Systematics and Biodiversity (2014), 1–12

Taylor & Francis
Taylor & Francis Group

Research Article

Phylogenetic analysis of the *Lacerta agilis* subspecies complex

CLAUDIA ANDRES[a]*, FRANZISKA FRANKE[b], CHRISTOPH BLEIDORN[a,c], DETLEF BERNHARD[a] & MARTIN SCHLEGEL[a,c]

[a]Universität Leipzig, Molekulare Evolution und Systematik der Tiere, Talstr. 33, 04103 Leipzig, Germany
[b]Universität Leipzig, Evolution und Entwicklung der Tiere, Talstr. 33, 04103 Leipzig, Germany
[c]German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5d, 04103 Leipzig, Germany

(Received 30 August 2013; revised 5 December 2013; accepted 18 December 2013)

The Sand lizard *Lacerta agilis* inhabits a huge area across Eurasia with several subspecies. Nine subspecies are generally approved, *L. a. agilis*, *L. a. argus*, *L. a. bosnica; L. a. chersonensis*, *L. a. exigua*, *L. a. grusinica*, *L. a. iorinensis*, *L. a. brevicaudata* and *L. a. boemica*, but several more have been described. The emergence of this large number of subspecies is connected with the phylogeographic history of this species, defined by geographic and climatic processes. A study revealing phylogenetic relationships of this species was already conducted with a broad taxon sampling and coverage. However, the latter study was based solely on the cytochrome b gene and, furthermore, the Balkan Peninsula inhabited by the subspecies *L. a. bosnica* was underrepresented. This peninsula is a centre of European herpetofaunal endemism with high levels of phenotypic and genotypic variation. Therefore, the inclusion of the subspecies *L. a. bosnica* is important to clarify the overall view of the phylogenetic relations within the *Lacerta agilis* subspecies complex and to investigate the level of population differentiation within this highly diverse area. Thus, the aim of the present study was a more thorough analysis of the Balkan Peninsula with a broader taxon sampling. Furthermore, we extended the available datasets by adding the mitochondrial d-loop region and by further samples of different areas of the distribution range apart from the Balkan Peninsula. Our study reveals that the Balkan Peninsula is apparently inhabited by several differentiated lineages, whereby the Central Greek lineage might deserve subspecies status. Furthermore, the distribution area of the two subspecies *L. a. agilis* and *L. a. argus* should be revised, as the results of our study raise doubt about the commonly assumed distribution of both subspecies. As the most important outcome our results support that *L. a. boemica* deserves species status.

Key words: Balkan Peninsula, cytochrome b, D-loop, *Lacerta agilis*, phylogeography

Introduction

The Sand lizard (*Lacerta agilis*) inhabits a large area ranging in west-east direction from the western border of France and eastern UK to Lake Baikal and North-western China (Fig. 1). The north–south distribution reaches from Sweden to Greece and Armenia. Within this huge distribution area nine approved subspecies exist that are commonly separated into two major clades (Bischoff, 1988). The western or Balkan clade comprises the four subspecies *L. a. agilis*, *L. a. argus*, *L. a. bosnica* and *L. a. chersonensis* and the distribution ranges from the UK and France to the Ukraine and eastern Russia. The eastern or Caucasian clade consists of the five subspecies *L. a. exigua*, *L. a. grusinica*, *L. a. iorinensis*, *L. a. brevicaudata* and *L. a. boemica* and the subspecies are distributed in the Caucasus and across Russia to China (Bischoff, 1988). Despite fundamental morphological and genetic differences between the eastern and western clade, mixed populations of interbreeding *L. a. chersonensis* and *L. a. exigua* exist in their contact zones (Zinenko *et al*., 2005). The approval of further subspecies remains ambiguous. Among them are the two subspecies *L. a. tauridica* and *L. a. garzoni*, inhabiting Crimea and Spain, respectively. *Lacerta agilis tauridica* was first described by Suchow in 1927 (Suchow, 1927; Kalyabina-Hauf *et al*., 2004) and is a representative of the eastern clade. Based on several morphological features like number of pores, number of nasal shields and postnasal shields Suchow stated that the Crimean specimens form a distinct subspecies (Suchow, 1927). However, after the description in 1927 Crimean sand lizards have been considered as belonging to the subspecies *L. a. exigua* for some unknown reason (Kalyabina-Hauf *et al*., 2004). Later on Kalyabina-Hauf *et al*. (2004) confirmed the subspecies status of *L. a. tauridica* with

*Correspondence to: Claudia Andres. E-mail: candres@uni-leipzig.de

ISSN 1477-2000 print / 1478-0933 online

http://dx.doi.org/10.1080/14772000.2013.878000

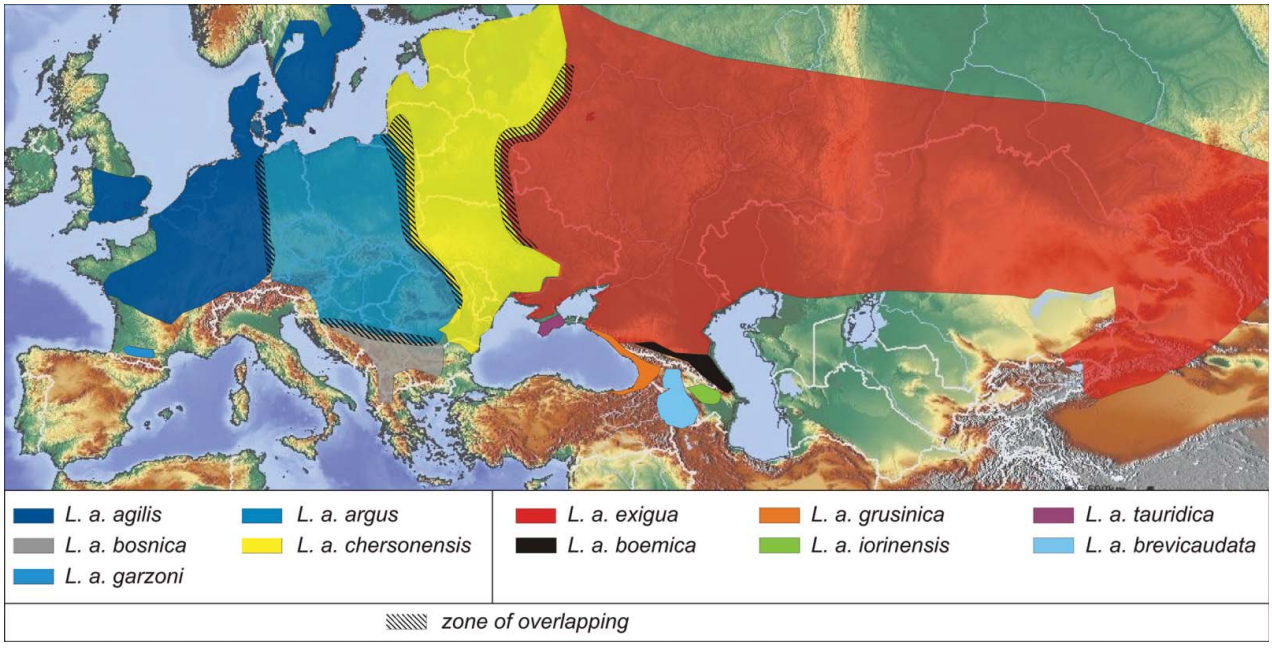

Fig. 1. Distribution range of *Lacerta agilis* with division into known subspecies according to Bischoff (1988).

morphological and genetic analysis based on the cytochrome b gene. Similarly the status of the subspecies *L. a. garzoni* is treated with caution. This subspecies was described in 1975 by Palacios & Castroviejo on the basis of specimens from only one isolated location in the Pyrenees, and genetic and morphological comparisons with specimens from Central France are missing (Palacios & Castroviejo, 1975; Amat, 2008).

The quite high number of four different subspecies within the small area of the Caucasus is explained by isolation of *L. agilis* populations through tectonic uplifts (Bischoff, 1988). A special consideration deserves *L. a. boemica*. Genetic analysis of the cytochrome b gene placed *L. a. boemica* as the sister group of all other members of the *L. agilis* complex (Kalyabina *et al*., 2001; Joger *et al*., 2006). This basal position indicates that *L. a. boemica* represents the oldest lineage of all subspecies of *L. agilis* which emerged in the Pliocene (Joger *et al*., 2006) and thus challenges the traditional grouping into two clades. Likewise, the separation of *L. a. bosnica* is assumed to already have happened in the Pliocene (Joger *et al*., 2006). Indeed it is assumed that the interspecific differentiation of several *Lacerta* species occurred within the Caucasian area (Bischoff, 1988; Kalyabina *et al*., 2001; Joger *et al*., 2006). Beginning from the Caucasus as a primary radiation centre the expansion started towards the eastern and western directions in several waves (Bischoff, 1988; Kalyabina *et al*., 2001). The age of the eastern clade cannot be estimated decisively (Kalyabina *et al*., 2001). However, the North Caspian radiation hypothesis which assumes a recent migration (Kalyabina *et al*., 2001; Joger *et al*., 2006) is supported by the low level of genetic divergence between the subspecies of the eastern clade (excluding *L. a. boemica*) and the huge expansion of the *L. a. exigua* subspecies (Kalyabina *et al*., 2001).

Better supported scenarios exist for the differentiation of the western clade. The Central European plain was colonized about 100 000 years ago, followed by the East European plain about 30 000–50 000 years ago (Kalyabina *et al*., 2001). The youngest populations are found in the Baltic region, which was colonized about 10 000–12 000 years ago (Kalyabina *et al*., 2001). Populations in the Central European plain migrated in a north–south direction triggered by glaciation events (Kalyabina *et al*., 2001; Böhme *et al*., 2007). During these glacial periods the populations were repeatedly forced back to refugees and isolated from each other (Bischoff, 1988). Assumed glacial refuge areas are the Crimea and South Caucasus for the eastern populations and the Pannonian Basin and the Balkans for the western populations (Bischoff, 1988; Zinenko *et al*., 2005). These areas later became centres of a postglacial radiation (Bischoff, 1988; Kalyabina *et al*., 2001). The Balkan Peninsula in particular is characterized by a complex geological history and palaeogeography and is still geologically active (Dzukic & Kalezic, 2004; Krystufek & Reed, 2004). The fact that the Balkan Peninsula is recognized as a postglacial distribution centre implicates the inclusion of the subspecies *L. a. bosnica* to reveal a more complete picture of the phylogeny of the *Lacerta agilis* subspecies complex. Hence, we conducted a broader sampling of the Balkan subspecies *L. a. bosnica* in order to provide more detailed information on potentially differentiated lineages within this subspecies. We furthermore aimed to extend the already existing data of

the *Lacerta agilis* complex by sampling new specimens across the distribution range. An additional molecular marker (mitochondrial D-loop region) was included to improve the phylogenetic reconstructions. With this new dataset we aimed to gain a more comprehensive insight into the genetic differentiation and phylogeography of *Lacerta agilis* with special attention on the subspecies *L. a. bosnica*.

Materials and methods

Study area

We sequenced a total number of 52 individuals, belonging to seven different subspecies originating from 16 countries (Table 1, Fig. 2). The sequences are deposited in GenBank; accession numbers and location of the sequenced individuals are listed in supplementary material (Table S6, online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2013.878000). The samples from Denmark, Hungary, Romania, Poland and three samples from Ukraine were not determined morphologically to the subspecies level. However, according to the distribution map of Bischoff (1988) the samples from Denmark most likely belong to *L. a. agilis*, the ones from Hungary to *L. a. argus* and were assigned accordingly. Since *L. a. argus* and *L. a. chersonensis* both coexist in eastern Poland, these samples can belong to either species or they can represent hybrids. The Romanian sample should belong to *L. a. argus*, but could potentially also represent *L. a. chersonensis* or a hybrid. Furthermore, we analysed samples from different parts of the Ukraine, whose morphological determination was unclear. Hence, we labelled the Polish, Romanian and Ukrainian samples as *L. agilis* ssp. To achieve a more comprehensive coverage of subspecies and distribution area we added the cytochrome b gene sequences from Kalyabina-Hauf & Ananjeva (2004) to our dataset.

DNA extraction, amplification and sequencing

All samples were tissue samples except the blood sample of the Romanian individual. The DNA extraction was carried out with the NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's protocol for all samples. Primers for the cytochrome b gene amplification were taken from Kalyabina *et al*. (2001) and primers for the D-loop region amplification from Crochet *et al*. (2004). The PCRs for both mitochondrial markers were carried out each in a total volume of 25 μl containing 0.2 mM of each dNTP, 2.5 μl of 10× Dream Taq^{TM}-Buffer including 25 mM $MgCl_2$, 1U Dream Taq^{TM} Green DNA polymerase, 0.4 μM for each forward and reverse primer and 0.5 μl DNA-extract and performed on an Eppendorf Mastercycler. The cytochrome b gene PCR was conducted under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 31 cycles of 45 s at 94°C, 60 s at 45°C and 120 s at 70°C and final extension for 10 minutes at 72°C. The D-loop region PCR consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 45 s at 94°C, 60 s at 53°C and 60 s at 72°C and final extension for 10 minutes at 72°C. The purified PCR products of the individuals were then either prepared for cycle sequencing with the Big DyeTerm v3.1 Cycle

Table 1. Analysed subspecies of the present study and their origin.

Subspecies	Country	Region	Sample size
L. a. agilis	Denmark	Rosnas, Koge, Hillerod	3
L. a. argus	Germany	Leipzig, Dresden	4
	Slovakia	Orlové	2
	Hungary	Kumposzer	1
L. a. bosnica	Bulgaria	Rila, Osogovo, Pirin	5
	Bosnia Herzegovina	Zelengora	2
	Montenegro	Durmitor, Prokletije, Maglic	5
	Greece	Varnous, Peristeri	4
	Serbia, Kosovo	Deravica	1
L. a. chersonensis	Bulgaria	Sofia	3
	Ukraine	Chernigiv region	1
	Moldavia	Leova / Hînceşti districts	1
	Russia	Luzhki, Zhernovka (both near Moscow)	3
L. a. brevicaudata	Armenia	Arayi Lerr	3
L. a. exigua	Ukraine	Kharkiv, Kherson region, Crimea	5
	China	North-West China	1
L. a. tauridica	Ukraine	Crimea	2
L. a. ssp.	Poland	Bialowieza, Bialystock	2
	Ukraine	Chernivtsi region, Volyn region, Transcarpathians	3
	Romania	Pelisor	1

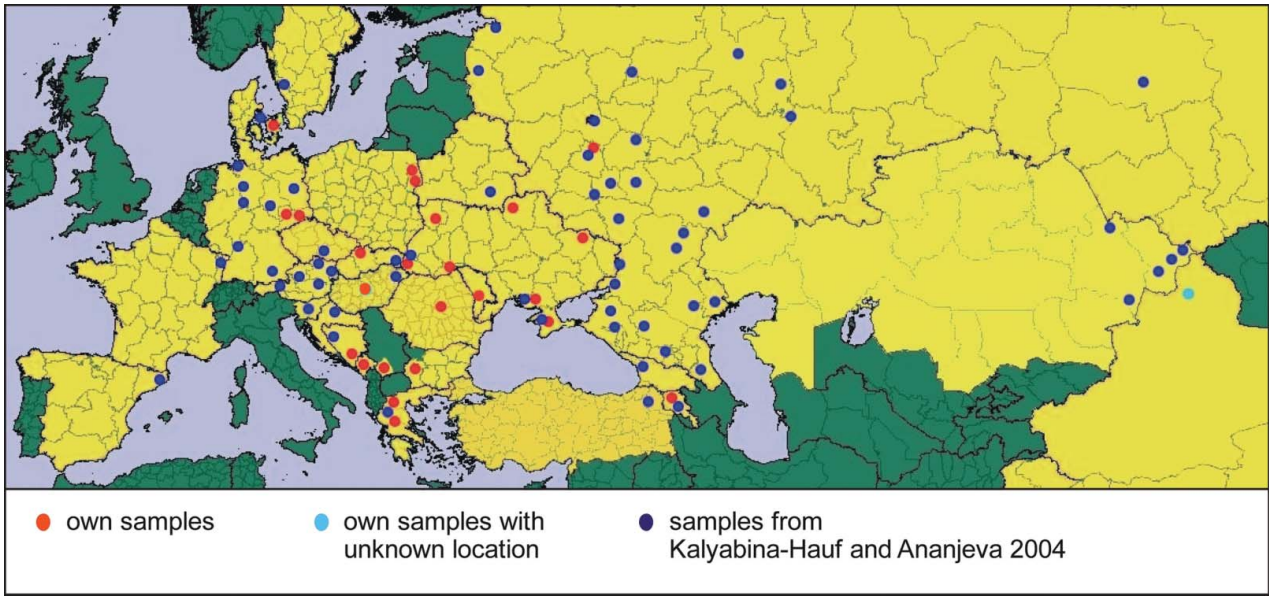

Fig. 2. Sampled locations of *Lacerta agilis*, red dots represent sampling locations of the utilized individuals, the exact sampling location of the Chinese specimen is not known and therefore represented by a light blue dot, dark blue dots represent sequences already published by Kalyabina-Hauf & Ananjeva (2004).

SeqKit; 100Rxn (Applied Biosystems) according to manufacturer's protocol and run on the ABI 3100 Genetic Analyzer or were sent to GATC Biotech AG (Konstanz, Germany) for sequencing. Both mitochondrial markers were sequenced in forward and reverse direction for all individuals.

Phylogenetic analysis

The obtained sequences were manually corrected in BioEdit 7.1.30 (Hall, 1999). Forward and reverse sequence of the same gene and individual were assembled to contigs. To avoid pseudogenes in the cytochrome b gene dataset contigs were translated into amino acids with the online translator of EMBL-EBI (http://www.ebi.ac.uk/Tools/st/) and manually checked for internal stop codons. The approved nucleotide sequences were then aligned online using Mafft 6 (Katoh *et al.*, 2002) with the FFT-NS-i method (slow; iterative refinement). Two alignments were conducted, one alignment containing all cytochrome b gene sequences of Kalyabina-Hauf & Ananjeva (2004) and of our study, and a second alignment containing the combined data of all cytochrome b gene and D-loop region sequences of our study.

Subsequently, mitochondrial gene trees were calculated for both datasets with Maximum likelihood (RaxML 7.0.4, Stamatakis, 2006), Maximum parsimony (PAUP 4.0b10, Swofford, 2002), Neighbour joining (PAUP 4.0b10, Swofford, 2002), and Bayesian inference (Mr. Bayes 3.2, Huelsenbeck & Ronquist, 2001). The chosen model of sequence evolution for all gene trees was the GTR model (General Time Reversible, Lanave *et al.*, 1984) employing a gamma distribution across sites. We chose this model because it is the only one available for Maximum likelihood reconstructions using RaxML. Clade stability was estimated using bootstrapping with 1000 pseudoreplicates for Maximum likelihood, Neighbour joining and Maximum parsimony.

For Bayesian inference we used MrBayes 3.2 (Huelsenbeck & Ronquist, 2001). The number of generations was set to 1 000 000 for the combined dataset and to 5 000 000 for the cytochrome b dataset, both with a sample frequency of 500. After the chains converged as indicated by a deviation of split frequencies below 5%, the burn-in was set to 850 for the cytochrome b gene reconstruction and to 120 for the combined analysis.

We calculated genetic divergence between subspecies and groups of subspecies. This calculation was carried out with MEGA 5.05 (Tamura *et al.*, 2011) on the basis of the K2P model (Kimura, 1980). Furthermore a cladistic haplotype aggregation analysis was conducted with TCS2.1.21 (Clement *et al.*, 2000).

Results

Sequence divergence

The sequencing yielded a 909 bp long fragment of the cytochrome b gene and a 422 bp long fragment of the D-loop region. The concatenated alignment of both mitochondrial markers of 52 specimens comprised 1331 bp including 172 variable sites, of which 147 were parsimony

informative. The sequences had an average nucleotide composition of T = 33%, C = 25.8%, A = 30% and G = 11.3%. The extended cytochrome b gene dataset including sequences from Kalyabina-Hauf & Ananjeva (2004) consisted of 245 sequences with a length of 909 bp including 194 variable sites of which 160 were parsimony-informative. The average nucleotide composition deviated slightly from the combined dataset (T = 31.5%, C = 28.9%, A = 27.4% and G = 12.2%).

Phylogenetic reconstructions of the *Lacerta agilis* complex

Gene trees inferred with Maximum likelihood are shown and bootstrap values/posterior probabilities of all utilized methods are given at the nodes (Figs 3–4). The remaining trees can be found in Figs S6–S13 (see supplemental material online). Furthermore, the genetic distances between and within the subspecies and groups of subspecies were calculated (Tables 2–5). Finally, a cladistic haplotype aggregation analysis was conducted to detect haplotypes and their correlation with each other within the dataset (Fig. 5).

Gene trees of cytochrome b gene. The extended dataset comprised the 10 subspecies *L. a. boemica*, *L. a. tauridica*, *L. a. bosnica*, *L. a. exigua*, *L. a. brevicaudata*, *L. a. grusinica*, *L. a. chersonensis*, *L. a. agilis*, *L. a. argus* and *L. a. garzoni* (Fig. 3). The monophyly of *Lacerta agilis* was confirmed in all analyses. *Lacerta a. boemica* represented the sister group of all other *L. agilis* subspecies in all reconstructions with high support.

The subspecies *L. a. chersonensis* and *L. a. agilis*/*L. a. argus* formed a highly supported clade in all reconstructions. The subspecies *L. a. chersonensis* could be recovered in all analysis. Remarkably, two individuals which were morphologically determined as *L. a. bosnica* clustered within the *L. a. chersonensis* clade (Fig. S14-1, see supplemental material online). These two individuals were sampled in the Pirin Mountains in Bulgaria. In this country *L. a. bosnica* coexists with *L. a. chersonensis*. In order to exclude the possibility of contamination or mislabelling, we sequenced the cytochrome b gene of four more samples from the same locality in the Pirin Mountains and all of them clustered within the *L. a. chersonensis* clade (not shown).

The two subspecies *L. a. agilis* and *L. a. argus* formed a distinct clade with high support in three of four analyses

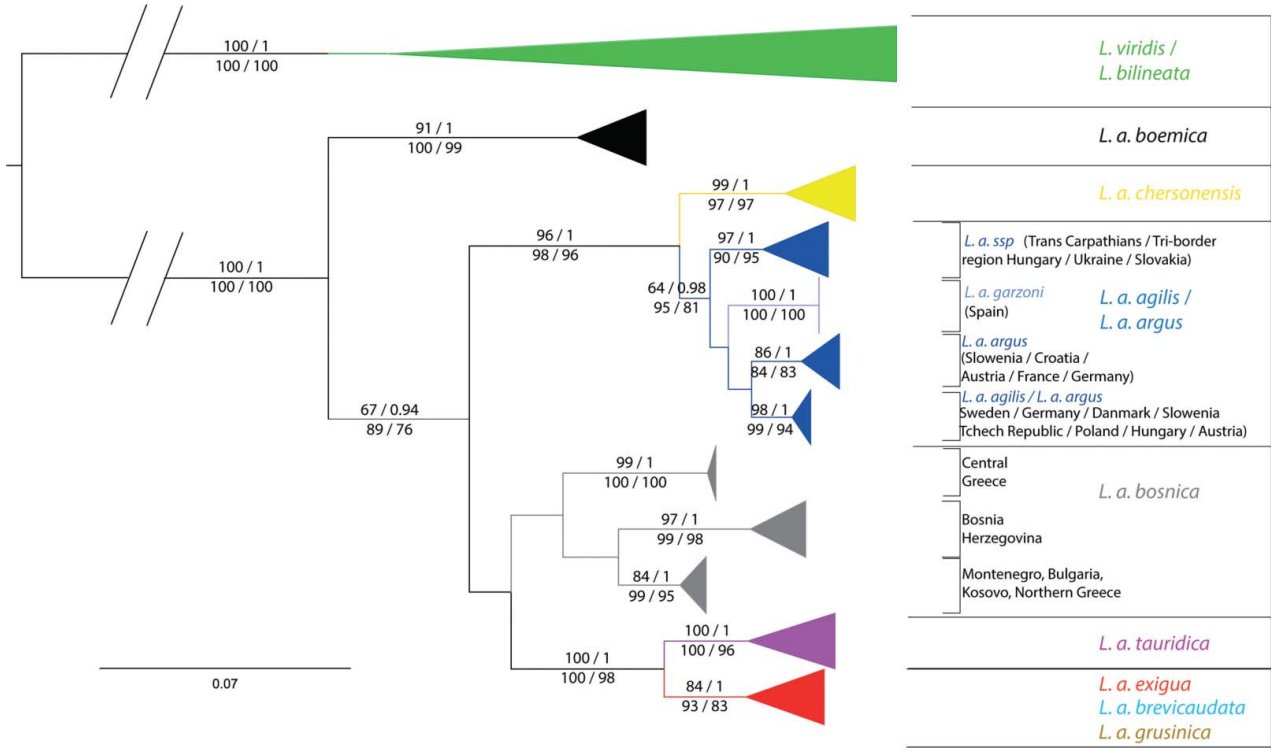

Fig. 3. Maximum likelihood phylogenetic reconstruction of the cytochrome b gene using the GTR + G + I model of sequence evolution. Bootstrap values and posterior probabilities are displayed for Maximum likelihood (upper left), Bayesian inference (upper right), Neighbour joining (lower left) and Maximum parsimony (lower right). Only bootstrap values higher than 80 and posterior probabilities higher than 0.95 are shown. When at least two of the four methods showed values above this limit, the values are also given for the remaining methods even if they are lower than the limiting value.

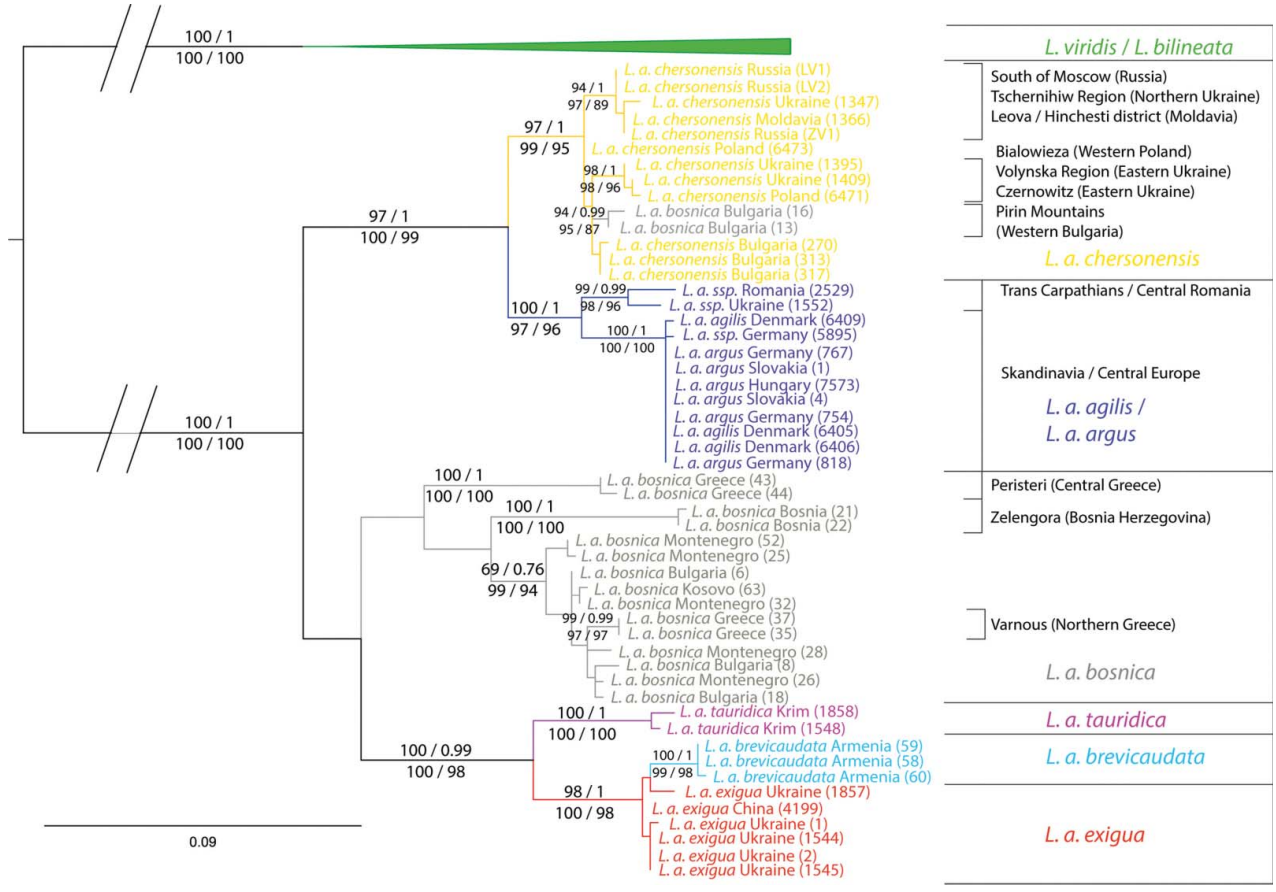

Fig. 4. Maximum likelihood phylogenetic reconstruction of the combined cytochrome b gene and d – loop data set using the GTR + G + I model of sequence evolution. Bootstrap values and posterior probabilities are displayed for Maximum likelihood (upper left), Bayesian inference (upper right), Neighbour joining (lower left) and Maximum parsimony (lower right). Only bootstrap values higher than 80 and posterior probabilities higher than 0.95 are shown. When at least two of the four methods showed values above this limit, the values are also given for the remaining methods even if they are lower than the limiting value.

comprising four distinct, well-supported lineages in all reconstructions, whereby the clade containing individuals from Romania, the Trans Carpathians and the tri-border region Ukraine, Slovakia and Hungary always branched off first (Fig. S14-2, see supplemental material online). This group contained all individuals formerly assigned to *L. a*. ssp., which were morphologically not determined to subspecies level. One lineage comprised all specimens from Kalyabina-Hauf & Ananjeva (2004) previously assigned to *L. a. argus* with samples from France, Germany, Austria, Slovenia and Croatia. Another cluster was formed by individuals from Kalyabina-Hauf & Ananjeva

Table 2. Calculated mean between group genetic distances for the cytochrome b gene (lower half) and the D-loop (upper half).

	Subspecies	1	2	3	4	5	6	7	8	9	10	11	12
1	*L. a. bosnica*		0.030	–	0.026	0.026	–	0.020	0.020	–	0.019	0.089	0.075
2	*L.a. brevicaudata*	0.049		–	0.006	0.017	–	0.032	0.032	–	0.030	0.081	0.075
3	*L. a. grusinica*	0.047	0.006		–	–	–	–	–	–	–	–	–
4	*L. a. exigua*	0.047	0.005	0,006		0.017	–	0.026	0.026	–	0.025	0.082	0.075
5	*L. a. tauridica*	0.049	0.025	0,024	0.024		–	0.027	0.027	–	0.025	0.074	0.064
6	*L. a. boemica*	0.065	0.072	0,071	0.069	0.074		–	–	–	–	–	–
7	*L. a. agilis*	0.063	0.066	0,066	0.062	0.067	0.074		0.002	–	0.003	0.086	0.080
8	*L. a. argus*	0.060	0.059	0,060	0.056	0.061	0.070	0.011		–	0.003	0.087	0.080
9	*L. a. garzoni*	0.064	0.068	0,068	0.065	0.069	0.073	0.023	0.024		–	–	–
10	*L. a. chersonensis*	0.061	0.062	0,062	0.059	0.067	0.077	0.038	0.035	0.041		0.083	0.076
11	*L. viridis*	0.152	0.162	0,162	0.158	0.161	0.154	0.164	0.154	0.167	0.157		0.042
12	*L. bilineata*	0.167	0.170	0,170	0.166	0.173	0.162	0.170	0.167	0.174	0.167	0.070	

Table 3. Calculated mean between group genetic distances for the cytochrome b gene and the D-loop for the lineages detected in the *L. a. agilis*/*L. a. argus* clade in the phylogenetic reconstructions.

Compared groups in *L. a. agilis/argus/garzoni* group	cytochrome b gene	D-loop
Agilis/Argus vs. *argus*	0.013	–
agilis tri-border /Trans Carp vs. *Argus*	0.026	–
Agilis tri-border/Trans Carp. vs. *Agilis/ argus*	0.025	0.002
Garzoni vs. agilis tri-border/Trans Carp	0.026	–
Garzoni vs. *Agilis/ argus*	0.020	–
Garzoni vs. *argus*	0.022	–
Trans Carpathians vs. Tri-Border-Region	0.010	–

(2004) assigned beforehand to *L. a. agilis* and newly sequenced specimens of the present study previously assigned to *L. a. argus* with samples from Sweden, Denmark, Germany, Czech Republic, Slovakia, Austria and Hungary (Fig. S14-2, see supplemental material online). In summary, the cytochrome b-trees did not recover the subspecies *L. a. agilis* and *L. a. argus* as separate evolutionary lineages.

The eastern clade represented by *L. a. exigua*, *L. a. brevicaudata*, *L. a. grusinica* and *L. a. tauridica* formed a highly supported group in all analyses. In all reconstructions the subspecies *L. a. tauridica* constituted the well-supported sister group to the cluster of *L. a. exigua*, *L. a. brevicaudata* and *L. a. grusinica*. However, in all analyses these three subspecies could not be resolved as separate groups (Fig. S14-3, see supplemental material online). In all analyses *L. a. bosnica* was detected as the sister-group to this eastern clade, however this relationship was only well supported with Bayesian inference. Similarly, monophyly of *L. a. bosnica* was only significantly supported by Bayesian inference. However, within this subspecies three lineages with high bootstrap support were detected (Fig. S14-4, see supplemental material online). Samples from Central Greece and from Bosnia each formed a separate lineage. The third group was composed of all remaining individuals from Montenegro, Bulgaria, Kosovo and Northern Greece, with samples from the latter region forming a well-supported subgroup.

Table 4. Calculated mean between group genetic distances for the cytochrome b gene and the D-loop for the lineages detected in the *L. a. bosnica* clade in the phylogenetic reconstructions, Bosnia I: specimens from Zelengora; Bosnia II: specimens from Bastasi, Bosansko Grahovo and Bugojno; Bosnica partly: specimens from Bulgaria, Montenegro, Kosovo, Northern Greece.

Compared groups in *L. a. bosnica* group	cytochrome b gene	D-loop
Central Greece vs. Bosnia I (Zelengora)	0.040	0.027
Central Greece vs. Bosnia II	0.034	–
Central Greece vs. Bosnica Partly	0.029	0.023
Bosnia I vs. Bosnia II	0.011	–
Bosnia I vs. Bosnica partly	0.033	0.016
Bosnica partly vs. Bosnia II	0.030	–

Table 5. Calculated mean within group genetic distances for the cytochrome b gene and the D-loop.

Within groups	cytochrome b gene	D-loop
L. a. bosnica	0.020	0.013
L. a. bosnica Partly	0.004	0.007
L. a. bosnica GR	0.000	0.003
L. a. bosnica Bosnia I (Zelengora)	0.001	0.000
L. a. bosnica Bosnia II	0.004	
L. a. chersonensis	0.005	0.001
L. a. exigua	0.002	0.001
L. a. brevicaudata	0.003	0.002
L. a. agilis	0.001	0.003
L. a. argus	0.007	0.000
L. a. ssp Tri-border-region / Trans Carpathians	0.009	0.000
L. a. ssp. Tri-border region	0.002	–
L. a. ssp. Trans Carpathians	0.006	–
L. a. boemica	0.007	–
L. a. tauridica	0.007	0.003
L. a. grusinica	0.006	–
L. a. garzoni	0.000	–
L. viridis	0.055	0.039
L. bilineata	0.089	0.031

Gene trees of cytochrome b gene and D-loop region. The analysis based on the concatenated dataset included *L. a. bosnica*, *L. a. tauridica*, *L. a. exigua*, *L. a. brevicaudata*, *L. a. chersonensis*, *L. a. argus* and *L. a. agilis*. All methods revealed almost identical reconstructions of the phylogenetic relationships (Fig. 4). In all analyses *Lacerta agilis* was confirmed as a monophyletic group with bootstrap values of 100%. The three subspecies of the eastern clade *L. a. tauridica*, *L. a. exigua* and *L. a. brevicaudata* formed a well-supported cluster, congruent to Kalyabina *et al*. (2004). Within this clade *L. a. tauridica* was the sister group to the clade of *L. a. brevicaudata* and *L. a.*

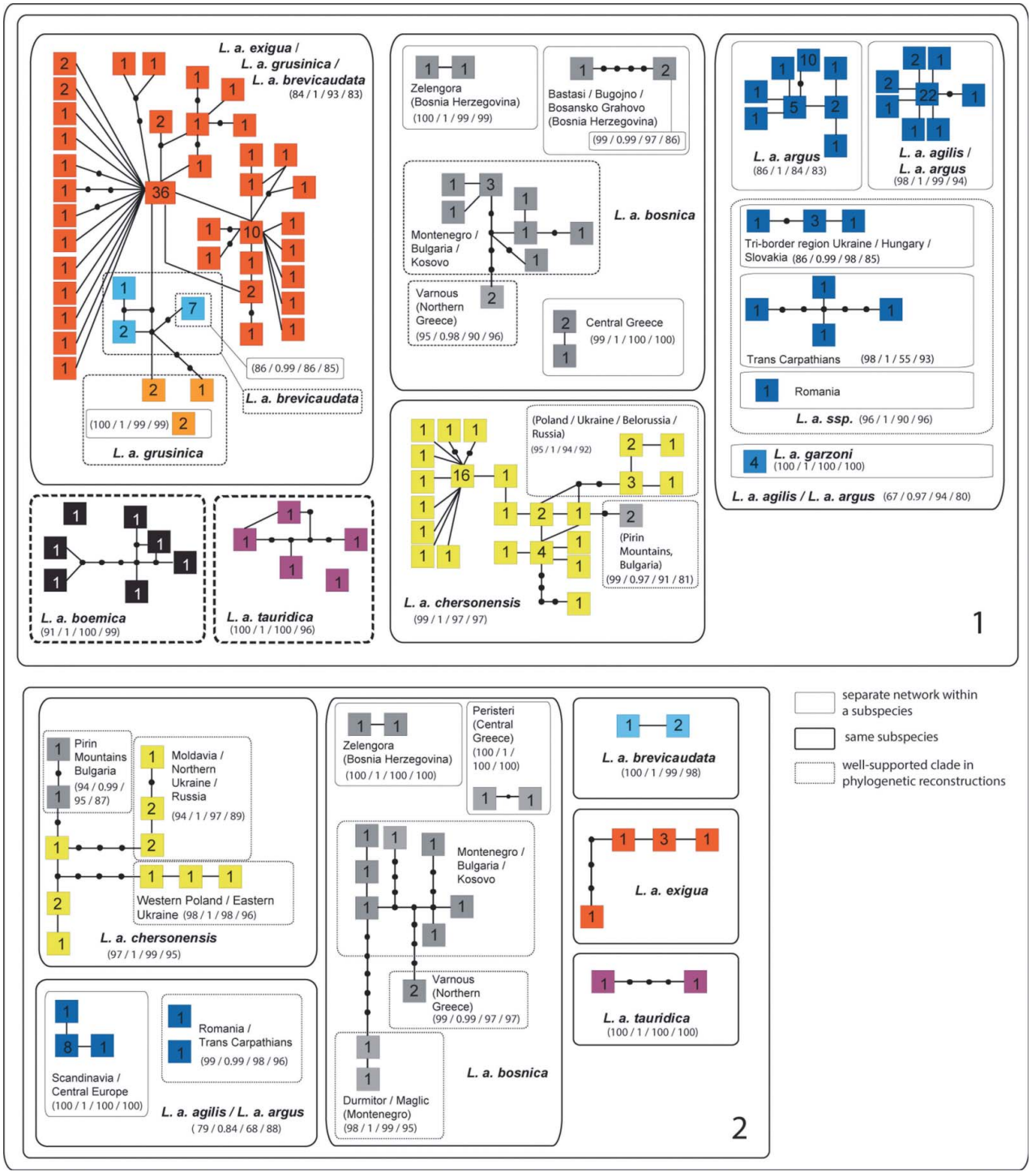

Fig. 5. Cladistic haplotype aggregation for the cytochrome b gene (1) and for the combination of cytochrome b gene and D-loop (2), each step is represented by a node, each haplotype is represented by a rectangle coloured accordingly to the assigned subspecies, numbers in rectangles represent amount of assigned individuals, numbers in parentheses indicate bootstrap support of phylogenetic analyses displayed for Maximum likelihood, Bayesian inference, Neighbour joining and Maximum parsimony, respectively.

exigua. Within this group the specimens of *L. a. brevicaudata* always formed one clade whereas *L. a. exigua* appeared paraphyletic except for the Maximum parsimony reconstruction.

The position of the *L. a. bosnica* specimens varied strongly between all reconstruction methods and none of them was supported significantly. Nevertheless, comparable to the extended cytochrome b gene dataset *L. a.*

bosnica showed in all analyses three distinct, highly supported lineages, one constituted by two individuals from Bosnia, one by two individuals from Central Greece, and two individuals from Northern Greece.

Furthermore, all methods supported the reciprocal monophyly of *L. a. chersonensis* and *L. a. agilis*/*L. a. argus*. As in the cytochrome b gene analyses the two individuals of *L. a. bosnica*, which were sampled in the Pirin Mountains, always clustered within the *L. a. chersonensis* clade (Fig. 4).

The monophyly of the two subspecies *L. a. agilis* and *L. a. argus* could not be validated (Fig. 4). Instead, the individuals from Romania and the Trans Carpathians, and the individuals from Denmark, Germany, Poland, Slovakia and Hungary formed two well-supported sister groups.

Mean group distances. The genetic distances calculated between and within groups are listed in Table 2 for both, cytochome b and D-loop region. The listed groups represent the morphology based subspecies assignments. The unassigned specimens from Poland (Bialowieza and Bialystock), Romania and Ukraine (Chernivtsi region, Volyn region) and the two individuals declared as *L. a. bosnica*, which clustered within the *L. a. chersonensis* clade, were excluded from calculations in order to avoid potentially false results.

The largest genetic distances in the cytochrome b gene were obtained between *L. a. boemica* and all other investigated subspecies and were as high as the genetic distance between the two outgroup-species *L. viridis* and *L. bilineata* (above 6.5%, Table 2). The lowest distances were found between *L. a. exigua*, *L. a. grusinica* and *L. a. brevicaudata* (0.5–0.6%), followed by *L. a. tauridica* (1.7%). However, the genetic distances of *L. a. exigua*, *L. a. grusinica*, *L. a. brevicaudata* and *L. a. tauridica* to the *L. a. bosnica* specimens showed almost equal values as the lineages recovered within the *L. a. bosnica* clade (Table 4).

Similarly, within the western clade the two subspecies *L. a. agilis* and *L. a. argus* showed the lowest genetic distance whereas. *L. a. chersonensis* showed the highest genetic distances to *L. a. agilis*, *L. a. argus* and *L. a. garzoni*.

The calculation of the genetic distances for the D-loop region revealed that the lowest genetic distances were found between *L. a. argus*, *L. a. agilis* and *L. a. chersonensis* (Table 2). All three showed the lowest genetic distances to *L. a. bosnica*, followed by *L. a. exigua*, *L. a. tauridica* and *L. a. brevicaudata*. In general, the genetic distances calculated for the D-loop region were lower than those of the cytochrome b gene. All groups of subspecies showed lower genetic distances between each other than to the outgroup species. The within group distances were for both genes significantly lower than the between-group distances (Table 5; cytochrome b gene: Welch F Test, df = 72.9, $F = 78.28$, $P = 3.635*10^{-13}$; –D-loop: one-way ANOVA, df = 45.51, $F = 35.61$, $P = 3.36*10^{-7}$).

Cladistic haplotype aggregation. Two haplotype networks were created, one on the basis of the cytochrome b gene sequences (Fig. 5-1) and a second one based on the cytochrome b gene and D-loop region (Fig. 5-2). The cladistic haplotype aggregation of the cytochrome b gene consisted of 16 haplotype networks with several subgroups within the networks. In general, the haplotype networks reflected the results of the gene trees. *Lacerta agilis exigua*, *L. a. brevicaudata* and *L. a. grusinica* could not be separated from each other, but rather formed a haplotype network distinct from all other samples. Each of the three subspecies *L. a. chersonensis*, *L. a. boemica* and *L. a. tauridica* constituted a separate haplotype network. The two individuals from the Pirin Mountains determined as *L. a. bosnica* always formed part of the *L. a. chersonensis* network. The three lineages detected in the *L. a. bosnica* subspecies were also found in the cladistic haplotype aggregation. Finally, all well-supported lineages within the *L. a. agilis* / *L. a. argus* clade were also detected in the haplotype network.

The haplotype network based on the combined alignment supported the combined sequence trees as well (Fig. 5-2). In total the analysis revealed 10 haplotype networks. The subspecies *L. a. exigua*, *L. a. brevicaudata*, *L. a. tauridica* and *L. a. chersonensis* each constituted a separate haplotype network. Again the specimens from Pirin Mountains were part of the *L. a. chersonensis* network. Unexpectedly, in the cladistic haplotype aggregation *L. a. exigua* represented an isolated network including all six samples contrary to the gene trees, in which this subspecies emerged paraphyletic. As in the cytochrome b gene network analysis, the three lineages of *L. a. bosnica* detected in the combined gene trees were also detected in the haplotype aggregation.

Discussion

Phylogenetic reconstructions of the *Lacerta agilis* complex

Gene trees and cladistic haplotype analyses of two mitochondrial markers within the *Lacerta agilis* complex yielded an overall consistent pattern of relationships. Our reconstructions recovered the monophyly of the species *L. agilis* and also the subspecies *L. a. chersonensis*, *L. a. boemica*, *L. a. tauridica* and *L. a. garzoni*. In contrast, the subspecies *L. a. exigua*, *L. a. brevicaudata*, *L. a. grusinica*, *L. a. bosnica*, *L. a. agilis* and *L. a. argus* were not confirmed in all reconstructions.

Lacerta a. boemica branched off first in all cytochrome b gene trees (Fig. 3) and formed a distinct network in the cladistic haplotype aggregation (Fig. 5-1). The taxonomic rank of *L. a. boemica* either as an archaic subspecies or as a separate species has been discussed before (Kalyabina *et al*., 2001; Joger *et al*., 2006). The genetic distances for

L. a. boemica to all other subspecies ranged between 6.5% and 7.7% for the cytochrome b gene (Table 2) and were distinctly higher than those between all other subspecies. Taking all evidence into account and following the phylogenetic species concept *sensu* Mishler & Theriot (2000) we suggest that *L. a. boemica* deserves species status and recommend further investigations.

Although not all subspecies could be confirmed, *L. a. exigua*, *L. a. grusinica*, *L. a. brevicaudata* and *L. a. tauridica* as representatives of the eastern clade, and *L. a. agilis*, *L. a. argus*, *L. a. garzoni* and *L. a. chersonensis* as representatives of the western clade were recovered (Figs. 3–4). According to Bischoff (1988) *L. a. bosnica* is also part of the western clade. However, in the present analyses all specimens of *L. a. bosnica* grouped with the eastern clade, albeit without statistical support. This is consistent with results published by Kalyabina-Hauf *et al*. (2004) and Joger *et al*. (2006). Furthermore, Joger *et al*. (2007) already assigned *L. a. bosnica* as a distinct Balkan subspecies to the eastern clade.

The subspecies *L. a. bosnica* was recovered in all tree reconstructions, however without significant support (Figs 3–4). This is in contrast to the result of Joger *et al*. (2006) where *L. a. bosnica* represented a well-supported clade. However, Joger *et al*. (2006) only examined individuals from Bosnia, Croatia and Greece. Interestingly, in the present study the individuals which have been sampled in these regions formed two distinct, highly supported lineages (Bosnia and Central Greece, respectively) separated from a third lineage formed by all other *L. a. bosnica* specimens from Bulgaria, Montenegro, Kosovo and Northern Greece with high genetic distances among them (2.9–4%) (Fig. S14-4, see supplemental material online). The Balkan Peninsula is characterized by high mountains and deep valleys representing barriers to dispersal. This leads to various restricted territories and high endemism comparable to the Caucasian region (Dzukic & Kalezic, 2004). The high level of diversity is probably benefited by its habitat heterogeneity, climatic variation and topographic diversity, and influenced by Pleistocene glaciation (Dzukic & Kalezic, 2004). These factors probably contributed to the development of distinct, genetically differentiated populations within the region. If tectonic uplifts lead to isolated populations and differentiation into several subspecies within a small area in the Caucasus (Bischoff, 1988), the same could have happened in the Balkan Peninsula which is also a tectonically active area (Dzukic & Kalezic, 2004). The results suggest that indeed markedly differentiated lineages exist, which might deserve subspecies status. Especially the samples from Central Greece show large genetic distances, and a profound revision including molecular and morphological studies of the Balkan subspecies is recommended.

The subspecies *L. a. exigua*, *L. a. brevicaudata* and *L. a. grusinica* were not recovered in the gene trees and showed low genetic distances of 0.5–0.6%. In contrast, the network analysis of the combined dataset including the fast-evolving D-loop region clearly separated *L. a. exigua* and *L. a. brevicaudata* although the genetic distance was equally low (0.6%, Table 2, Fig. 5-2). Our findings suggest that considerably differentiated populations exist within the distribution area of these subspecies, but they also raise doubt on their taxonomic validity. In contrast, the subspecies status of *L. a. tauridica,* which was already revalidated by Kalyabina-Hauf *et al*. (2004), could also be confirmed in the present study.

In all analyses *L. a. chersonensis* and *L. a. agilis*/*L. a. argus* were found to be sister taxa. Remarkably, the two individuals sampled in the Pirin Mountains in Bulgaria, which were morphologically determined as *L. a. bosnica* always clustered within the *L. a. chersonensis* clade. Usually the two subspecies are separated by altitude with *L. a. bosnica* preferably inhabiting mountainous regions between 850 and 2800 m and *L. a. chersonensis* appearing from sea level up to 1500 m (Biserkov, 2007). Normally, *L. a. chersonensis* does not occur in such heights within the Pirin Mountains. This result is interesting, as all individuals sampled in the Pirin Mountains represent morphologically pure *L. a. bosnica* specimens. However, the cytochrome b gene as well as the D-loop region are inherited exclusively maternally. Hence, possible recent or historical hybridization events between subspecies cannot be detected with these marker genes. We suggest therefore additional analyses with nuclear genes to clarify this finding.

The *L. a. agilis*/*L. a. argus* clade was supported by all analyses of the combined dataset. The geographic border between these two subspecies is assumed to run between eastern and western Germany starting between Kiel and Lübeck, going down to Munich and crossing Austria (Bischoff, 1988). However, Elbing *et al*. (1996) opposed that at least in Germany only one subspecies (*L. a. agilis*) exists. Indeed the differentiation of these subspecies seems to be more complicated. Kalyabina-Hauf *et al*. (2004) already showed that besides the two subspecies *L. a. argus* and *L. a. agilis* another group exists which they called the Carpathian group with samples from Western Hungary, the Trans Carpathians and Western Slovakia. This group was confirmed in the present study (Table 3, Figs 3–4). Furthermore, the specimens from Spain formed a distinct clade within the *L. a. agilis*/*argus*-complex, in congruence with the findings of Kalyabina-Hauf *et al.* (2004).

Concerning the specimens which were assigned previously to either *L. a. argus* or *L. a. agilis* the results differed in some aspects from Kalyabina-Hauf & Ananjeva (2004) and Kalyabina-Hauf *et al*. (2004). In the present study, the specimens determined as *L. a. argus* by Kalyabina-Hauf *et al*. (2004) formed a well-supported distinct

clade congruent with their analyses. However, Kalyabina-Hauf *et al*. (2004) also detected a *L. a. agilis* clade, which was not the case in the present study. In contrast, all specimens assigned to *L. a. agilis* by Kalyabina-Hauf *et al*. (2004) formed a fourth, highly supported clade, which included the *L. a. agilis* specimens from Denmark, sequenced in the present study, as well as the newly analysed *L. a. argus* specimens from Hungary, Slovakia and Germany (Fig. S14-1, see supplemental material online). Indeed, the distinction of both subspecies on the basis of morphological characteristics is very subtle and a misidentification of the analysed specimens is possible. However, our data obviously shows the existence of two well-supported lineages in the *L. a. agilis*/*L. a. argus* clade, the first containing specimens from Austria, Croatia, Slovenia, France and Germany labelled as *L. a. argus* and the second with individuals from Germany, Czech Republic, Sweden, Austria, Hungary, Slovakia and Denmark labelled as *L. a. agilis*/*L. a. argus*. Our results suggest a comprehensive revision of the distribution of both subspecies.

Phylogeography of *Lacerta agilis*

It is assumed that the expansion of *Lacerta agilis* started from the Caucasus towards the Balkan Peninsula, with both areas also known to be distribution centres and refuge areas during the glaciation period (Bischoff, 1988; Kalyabina *et al*., 2001). Thus, *L. a. boemica* located in the Caucasus represents the oldest lineage of *Lacerta agilis* which probably originated in the Pliocene (Kalyabina *et al*., 2001; Joger *et al*., 2006). In the present study this assumption was supported by the basal branching position in the gene trees and further by the high genetic distances compared with all other investigated subspecies. Possibly, the distribution went on in the first place towards the Balkan Peninsula with the differentiation of the *L. a. bosnica* subspecies also during the Pliocene (Joger *et al*., 2006).

The phylogeographic history of *L. a. bosnica* remains ambiguous. However, the majority of the analyses recovered a weakly supported sister group relationship with *L. a. tauridica*/*L. a. exigua*/*L. a. brevicaudata*/*L. a. grusinica*. This finding is congruent with the phylogenetic reconstruction in Kalyabina-Hauf *et al*. (2004) and Joger *et al*. (2006), with the exception that in their studies this relationship was well supported. This sister group relationship would indicate that the Central European and eastern European Plain subspecies (*L. a. agilis*, *L. a. argus*, *L. a. chersonensis*) did not arise from the Balkan Peninsula, but from the Caucasus. *Lacerta agilis bosnica* would then represent a distinct lineage within the 'eastern clade', phylogeographically older than *L. a. agilis*, *L. a. argus* and *L. a. chersonensis*. This hypothesis was also supported by Joger *et al*. (2007).

Conclusions

In general, differentiation of the *L. agilis* complex into different morphological subspecies could be supported with two mitochondrial markers. However, only the subspecies *L. a. chersonensis*, *L. a. tauridica*, *L. a. garzoni*, and *L. a. boemica* were well supported. The genetic distance of *L. a. boemica* and its reciprocal monophyly with all other subspecies of *L. agilis* suggest that this taxon deserves species status. Furthermore, the re-evaluation of Kalyabina-Hauf *et al*. (2004) that *L. a. tauridica* constitutes a distinct subspecies could be supported by our data. The analyses of *L. a. exigua*, *L. a. brevicaudata* and *L. a. grusinica* showed that the three subspecies are genetically similar and could not be separated from each other. Furthermore, the distribution area of the two subspecies *L. a. argus* and *L. a. agilis* needs to be reviewed. The results of the present study support the existence of two differentiated lineages, but their distribution pattern is contradictory to the preliminary one presented in Bischoff (1988). Finally, additional data is necessary to resolve the position of the *L. a. bosnica* subspecies. Apparently, the Balkan Peninsula is inhabited by distinct populations showing a strong differentiation among each other but the subspecies itself was not significantly supported in the present study. For further clarification if these differences can be accounted for by the existence of different subspecies, a broader sampling together with morphological and ecological investigations will be necessary.

Acknowledgements

We would like to thank Dr Pavel Stoev (National Museum of Natural History, Sofia), Dr Nikolay Tzankov (National Museum of Natural History, Sofia), Alexander Westerström (Department of Physics, Stockholm University), Dr Uwe Fritz (Senckenberg Naturhistorische Sammlungen Dresden), Peter Lesny (Universität Bonn, Institut für Evolutionsbiologie und Zooökologie), Nadezda Rimskaya-Korsakova (Faculty of Biology, Lomonosov Moscow State University Russia) and Oleksandr Zinenko (Museum of Nature at V. N. Karazin Kharkiv National University) for helping in collecting specimens or for providing samples for the analyses of the present study. We furthermore would like to thank Michéle Detzner and Annemarie Geissler for the support in the laboratory analyses. This work was supported by special funds of the rectorate of the University of Leipzig.

References

Amat, F. 2008. Lagarto ágil – *Lacerta agilis* Linnaeus. In: Carrascal, L. M. & Salvador, A., Eds., *Encicloped. Museo Nacional de Ciencias Naturales*, Madrid, Spain.

Bischoff, W. 1988. Zur Verbreitung und Systematik der Zauneidechse, *Lacerta agilis* LINNAEUS, 1758. *Mertensiella* **1**, 11–30.

Biserkov, V. 2007. *A Field Guide to Amphibians and Reptiles of Bulgaria* (V. Biserkov, Ed.). Sofia, Green Balkans, Bulgaria.

Böhme, M.U., Fritz, U., Kotenko, T., Ljubisavljević, K., Tzankov, N. & Berendonk, T.U. 2007. Phylogeography and cryptic variation within the *Lacerta viridis* complex (Lacertidae, Reptilia). *Zoologica Scripta* **36**, 119–131.

Clement, M., Posada, D. & Crandall, K.A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657–1659.

Crochet, P., Chaline, O., Surget-Groba, Y., Debain, C. & Cheylan, M. 2004. Speciation in mountains: phylogeography and phylogeny of the rock lizards genus Iberolacerta (Reptilia: Lacertidae). *Molecular Phylogenetics and Evolution* **30**, 860–866.

Dzukic, G. & Kalezic, M.L. 2004. The biodiversity of amphibians and reptiles in the Balkan Peninsula. In: Griffiths, H.I., Krystufek, B. & Reed, J.M., Eds., *Balkan Biodiversity : Pattern and Process in the European Hotspot*. Springer, Berlin, Germany, pp. 167–177.

Elbing, K., Günther, R. & Rahmel, U. 1996. Zauneidechse – *Lacerta agilis* LINNAEUS, 1758. In: Günther, R. (Ed.), *Die Amphibien und Reptilien Deutschlands*. Gustav Fischer Verlag, Jena, Germany, pp. 535–557.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Huelsenbeck, J.P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.

Joger, U., Guicking, D., Kalyabina-Hauf, S., Lenk, P., Nagy, Z. T. & Wink, M. 2006. Phylogeographie, Artbildung und postpleistozäne Einwanderung mitteleuropäischer Reptilien. *Zeitschrift für Feldherpetologie* Supplement: 29–59.

Joger, U., Fritz, U., Guicking, D., Kalyabina-Hauf, S., Nagy, Z.T. & Wink, M. 2007. Phylogeography of western Palaearctic reptiles – spatial and temporal speciation patterns. *Zoologischer Anzeiger – A Journal of Comparative Zoology* **246**, 293–313.

Kalyabina-Hauf, S.A. & Ananjeva, N.B. 2004. Phylogeography and intraspecies structure of wide distributed sand lizard, *Lacerta agilis* L. 1758 (Lacertidae, Sauria, Reptilia) (case study of mitochondrial cytochrome b gene). Zoological Institute, Russian Academy of Sciences, Saint-Petersburg, Russia.

Kalyabina, S.A., Milto, K.D., Ananjeva, N.B., Legal, L., Joger, U. & Wink, M. 2001. Phylogeography and systematics of *Lacerta agilis* based on mitochondrial cytochrome b gene sequences: first results. *Russian Journal of Herpetology* **8**, 149–158.

Kalyabina-Hauf, S.A., Milto, K.D., Ananjeva, N.B., Joger, U., Kotenko, T.I. & Wink, M. 2004. Reevaluation of the status of *Lacerta agilis tauridica* SUCHOV, 1926. *Russian Journal of Herpetology* **11**, 65–72.

Katoh, K., Misawa, K., Kuma, K. & Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**, 3059–3066.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Molecular Evolution* **16**, 111–120.

Krystufek, B. & Reed, J.M. 2004. Pattern and process in Balkan biodiversity – an overview. In: Griffiths, H.I., Krystufek, B. & Reed, J.M., Eds., *Balkan Biodiversity : Pattern and Process in the European Hotspot*. Springer, Berlin, Germany, p. 5.

Lanave, C., Preparata, G., Saccone, C. & Serio, G. 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution* **20**, 86–93.

López-García, J.M., Blain, H.-A., Allué, E., Bañuls, S., Bargalló, A., Martín, P., Morales, J.I., Pedro, M., Rodríguez, A., Solé, A. & Oms, F.X. 2010. First fossil evidence of an "interglacial refugium" in the Pyrenean region. *Die Naturwissenschaften* **97**, 753–761.

Mishler, B.D. & Theriot, E.C. 2000. The phylogenetic species concept (*sensu* Mishler and Theriot): monophyly, apomorphy, and phylogenetic species concepts. In Wheeler, Q.D. & Meier, R., Eds., *Species Concepts and Phylogenetic Theory. A Debate.* Columbia University Press, New York, USA, pp. 44–54.

Palacios, F. & Castroviejo, J. 1975. Descripción de una nueva subspecie de lagarto agil (*Lacerta agilis garzoni*) de los Pirineos, Doñana. *Acta Vertebrata*, *Sevilla* **2**, 5–24.

Schmitt, T. 2007. Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology* **4**, 11.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690.

Suchow, G.F. 1927. Die Zauneidechse aus der Krim (Lacerta agilis tauridica subsp. nov.). Mémoires de la Classe des Sciences Physiques et Mathématiques de lÀcadémie des Sciences de l'Ukraine **IV**, 327–331.

Swofford, D.L. 2002. *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.* Sinauer Associates, Sunderland, MA, USA.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.

Zinenko, O.I., Drabkin, P.L. & Rudyk, O.M. 2005. Contact zone between two subspecies of the sand lizard: *Lacerta agilis exigua* eichw., 1831 and *Lacerta agilis chersonensis* andr., 1832 in three regions of the left-bank Ukraine. In: Ananjeva, N. & Tsinenko, O., Eds., *Herpetologia Petropolitana, Proceedings of the 12th Ordinary General Meeting of the Societas Europaea Herpetologica, 12–16 August 2003, Saint-Petersburg, Russia, Russian Journal of Herpetology,* 12 (Supplement), pp. 109–112.

Associate Editor: Elliot Shubert