical support. In the concatenated dataset phylogenetic analyses, it forms a polytomy with both Clades 1 and 2. On the other hand, most nuclear haplotype networks show that the private alleles of *cyprica* are closer to Clade 2 rather than Clade 1.

EVOLUTIONARY HISTORY

The diversification of '*L. stellio*' took place in the Middle Miocene when a global cooling trend and fluctuating CO₂ levels led to the expansion of arid ecosystems

and subsequent evolutionary shift in many plant and animal taxa (Kürschner et al., 2008). Apart from this drastic climatic change, the split between *L. stellio* and *L. caucasia* could have been initiated by the division of Anatolia by the central Paratethys during the Early Serravallian (13.8 Mya) (Rögl, 1999a, b) and maintained by the Lesser Caucasus and the Zagros mountain ranges, limiting the range of *L. stellio* to the west and *L. caucasia* to the east of these mountains. The opening of eastern Anatolia to the central Paratethys has shaped the evolution of other reptile species found in this area, such as the Anatolian lizards of the genus *Apathya* Méhely, 1907 (Kapli et al., 2013).

Further diversification during the Plio-Pleistocene transition led to the formation of three distinct evolutionary groups: Clade 1, Clade 2 and *cyprica*. This period, from Late Pliocene to Middle Pleistocene, is known as the second orogenic phase of the Middle East and the Levant, when intense tectonic movements led to the uplifting of mountain masses and the creation of folds and faults along the northern, eastern and western boundaries of the Arabian Plate (Lateef, 2007). The mountains of northern Lebanon, the Palmyrides mountain belt and the Bitlis–Pütürge Massifs, have probably contributed to further diversification of the two major clades (1 and 2), limiting Clade 1 to the Near East and Clade 2 mostly to Anatolia. A similar pattern of differentiation between Anatolian and Near Eastern populations has also been reported for other reptiles, such as the striped skink, *Heremites vittatus* (Olivier, 1804) (Baier et al., 2017).

Several other mountain ranges have either promoted or halted diversification by acting as local refugia or barriers to dispersal. Within Clade 1, *salehi* occurs exclusively on the granite mountains of the southern Sinai, whereas the *Jordan* subclade is limited to the highlands east of the Wadi Araba–Jordan valley (Fig. 3). Located along the fault between Africa and Arabia, the Wadi Araba represented an obstacle to animal dispersal between the two and thus has shaped the evolutionary history of other lizard species as well; for example, of the genus *Mesalina* Gray, 1838 (Kapli et al., 2008, 2014). On the other hand, a water barrier could have contributed to the separation of *brachydactyla* from its sister *Jordan–picea* subclade. That would be the Sedom Lagoon, formed by ingression of the Mediterranean Sea water into the Dead Sea depression during Late Pliocene–Early Pleistocene, and extending from the broader area of the Galilee Sea to the Jordan–Arava valley (Stein, 2001). A similar pattern of divergence, shaped by the presence of the Sedom Lagoon, is exhibited by chameleons inhabiting the Gilboa highlands (Yaacov et al., 2012).

Within Clade 2, the Syrian coastal mountain range and the adjacent Ghab plain keep the two 'Near East' subclades, *Lebanon* and *Hatay*, separate from each

other. Further north, this division is sustained by the Amanos (Nur) mountains and the adjacent Amik plain. The two are further isolated from the rest of Clade 2 by the Anatolian Diagonal mountains. This complex geomorphological landscape has acted as a biogeographic barrier for several other lizard species belonging to *Heremites* (Baier *et al.*, 2017), *Apathya* (Kapli *et al.*, 2013) and *Lacerta* (L., 1758) (Ahmadzadeh *et al.*, 2013). The Taurus massif limited the westward expansion of *stellio* TR in southern Anatolia. On its western side, *daani* and its sister-subclades (*W Taurus* and *Symi–Kaş*) replace *stellio*, extending to the East Aegean islands and some of the Cyclades (Paros and Naxos).

The general pattern of *daani* in the west and *stellio* in the east is disrupted by the presence of both on the Cyclades (*stellio* on Mykonos and Delos, and *daani* on Naxos and Paros), raising questions about their origin. The Cyclades were separated from the E. Aegean islands by an extensive sea transgression initiated in the end of the Middle Miocene (12 Mya), leading to the fragmentation of the Aegean area, and the creation of the northern and eastern Aegean Sea in the Tortonian (Fassoulas, 2018 and references therein). This Aegean Sea Barrier has shaped the evolution of several groups of organisms. Many taxa were unable to cross it, but only a handful succeeded (Lymberakis & Poulakakis, 2010; Poulakakis *et al.*, 2015; Kornilios *et al.*, 2019). Taking into account (1) the sister-relationship of *stellio* from Mykonos–Delos and *stellio* from south-eastern Anatolia, (2) that two mitochondrial haplotypes from south-eastern Anatolia cluster with the Cycladic ones, and (3) the fact that both Delos and the gulf of Alexandretta (Iskenderun) hosted important ports during ancient times (Akar, 2009; Kron, 2013), the presence of *stellio* in the Cyclades could be attributed to human-mediated dispersal. This result contradicts Brammah *et al.* (2010) according to which the two subspecies derived from a single ‘*stellio*-type’ species occurring in the Protocycladic Block. Whether *daani* crossed the Aegean Sea Barrier by natural or human dispersal awaits further research. Human-mediated dispersal, both in the past and in the present, explains the presence of *daani* in Thessaloniki and Crete (Klaptocz, 1910; Spaneli & Lymberakis, 2014), of *stellio* in Corfu (Koch, 1932) and *brachydactyla* in Giza and Cairo (Flower, 1933). It could also explain the disjunct distribution of the *Symi–Kaş* subclade, as it comprises specimens from south-western Turkey and Kastellorizo and *Symi* from Greece, the latter being geographically more distant from the rest, but connected to Kastellorizo via ship transport. Finally, looking at the fossil record, the absence of post-Pliocene agamid remains from the Balkans suggests that the extant Greek populations do not represent a relict widespread European population, but are the result of

a more recent, anthropochorous or natural dispersal from the east (Delfino *et al.*, 2008), corroborating the results of this study.

Climatic fluctuations during the Pleistocene, acting synergistically with mountain uplifting, led to the burst of diversification within the two major clades (1 and 2). Cold and dry glacial periods were followed by hotter and warmer interglacial periods (Webb & Bartlein, 1992), causing repeated contraction of species to refugia and outward re-expansion, thus promoting speciation in many different animal groups (Hewitt, 1996; Avise *et al.*, 1998). Apart from the Iberian, the Italian and the Balkan peninsulas (Blondel, 2010 and references therein), Anatolia also acted as a major glacial refugium (Rokas *et al.*, 2003; Gündüz *et al.*, 2007; Mutun, 2010; Bilgin, 2011; Arslan *et al.*, 2020). Indeed, Clade 2 seems to have persisted and diverged in several areas of southern Anatolia that served as local refugia for other reptile species during the Pleistocene, such as *Lacerta trilineata* Bedriaga, 1886 (Ahmadzadeh *et al.*, 2013; Sagonas *et al.*, 2014), *Ophisops elegans* Ménétries, 1832 (Kyriazi *et al.*, 2008) and *Heremites vittatus* (Baier *et al.*, 2017). Moreover, the diversification pattern revealed by this study is almost identical to the one exhibited by the Turkish worm lizard *Blanus strauchi* (Bedriaga, 1884) (Sindaco *et al.*, 2014), the Eurasian blindsnake *Xerotyphlops vermicularis* (Merrem, 1820) (Kornilios *et al.*, 2011, 2012), the Anatolian lizard *Anatololacerta anatolica* (Werner, 1900) (Bellati *et al.*, 2015; Karakasi *et al.*, 2021) and the snake-eyed skink *Ablepharus kitaibelii* Bory de Saint-Vincent, 1833 (Skourtanioti *et al.*, 2016). The biogeographical subregions or biodiversity hotspots of Anatolia shaping this common evolutionary pattern and hosting distinct evolutionary lineages are: Cilicia and Pamphylia, on the eastern and western sides of the Taurus, respectively, and Lycia in south-western Anatolia.

The rougtail rock lizards of Cyprus formed a well-supported and distinct phylogenetic lineage. It diversified in the Early Pleistocene (2.4 Mya), soon after the split of Clade 2 from Clade 1 + *cypriaca* (2.7 Mya). Cyprus is an oceanic island that took its present form during the last 2.6 Myr, and was never connected to the mainland (Simmons, 1999; Aksu *et al.*, 2005). Therefore, colonization of Cyprus by various amphibian and reptile species has been attributed mainly to human or natural overseas dispersal, starting in the Miocene and continuing through the Pleistocene (Böhme & Wiedl, 1994; Simonato *et al.*, 2007; Poulakakis *et al.*, 2013; Baier *et al.*, 2017). The time of divergence estimated for *cypriaca* suggests that it colonized the island via overseas dispersal rather than human-mediated transport. However, the origin of the ancestor population cannot be specified in this study as there are affinities with both major clades. In some analyses it appears closer to Clade 1

(BI of mtDNA and calibrated species-tree) but with low support, whereas in others it is closer to Clade 2 (nuDNA haplotype networks). The greatly overlapping divergence-time ranges of Clades 1, 2 and *cypriaca* imply that the first colonizers of Cyprus could belong to the common ancestor of all three lineages. On that note, some amphibian and reptilian taxa inhabiting Cyprus are closer to their Near Eastern counterparts (Kornilios *et al.*, 2012; Baier *et al.*, 2017), while others show a greater affinity to Turkish populations (Gvoždík *et al.*, 2010; Skourtanioti *et al.*, 2016).

TAXONOMIC IMPLICATIONS

This study reveals that '*L. stellio*' comprises three species with high intraspecific diversity that correspond to Clade 1, Clade 2 and *cypriaca*. Therefore, we propose the following taxonomy:

LAUDAKIA VULGARIS (SONNINI & LATREILLE, 1802) STAT. NOV.

Included taxa: *Laudakia s. picea* (Parker, 1935), *L. s. brachydactyla* (Haas, 1951), *L. s. vulgaris* (Sonnini & Latreille, 1802), *L. s. salehi* (Werner, 2006) and the Jordan phylogenetic subclade.

Type specimen: *Agama stellio vulgaris* (Sonnini & Latreille, 1802), FMNH 153134 (neotype) Crochet *et al.* (2006) (Field Museum of Natural History, Chicago, USA).

Type locality: El Amiria, Alexandria, Lower Egypt, by present neotype designation.

Distribution: All localities from Egypt, Jordan, Israel and south-western and western Syria (east to Palmyra).

Remarks: According to the principle of priority of the ICZN (International Code of Zoological Nomenclature), this distinct genetic lineage was named *vulgaris* as that is the oldest available name. It includes the morphological subspecies of *L. s. picea*, *L. s. brachydactyla*, *L. s. vulgaris* and *L. s. salehi*, as well as a new phylogenetic subclade that corresponds to the Jordan populations from the eastern side of the Wadi–Araba valley, previously reported as an unnamed subspecies. The subspecific description of the latter is under preparation, but we have to note that the subspecific status of *L. s. vulgaris* and *L. s. salehi* is ambiguous and requires further investigation.

LAUDAKIA STELLIO (LINNAEUS, 1758) S.S.

Included taxa: *Laudakia s. stellio* (Linnaeus, 1758), *L. s. daani* (Beutler & Frör, 1980) and the subclades

from the western Taurus, *Symi–Kaş*, Anatolia, Lebanon and Hatay, as shown in this study (Figs 2, 4).

Type specimen: *Laudakia stellio stellio* (Linnaeus, 1758), ZFMK 2063 (neotype), Crochet *et al.* (2006) (Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany).

Type locality: Delos Island, Cyclades, Greece.

Distribution: All localities from Greece (Cyclades, Crete, Thessaloniki, Corfu and East Aegean islands) and Turkey, north-western Syria and northern Lebanon.

Remarks: According to the principle of priority of the ICZN, this distinct genetic lineage should be named *stellio* because that is the oldest available name. It includes already described morphological subspecies and other cryptic lineages of Clade 2, revealed by this study: the nominal *L. s. stellio* and its sister *Anatolia* subclade, *L. s. daani* and its sister *WTaurus–Symi–Kaş* subclades, as well as the *Lebanon* and *Hatay* subclades. The subspecific status of *L. s. stellio* and *L. s. daani* is retained, while the elevation of the *Lebanon* and *Hatay* lineages to the subspecies level is under preparation.

LAUDAKIA CYPRIACA (DAAN, 1967) STAT. NOV.

Included taxa: *Laudakia s. cypriaca* (Daan, 1967).

Type specimen: *Agama stellio cypriaca* Daan, 1967. BMNH 1930.10.5.6. (holotype) (Natural History Museum, London, UK).

Type locality: Limassol, Cyprus.

Distribution: Endemic to Cyprus.

Remarks: This species exhibits distinctive morphology, ecology and behaviour, all of which further corroborate molecular evidence and support its elevation to species rank.

CONCLUSION

This is the first comprehensive genetic study shedding light on the evolution of the '*L. stellio*' species complex, using both mitochondrial and nuclear markers, bridging the gap between genetic divergence and morphology-based taxonomy. Our findings confirm that the reported phenotypical diversity matches the genetic differentiation of several evolutionary entities encompassed within '*L. stellio*' and highlight the importance of Anatolia and

the Levant as glacial refugia in the past that turned into biodiversity hotspots in the present.

According to the present study, albeit monophyletic, '*L. stellio*' represents not just one, but three distinct, well-supported evolutionary entities that should be considered distinct species. The Cypriot population, previously considered a subspecies, is elevated to species level, *L. cypriaca* (Daan, 1967), given its distribution, divergence time, genetic differentiation and morphological features. Its phylogenetic relationship with the other two species remains unclear since clade divergence seems to have happened in a narrow time period. Further investigations focusing on population-level analyses, using more specimens from the neighbouring mainland (Turkey, Syria, Lebanon and Israel), should resolve the details of this three-partite divergence. The other two species correspond to the two major phylogenetic Clades 1 and 2. The first (*L. picea*) includes the populations from North Africa, Jordan, Israel and south-western Syria, corresponding to *L. s. picea*, *L. s. brachydactyla*, *L. s. vulgaris* and *L. s. salehi*. The status of the currently recognized subspecies *L. s. vulgaris* and *L. s. salehi* needs to be further explored using more samples from the type localities. The second (*L. stellio*), comprises *L. s. stellio*, *L. s. daani* and populations from Lebanon and Hatay, whose morphological and ecological divergence has already been highlighted by numerous researchers. The elevation of the latter two, as well as the *Jordan* subclade, to subspecies status is under preparation. Future research should focus on the population-level phylogeographic analysis that will help towards clarification of divergence patterns of these variable lizards at a finer temporal and spatial scale.

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DATA AVAILABILITY

The data underlying this article are available in GenBank Nucleotide Database at <https://www.ncbi.nlm.nih.gov/genbank>, and can be accessed with accession numbers given in File S1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

File S1. Specimens and outgroups, mtDNA and concatenated DNA datasets, GenBank accession numbers, primers and PCR condition information, 95% HPD intervals of divergence times (.xlsx).

File S2. PTP results (.txt).

File S3. PartitionFinder2 results (.docx).

File S4. BEAUti2.xml file (.xml).

File S5. Calibration points used in the StarBeast2 analysis from Leaché *et al.* (2014). Divergence times (Myr) are presented in red above branches and the 95% HPD interval (Myr) is presented in black below branches (.pdf).

File S9. Codes of haplotypes corresponding to samples used in this study, inferred from the six nuclear loci (.xlsx).