Phylogeography and cryptic variation within the *Lacerta viridis* complex (Lacertidae, Reptilia)

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It is well known that the current genetic pattern of many European species has been highly influenced by climatic changes during the Pleistocene. While there are many well known vertebrate examples, knowledge about squamate reptiles is sparse. To obtain more data, a range-wide sampling of *Lacerta viridis* was conducted and phylogenetic relations within the *L. viridis* complex were analysed using an mtDNA fragment encompassing part of cytochrome *b*, the adjacent tRNA genes and the noncoding control region. Most genetic divergence was found in the south of the distribution range. The Carpathian Basin and the regions north of the Carpathians and Alps are inhabited by the same mitochondrial lineage, corresponding to *Lacerta viridis viridis*. Three distinct lineages occurred in the south-eastern Balkans — corresponding to *L. v. viridis*, *L. v. meridionalis*, *L. v. guentherpetersi* — as well as a fourth lineage for which no subspecies name is available. This distribution pattern suggests a rapid range expansion of *L. v. viridis* after the Holocene warming, leading to a colonization of the northern part of the species range. An unexpected finding was that a highly distinct genetic lineage occurs along the western Balkan coast. Phylogenetic analyses (Bayesian, maximum likelihood, maximum parsimony) suggested that this west Balkan lineage could represent the sister taxon of *Lacerta bilineata*. Due to the morphological similarity of taxa within the *L. viridis* complex this cryptic taxon was previously assigned to *L. v. viridis*. The distribution pattern of several parapatric, in part highly, distinct genetic lineages suggested the existence of several refuges in close proximity on the southern Balkans. Within *L. bilineata sensu stricto* a generally similar pattern emerged, with a high genetic diversity on the Apennine peninsula, arguing for two distinct refuges there, and a low genetic diversity in the northern part of the range. Close to the south-eastern Alps, three distinct lineages (*L. b. bilineata*, *L. v. viridis*, west Balkan taxon) occurred within close proximity. We suggest that the west Balkan lineage represents an early offshoot of *L. bilineata* that was isolated during a previous Pleistocene glacial from the more western *L. bilineata* populations, which survived in refuges on the Apennine peninsula.

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Introduction

The current divergence and differentiation of many European species have been highly influenced by the rapid climatic changes during the Pleistocene and the fluctuation of suitable habitats. The resultant distribution patterns have been reviewed in classic biogeographical works (Reinig 1937; Holdhaus 1954; De Lattin 1957, 1967; Frenzel 1960, 1968) as well as more recent phylogeographical studies (Hewitt 2001; Walter & Epperson 2001; Petit *et al*. 2003). These have made a major contribution to our understanding of range expansions or restrictions.

Most of these studies demonstrate that the impact of the last glaciations (25 000–10 000 BP) within Europe led to a south–north gradient of genetic diversity within many species (e.g. Gollmann *et al*. 1993; Assmann *et al*. 1994; Hewitt 2001), caused mainly by the colonization of northern areas after glaciations from several southern, Mediterranean refugia. This gradient of genetic diversity is explained by recurrent founder effects during recolonization followed by population growth and spread of the reduced genetic diversity further northwards (Nei *et al*. 1975; Highton & Webster 1976; Hewitt 2004).

Within Europe, three main refugial areas are postulated, corresponding to the three southern peninsulas, i.e. the Iberian, Apennine and Balkan peninsulas (de Lattin 1967; Taberlet *et al*. 1998; Hewitt 1999; Gómez & Lunt 2004). Holocene range expansions from the western and eastern refuges led to a common distribution pattern found in many central European biotas, corresponding to a western and eastern species or subspecies (Taberlet *et al*. 1998). Well-known examples exist for many vertebrates such as the fire-bellied toads (*Bombina variegata* and *B. bombina*; Arntzen 1978), crows (*Corvus corone* and *C. cornix*; Meise 1928) and hedgehogs (*Erinaceus europaeus* and *E. concolor*; Santucci *et al*. 1998). However, well documented examples of reptiles within this context are very rare. The green lizards *Lacerta viridis* and *L. bilineata* provide an ideal model for investigating these processes and patterns of genetic differentiation and speciation, because they correspond well with the above described distribution pattern, with *L. viridis* the eastern and *L. bilineata* the western representative.

The genus *Lacerta sensu stricto* is currently divided into eight species: *L. agilis*, *L. schreiberi*, *L. trilineata*, *L. strigata*, *L. pamphylica*, *L. media*, *L. bilineata* and *L. viridis*. For decades, *L. bilineata* and *L. viridis* were regarded as synonyms (Böhme 1978; Nettmann & Rykena 1984). Based on hybridization experiments, indicating a reduced viability of captive-bred hybrids, *L. bilineata* was elevated to full species level (Rykena 1991, 2001). Recent molecular studies supported the separation of *L. viridis* and *L. bilineata* (Amann *et al*. 1997; Brückner *et al*. 2001; Joger *et al*. 2001). Within *L. viridis* and *L. bilineata*, several subspecies are recognized. Most of the

L. viridis subspecies (*L. v. meridionalis*, *L. v. guentherpetersi*, *L. v. infrapunctata*, *L. v. paphlagonica*) are confined to the southern part of the Balkan peninsula or Turkey, while one subspecies, *L. v. viridis*, inhabits a wider range, spanning from the Balkan peninsula northwards across the Carpathian Basin to the, in part isolated, peripheral populations in Ukraine, the Czech Republic and eastern Germany (Fig. 1).

For the western European species *L. bilineata* the Apennine peninsula is assumed to represent the glacial refuge (Böhme 1978). However, the subspecific differentiation within *L. bilineata* is still poorly understood. The main part of the species range (Spain, France and Italy) is inhabited by *L. b. bilineata*, while several subspecies (*L. b. chloronota*, *L. b. chlorosecunda*, *L. b. fejervaryi*) are thought to occur in Italy (Keller & Vassilakaki 2001; Brückner *et al*. 2001), suggesting that several distinct glacial refuges might have existed.

It has been proposed that *L. viridis* and *L. bilineata* hybridize in a zone of secondary contact in the western Balkans, resulting in an introgression of *L. viridis* nuclear genes into the *L. bilineata* gene pool (Amann *et al*. 1997; Joger *et al*. 2001). However, a recent study focusing on the phylogenetic relationships of species of *Lacerta s.s.* using mtDNA and nDNA sequence data (Godinho *et al*. 2005) places the species status of *L. bilineata* and *L. viridis* in doubt. Godinho and coworkers found a closer phylogenetic relationship between *L. bilineata* and *L. viridis* than between any other *Lacerta* species; moreover, *L. viridis* was demonstrated to be paraphyletic with respect to *L. bilineata*.

In order to obtain more information about the phylogeographical pattern of *L. viridis* and the complex relationships between *L. viridis* and *L. bilineata*, we analysed the most variable part of the mitochondrial genome encompassing the cytochrome *b* gene (cyt *b*) and the highly variable part of the noncoding control region (CR, 5′-region). Both mtDNA fragments have so far been used successfully for phylogeographical purposes (e.g. Lenk *et al*. 1999; Haring *et al*. 2000; Seddon *et al*. 2001; Babik *et al*. 2004; Böhme *et al*. 2006). In contrast to previous molecular studies on green lizards, we included samples throughout the whole species range of *L. viridis* and compared them with crucial samples of *L. bilineata*. Special emphasis was given to populations of the putative contact zone with *L. bilineata*.

Materials and methods

Population sampling and DNA isolation

We collected blood and tissue samples from populations throughout the species range of *L. viridis* (Fig. 1, Table 1). Blood samples (5–10 μL) were taken by frontal leg vein puncture with syringes and were stored in a special EDTA-Thymol buffer at −20 °C. Tissues (0.5 cm) were taken from tail tips and preserved in 80% ethanol. Total genomic DNA
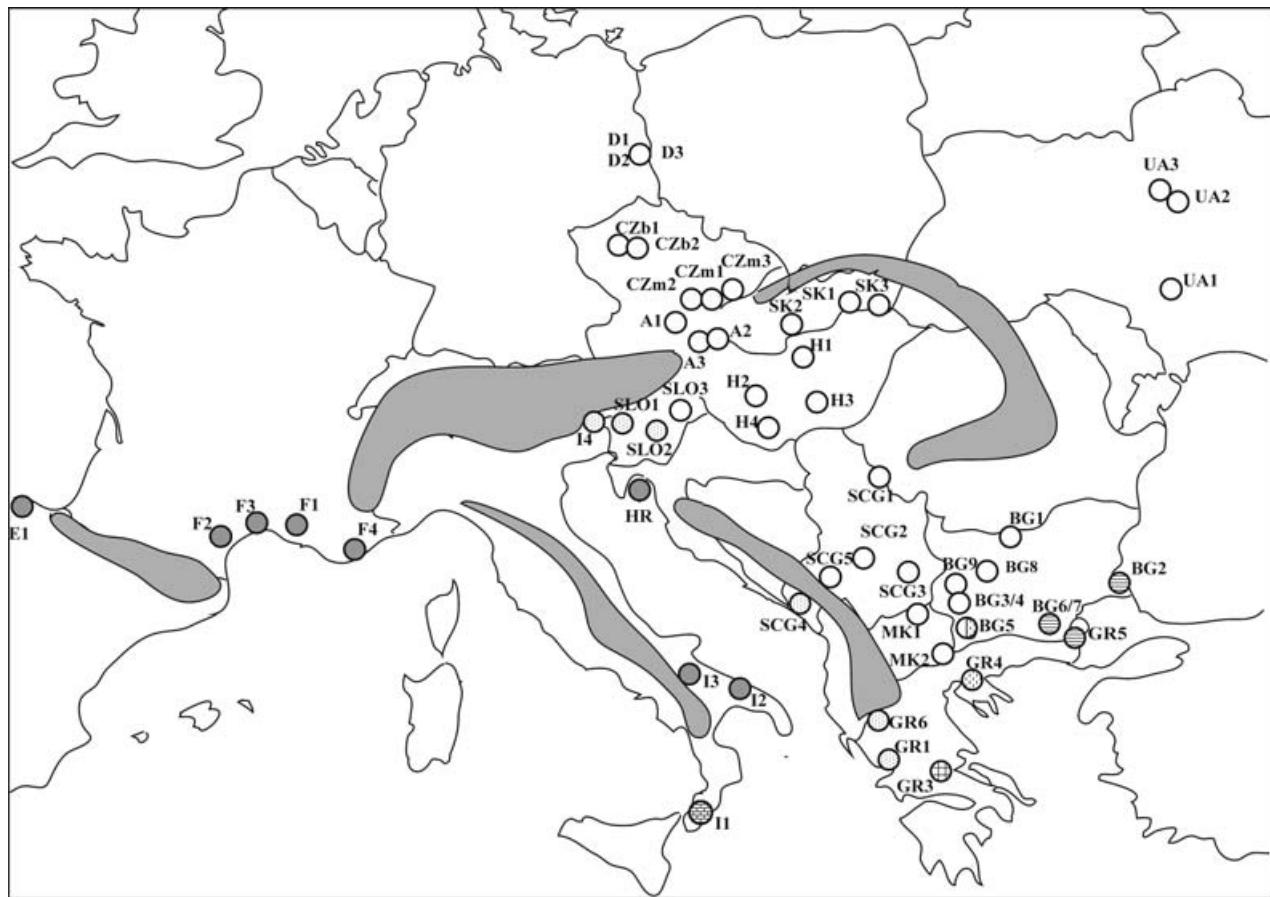
Fig. 1 Geographic distribution of sampled populations. Population codes refer to Table 1. Patterns indicate lineages revealed by phylogenetic analyses (Fig. 2): ○, lineage I (*L. v. viridis*); ⊖, lineage II (*L. v. meridionalis*); ⦶, lineage III (*L. viridis* ssp.); ⊕, lineage IV (*L. v. guentherpetersi*); ⬤, lineage V *s.l.* (*L. b. bilineata*, *L. b. fejervaryi* and *L. b. chlorosecunda*); ⊞, lineage VI (*L. b. chloronota*); ⊛, lineage VII (*L. bilineata* ssp.).

was extracted from 99 lizards using Nucleospin Tissue Kit (Machery & Nagel), following the manufacturer's protocol.

Amplification and sequencing
A fragment of mitochondrial DNA, encompassing the partial cyt *b*, tRNA Thr, tRNA Pro genes and part of the control region (CR) was amplified. Amplification was performed using primers LvF and LvR1 (Böhme *et al*. 2006) under the following conditions: 1 × buffer (Sigma), 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 15 pmol of each primer and 1 U Taq polymerase (Sigma). PCR was performed on an Eppendorf Mastercycler for 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min. Purified PCR products were prepared for cycle sequencing using Terminator Ready Reaction Mix 'Big Dye' 3.1 (Applied Biosystems, Böhme *et al*. 2006) following the manufacturer's protocol. Products were analysed on an ABI 3100 DNA Sequencer. Partial sequences and internal sequences were assembled using the programme

Dnasis 7.0 (Hitachi Software). Sequences were checked by visual inspection using Bioedit 7.1 (Hall 1999). Resulting sequences differed in final lengths, caused by a distinct repeat number of a minisatellite within the amplified part of the mitochondrial control region (Böhme *et al*. 2006).

Phylogenetic analysis
Teira dugesii (*AY147878* Brehm *et al*. 2003) was defined as outgroup to the residual lacertid species used in the dataset (*L. bilineata*, *L. viridis*, *L. agilis*). In total, 100 sequences were included in the phylogenetic analyses. Alignment was done with Clustal 1.83 implemented in Mega 3.1 (Kumar *et al*. 2004). Alignment gaps caused by different repeat lengths and other indels were excluded from the phylogenetic analyses. The hierarchical likelihood ratio test implemented in Model test 3.7 (Posada & Crandall 1998) was used to select the most appropriate DNA substitution rate. The best fitting model was obtained by the Akaike information

Table 1 Samples and populations analysed in this study. Voucher numbers refer to tissue samples in the collections of: ZSL — the University of Leipzig; MTD — TD — the Museum of Zoology, Dresden; Y — the Natural History Museum, Vienna; C-A and M — the University of Bremen. Abbreviations: Pop, population labelling; SNo, number of individuals.

	Region	Pop	Location	SNo	Accession numbers	cyt *b* haplotypes	Vouchers
Lacerta viridis clade							
L. v. viridis	Germany	D1	Brandenburg	3	AM087289, AM087329, AM87297	c1, c12, c13	ZSL310G, ZSL311G, ZSL312G
		D2	Brandenburg	2	AM087330, AM087328	c1, c13	ZSL316G, ZSL317G
		D3	Brandenburg	3	AM087297, AM087294	c1	ZSL313G, ZSL314G, ZSL315G
	Czech Rep.	CZb1	Bohemia, Karlik	1	AM292928	c1	ZSL320G
		CZb2	Bohemia, Krivoklad	2	AM087299, AM087300	c1	ZSL318G, ZSL319G
		CZm1	Moravia, Pavlovske kopce	2	AM087308, AM087309	c1	ZSL321G, ZSL322G
		CZm2	Moravia, NP Podyji	2	AM087312, AM087326	c1	ZSL323G, ZSL324G
		CZm3	Moravia, Bzenec	2	AM087315, AM087290	c1	ZSL325G, ZSL326G
	Slovakia	SK1	Slovak Carst	2	AM087310, AM087303	c1, c10	ZSL304G, ZSL305G
		SK2	Cabrad	2	AM292949, AM292950	c1, c8	ZSL306G, ZSL307G
		SK3	Tajba	2	AM292951, AM292952	c1	ZSL308G, ZSL309G
	Austria	A1	Weißenkirchen	2	AM087315, AM292929	c1, c2	ZSL327G, ZSL328G
		A2	Hundsheim	2	AM087305, AM087319	c14	
		A3	Gumpoldskirchen	2	AM087319, AM087314	c1	ZSL329G, ZSL330G
	Slovenia	SLO3	Maribor	1	AM292954	c1	ZSL331G
	Hungary	H1	Gödöllő	3	AM087321, AM087301, AM087302	c1	ZSL332G, ZSL333G, ZSL334G
		H2	Balaton, Tihany	1	AM292937	c7	ZSL335G
		H3	Bugac Puszta	3	AM087314, AM292938, AM292939,	c1	ZSL336G, ZSL337G, ZSL338G
		H4	Pecs	1	AM292936	c1	ZSL339G
	Serbia	SCG1	Vojvodina, Vrsacki breg	3	AM292933, AM292934, AM292935	c4, c5, c6	ZSL340G, ZSL341G, ZSL342G
		SCG2	Baljevac	2	AM292946, AM292947	c11	ZSL343G, ZSL344G
		SCG3	Leskovac	1	AM292948	c1	ZSL345G
	Macedonia	MK1	Stracin	2	AM292930, AM292932	c1, c3	ZSL346G, ZSL347G
		MK2	Dojran	1	AM292931	c1	ZSL348G
	Montenegro	SCG5	Radovici, Bijelo Polje	1	AM292953	c15	ZSL349G
	Bulgaria	BG1	D. Pleven, Muselievo	1	AM292955	c16	ZSL350G
		BG3	D. Dupnitica, Saparevo	1	AM292956	c1	ZSL351G
		BG4	D. Dupnitica, Smotchevo	1	AM292957	c1	ZSL352G
		BG8	D. Etropole, Lopian	1	AM292959	c1	ZSL353
		BG9	D. Sofia, Vladaya	1	AM292960	c27	ZSL354
	Ukraine	UA1	near Pervomays'k, on Southern Bug	3	AM292940, AM292941, AM292942	c1	ZSL355G, ZSL356G, ZSL357G
		UA2	near Kaniv on Dnieper	3	AM292943, AM292944, AM292945	c1, c9	ZSL358G, ZSL359G, ZSL360G
		UA3	70 km south-east of Kiev on Dnieper	1	AM292945	c1	ZSL361G
L. v. meridionalis	Bulgaria	BG2	D. Burgas, Reserve Ropotamo	1	AM292961	c17	ZSL362G
		BG5	D. Bladoevgrad, Kresna Gorge	4	AM292958, AM292966, AM292967, AM292968	c1, c18, c19	ZSL363G, ZSL364G, ZSL365G, ZSL366G
		BG6	D. Kardtzali, Studen Kladenetc	1	AM292963	c20	ZSL367G
		BG7	D. Ivailovgrad, Meden Buk	1	AM292964	c28	ZSL368G
	Greece	GR5	Evros, Gianuli	2	AM87228, AM292962	c25, c26	YE 1, YE2
		GR4	Thessaloniki, Zangliveri	1	AM292965	c24	YZ 9
L. v. guentherpetersi	Greece	GR3	Fthiotida, Iti-Mountains	2	AM292969, AM292970	c22, c23	YGJ 1, YGJ 2
L. bilineata clade							
L. b. bilineata	Spain	E1	Bilbao	1	AM292988	c37	YBJ1
	France	F1	Bouchesdu-Rhône, Eygalières	1	AM292987	c36	MTD-TD 2543
		F2	Herault, Aigues Vives	1	AM292986	c36	MTD-TD 2544
		F3	Herault, Cournonterral	1	AM292985	c36	MTD-TD 2546
		F4	Var, Plan de La Tour	1	AM292984	c35	MTD-TD 2548
	Croatia	HR	Cres Isl.	1	AM292989	c36	YCR
L. b. chloronota	Italy	I1	Aspromonte	1	AM292992	c40	C46
L. b. chlorosecunda	Italy	I2	Noci	1	AM292991	c39	A8
L. b. fejervaryi	Italy	I3	Melfi	1	AM292990	c38	M10
L. bilineata ssp.?	Montenegro	SCG4	Ceklici area (Ubine, Vucji do, Kuscista, Rzani do)	5	AM292975, AM292980, AM292981, AM292982, AM292983	c32	ZSL379G, ZSL380G, ZSL381G, ZSL382G, ZSL383G

Table 1 *Continued.*

	Region	Pop	Location	SNo	Accession numbers	cyt *b* haplotypes	Vouchers
L. bilineata ssp.?	Slovenia	SLO1	Lake Bohinij	3	AM292977, AM292978, AM292979	c33, c34	ZSL369G, ZSL370G, ZSL371G
		SLO2	Kamence	2	AM292976	c32	ZSL372G, ZSL373G
	Italy	I4	Friuli, Musi	1	AM292993	c32	YF1
	Grecce	GR1	Arta	2	AM292971	c21	YAR 5, YAR 6
		GR6	Epirus, Zagoria area	3	AM292972, AM292973, AM292974	c29, c30, c31	ZSL374G, ZSL375G, ZSL376G
Lacerta agilis	Romania		captive specimen	1	AM087227	—	ZSL377G
	Germany		Bernburg	1	AM292994	—	ZSL378G
Teira dugesii					AY147878	—	—

criterion (AIC; Akaike 1974), which selected the transitional model (TIM + I + G, ln L = –10563.1689) with base frequencies of A = 0.2897, C = 0.2732, G = 0.1117, T = 0.3254, proportion of invariable sites of I = 0.5208 and a gamma distribution shape parameter of variable sites of G = 0.6106.

Based on this information about the category of the evolutionary model, the best fitting parameters were estimated for a comparatively complex model using PhyML 2.4.4 (Guindon & Gascuel 2003). The integrated operation of the programme revealed for the chosen GTR model base frequencies of A = 0.2879, C = 0.2731, G = 0.1142, T = 0.3248 and a gamma distribution shape parameter of variable sites of G = 0.187. The log likelihood value for the GTR model calculated by PhyML (ln L = –10524.5575) was much higher than revealed for the TIM model under the AIC using Modeltest 3.7. Therefore the parameters revealed by the PhyML statistics were used for all subsequent calculations.

A maximum likelihood (ML) tree was calculated with PhyML and the robustness of the phylogeny was tested by 1000 bootstrap replications. Furthermore, we used PAUP* 4.0 b10 (Swofford 2002) to calculate a maximum parsimony tree (MP) with heuristic search using 10 stepwise additions of sequences and the TBR branch swapping option. To test the robustness of the MP bifurcations, bootstrap analysis with 2000 replicates was conducted. Additionally, a Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The analysis was run with the best-fit model as inferred by PhyML for 1 000 000 generations, with a sampling frequency of 100 generations. The burn-in was set to 50 000 generations after testing for stable likelihood values using the integrated statistics of MrBayes. From the 10 000 trees found, a subset of trees was determined that was used for building a consensus tree by inspecting likelihood values of trees saved by MrBayes.

Network estimation

As gene evolution within species cannot always be accurately described by bifurcating phylogenetic trees (Posada & Crandall 2001), we calculated a medium joining network for *L. bilineata* and *L. viridis* using Network 4.1.1.2 (Bandelt *et al*. 1999). The network for the complete data set, including sequences of the control region, contained too much variation, as most haplotypes were unique and corresponded to single individuals. Using the complete sequence information for network estimation would mask the basic relationships between haplotype lineages. Therefore, we used a modified data set consisting of cyt *b* sequences (851 bp) only, resulting in a smaller but more concise haplotype network.

Differentiation level

Nucleotide diversity within and between the revealed lineages was calculated using DnaSP 4.10.4 (Rozas *et al*. 2003). To obtain more information on divergence within distinct lineages, we counted the average number of nucleotide differences (K) and determined the nucleotide diversity (π) as well as the haplotype diversity (Hd). Diversity between lineages was analysed by calculating the mean between group diversity (D_{xy}; Nei 1987) for two different datasets, the partial sequence of (i) cyt *b* and (ii) the noncoding control region. Calculations of nucleotide diversity (π) and mean between group diversity (D_{xy}) were done using the Jukes and Cantor method (Nei 1987) integrated in DnaSP 4.10.4. Within all calculations, sites with alignment gaps were excluded in concordance to the other phylogenetic analyses described above.

Results

Sequence characteristics

We analysed a mitochondrial fragment encompassing 851 bp of cyt *b*, 67 bp of tRNA Pro, 69 bp of tRNA Thr and a part of the control region (CR), which varied in length. The sequences have been deposited in GenBank under the accession numbers listed in Table 1. In summary, 2760 sites were aligned; the total number of sites excluding alignment gaps was 2432 bp. Within the whole dataset we found 1762 constant and 670 variable positions; 377 of these were parsimony informative. If alignment gaps were not considered, 80 haplotypes were detected within the whole dataset caused mainly by base changes within the 5'-repeat region of the control region. Overall nucleotide diversity of the whole

sequence set was T = 33.3%, C = 25.5%, A = 29.6% and G = 11.7%, which corresponds well with the values found in other vertebrate species (Janke & Arnason 1997; Janke *et al.* 2001).

The protein-coding cyt *b* sequence differed from the noncoding CR sequence in base content, portion of variable sites and the ts/tv ratio. The cyt *b* (851 bp) contained 276 variable positions, of which 191 were parsimony informative. No insertions or deletions were detected within the cyt *b* fragment. The sequence variation was mainly caused by transitions (ts/tv ratio = 5.8). The GC content was 40.7%, the AT content was 59.4%. We detected 43 different cyt *b* haplotypes. Within the CR (1740 bp) we found only 429 variable sites, of which 204 were parsimony informative. In contrast to cyt *b*, the number of transitions was nearly equal to the number of transversions (ts/tv ratio = 1.5). Compared to the cyt *b*, the base composition of the noncoding control region showed a lower GC content of 34.6% and a higher AT content of 65.4%. The CR of *L. bilineata*, the west Balkan lizards and *L. viridis* possessed distinct, diagnostic repeat motifs that differed within each of these groups only in single nucleotides (data not shown). All *L. viridis* samples were, in addition to the diagnostic repeat motif, characterized by a unique 11 bp long insertion in the CR that was lacking in *L. bilineata*, the west Balkan lineage and the outgroup species *Lacerta agilis* and *Teira dugesii*.

Phylogeny

Based on the complete sequence alignment of 2760 bp, all tree-building methods recovered the same basal relationships of defined haplotype lineages and clades (Fig. 2). However, the position of individual sequences within the terminal branches of the haplotype lineages differed between the different tree-building methods (data not shown). To describe the tree topology, a Bayesian tree including bootstrap values for ML and MP analyses serves as an example (Fig. 2). All analyses resulted in two major clades, roughly corresponding to *L. viridis* and *L. bilineata*. Sequences from the west Balkans, representing populations that were traditionally assigned to *L. viridis* (Nettmann 2001), were found to be located outside the *L. viridis* clade. Instead of the expected grouping with *L. viridis*, these sequences fell within the clade of *L. bilineata* and therein constituted the sister group of *L. bilineata* sequences from western Europe, the western Mediterranean and the Croatian island, Cres.

In the following, we will use the term '*Lacerta viridis* clade' for the major clade exclusively comprising sequences of *L. viridis* and the term '*Lacerta bilineata* clade' for the second major clade containing sequences of *L. bilineata* and from the west Balkans (Fig. 2).

Within the *L. viridis* clade, four lineages (I–IV) occurred; these corresponded well with the distinct subspecies, with

one exception. Lineage I comprised all sequences of *L. v. viridis* (from the border of Macedonia northwards across the Carpathian Basin to eastern Germany and Ukraine). The sister group of lineage I was lineage II, containing sequences of *L. v. meridionalis* (north-eastern Greece, southern Bulgaria and the Black Sea region of Bulgaria). Together, lineages I and II constituted the sister group of a third, well-supported, lineage (III) comprising one sequence from the Thessaloniki region (Zangliveri, GR4) and three out of four sequences from south-western Bulgaria (Kresna Gorge, B5). *Lacerta viridis guentherpetersi* from Fthiotida (Mayer & Beyerlein 2001; Godinho *et al.* 2005) constituted a further lineage within *L. viridis* (IV) that was basal to a clade containing the lineages ((I + II) + (III)).

An even more complicated pattern was found in the *L. bilineata* clade. As outlined above, we found this clade comprising sequences from west Balkan populations that were previously assigned to *L. viridis*. Additionally, another monophyletic group including all currently recognized subspecies of *L. bilineata* (*L. b. bilineata* V, *L. b. fejervaryi*, *L. b. chlorosecunda*, *L. b chloronota* VI) was found, although it was not well supported. This group, containing sequences from western Europe, the western Mediterranean and the Croatian island Cres was closely related to a second well-supported west Balkan lineage (VII), corresponding to sequences from western Greece and the eastern Adriatic coast.

The median joining network based on a limited dataset consisting only of 851 bp of cyt *b* (Fig. 3) underscored the fact that haplotypes within lineage I (*L. v. viridis*) have no geographical structure. The most common haplotype (c1) was found to be distributed all over the subspecies' range (Table 1); only some individuals possessed unique haplotypes differing in single nucleotides. A narrow relationship was found between haplotypes of lineages I and II, while the other lineages were clearly distinct.

Clade and lineage differentiation

The protein-coding and noncoding regions of the analysed mitochondrial fragment differed extensively regarding the pairwise between-group sequence divergences (Table 2). Within the cyt *b* sequence a two times higher level of between-clade differentiation (D_{xy}) occurred in comparison to the noncoding control region. A low level of genetic differentiation (cyt *b* circa 1%, CR < 1%) was found between *L. v. viridis* (I) and *L. v. meridionalis* (II), between *L. b. bilineata sensu stricto* (V *s.s.*) and the lineage *L. b. bilineata sensu lato* (V *s.l.*, *L. b. bilineata*, *L. b. fejervaryi*, *L. b. chlorosecunda*). All other lineages showed a higher genetic differentiation of 3.2–8.4% for cyt *b* and 1.7–4.1% within the CR. The average between-group sequence divergence (D_{xy}) of the *L. viridis* clade and the *L. bilineata* species (lineages V *s.l.*, VI) was 6.9% for cyt *b*
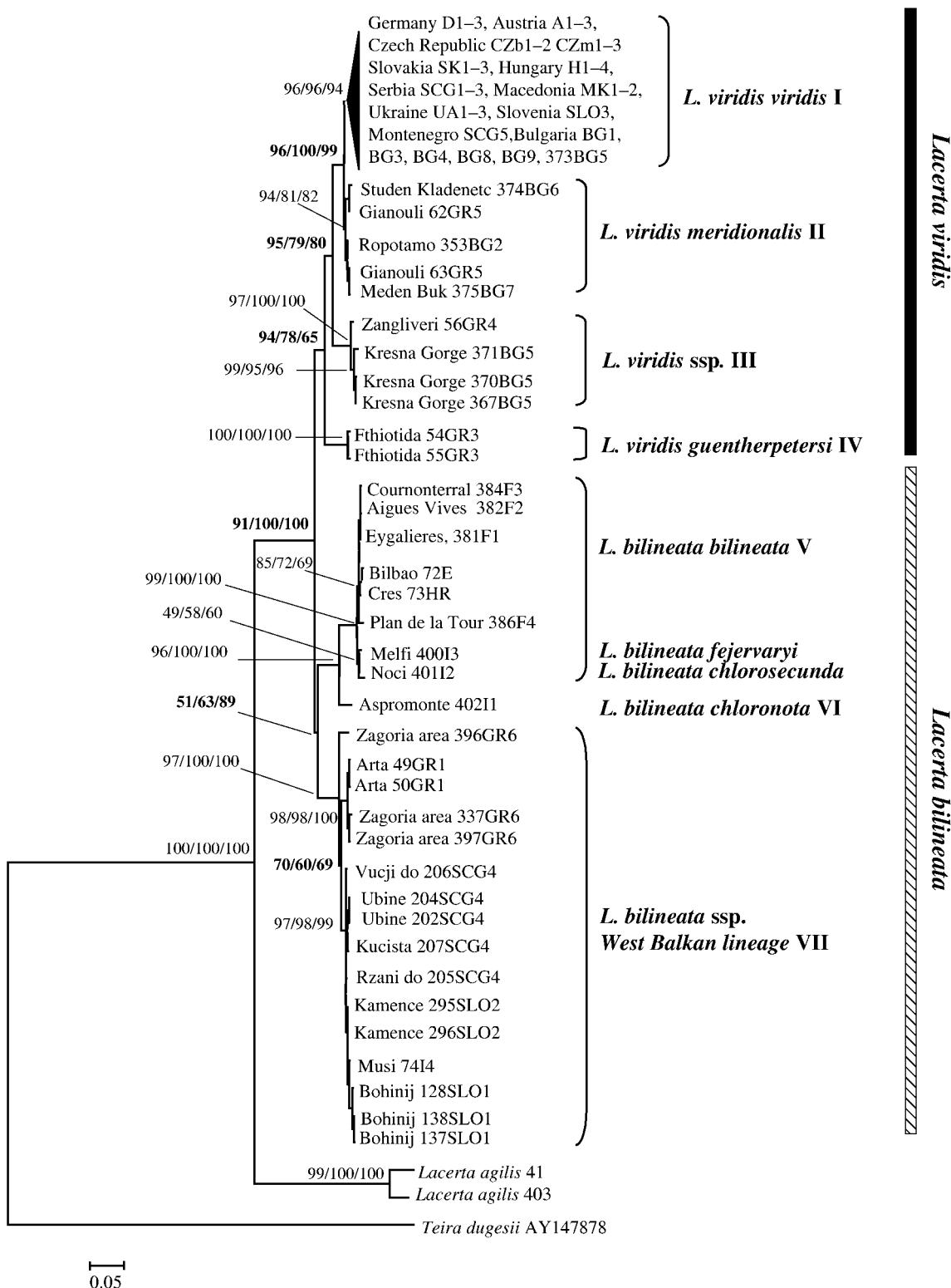
Fig. 2 Bayesian phylogram inferred from a 2760 bp long mtDNA fragment (cyt *b*, tRNA Thr, tRNA Pro, control region) for *Lacerta bilineata* and *L. viridis*. Lineages are indicated by different roman numbers. Values for Bayesian support and bootstrap support from maximum likelihood (ML, 1000 replicates) and maximum parsimony (MP, 2000 replicates) analyses are indicated on bifurcations.
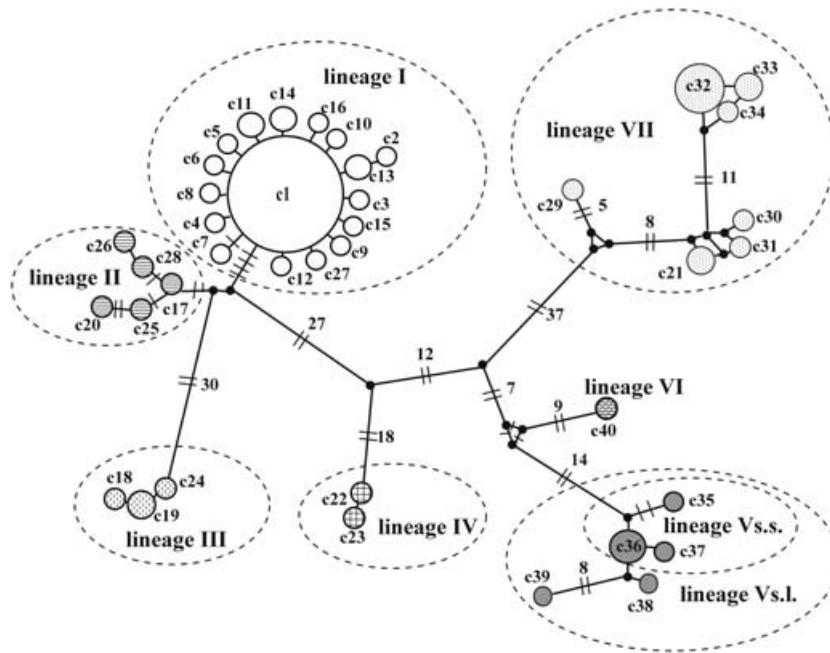
Fig. 3 Median-joining network of cyt *b* haplotypes of *Lacerta bilineata* and *L. viridis* (851 bp). Patterned circles represent observed haplotypes; black dots, missing intermediates. Patterns refer to Fig. 1. Circle sizes are proportional to haplotype frequency. Haplotypes correspond to information in Table 1. Each branch between circles represents a single mutational step, perpendicular hash marks represent single changes; more than four changes are indicated by numbers.

Table 2 Pairwise between-group sequence divergence (D_{xy}) shown for the protein-coding region of cytochrome *b* gene (cyt *b*, 1–851 bp) in the upper part of the matrix and for the noncoding control region (CR, 1021–2760 bp) in the lower part. Clade numbers correspond to numbers within Figs 2 and 3. I, *L. v. viridis*; II, *L. v. meridionalis*; III, *L. viridis* ssp.; IV, *L. v. guentherpetersi*, V *s.s. L. b. bilineata* V *s.l. L. b. bilineata*, *L. b. fejervaryi*, *L. b. chlorosecunda*; VI, *L. b. chloronota*; VII, *L. bilineata* ssp., west Balkans.

Lineage	I	II	III	IV	V *s.s.*	V *s.l.*	VI	VII
I	—	0.012	0.044	0.061	0.068	0.068	0.073	0.078
II	0.007	—	0.042	0.061	0.069	0.069	0.068	0.081
III	0.017	0.018	—	0.061	0.066	0.067	0.065	0.084
IV	0.023	0.019	0.024	—	0.067	0.069	0.059	0.081
V *s.s.*	0.039	0.033	0.038	0.035	—	0.003	0.032	0.079
V *s.l.*	0.039	0.035	0.038	0.035	0.002	—	0.033	0.079
VI	0.038	0.033	0.041	0.036	0.020	0.020	—	0.075
VII	0.024	0.021	0.026	0.026	0.032	0.032	0.028	—

lineage diversity. In particular, the level of sequence diversity (π) appeared very low compared to the lineages confined to the southern distribution range, for example the west Balkan lineage VII or lineages II or IV (*L. v. meridionalis* and *L. v. guentherpetersi*). Overall haplotype diversity (Hd) was high within all lineages, but was reduced if we only considered haplotypes of the protein-coding sequence. Again, the southern lineages (II, III, IV, VII) exhibited higher levels of diversity.

Discussion

The approximately two times higher level of sequence differentiation in the protein-coding cytochrome *b* gene compared to the noncoding control region between the two studied species is not particularly surprising. Although in most species the mitochondrial control region is generally the most variable part of the mitochondrial DNA, comparably low variation within this region was also observed in other studies between closely related species (e.g. Brown *et al*. 1986; Bernatchez & Danzmann 1993; Crochet & Desmarais 2000; Ruokonen & Kvist 2002; Tang *et al*. 2006). Increased functional constraints, such as the ability to fold into secondary structures as a functional basis for H-strand replication and the termination of RNA transcription, were assumed to be reasons for a reduced genetic variation in the control region (Brown *et al*. 1986). Nevertheless, we found specific repeat motives and insertions within this noncoding region.

Phylogeography

We found two major haplotype clades representing the two distinct species *L. viridis* and *L. bilineata*. Within each of

and 3.8% for the CR. The west Balkan lineage (VII) showed a similar level of differentiation compared to *L. viridis* (D_{xy}, cyt *b* = 7.9%, CR = 2.4%) and to *L. bilineata* (D_{xy}, cyt *b* = 7.9%, CR = 3.1%). This emphasized the distinctness of the west Balkan lineage.

Nucleotide diversity (π) within the complete sequence (cyt *b*, tRNAs, CR), within the protein-coding part (cyt *b*) and the noncoding part was low for all lineages ($\pi < 1\%$). Nevertheless, the most widely distributed lineages I (*L. v. viridis*) and V *s.s.* (*L. b. bilineata s.s.*) showed comparably low levels of within

these clades we detected several haplotype lineages corresponding to the different subspecies of the eastern green lizard *L. viridis* and the western green lizard *L. bilineata*. The discovery of the hitherto unknown west Balkan lineage within the *L. bilineata* clade was unexpected.

In both species clades the greatest haplotype variation and differentiation were found in their southern ranges (Balkan and Apennine peninsulas). Within the *L. viridis* clade four distinct lineages were found, three of them (II–IV) restricted to the southern Balkan peninsula. The fourth lineage (I) occurred in the southern Balkans as well and occupied the entire northern parts of the range, suggesting a Holocene range expansion. The high level of differentiation between lineages II and IV (cyt *b* divergence > 6%, Table 2, Fig. 3) suggests that these haplotypes represent long persisting and highly distinct lineages that were restricted to several microrefuges during the last glaciations within Europe. Lineage I corresponded well with the geographical distribution of *L. v. viridis*, which seems to be adapted to a wider ecological range than other subspecies of *L. viridis*. The low level of genetic distance (Figs 2 and 3) and haplotype diversity (Table 3, < 1%) within this lineage suggests that the process of recolonization and migration took place very rapidly, starting from a common genetic pool for this subspecies.

Table 3 Within-lineage genetic diversity for the complete mtDNA fragment, including adjacent tRNAs and the protein-coding cytochrome *b* gene (cyt *b*) and the noncoding control region (CR) independently. Given only for the lineages with more than one haplotype: NoI, number of individuals; NoH, number of haplotypes; nucleotide diversity π (JC); K, average of nucleotide differences; Hd, haplotype diversity.

Lineage	NoI		NoH	π in % (± SD)	K	Hd (± SD)
L. v. viridis I	61	total	49	0.29 ± 0.03	7.4	0.97 ± 0.02
		cyt *b*	17	0.08 ± 0.02	0.7	0.53 ± 0.08
		CR	46	0.43 ± 0.04	6.6	0.96 ± 0.02
L. v. meridionalis II	5	total	5	0.42 ± 0.09	10.6	1.00 ± 0.13
		cyt *b*	5	0.47 ± 0.12	4.0	1.00 ± 0.13
		CR	5	0.39 ± 0.08	6.0	1.00 ± 0.13
L. viridis ssp.? III	4	total	4	0.39 ± 0.11	10.0	1.00 ± 0.18
		cyt *b*	3	0.12 ± 0.04	1.0	0.83 ± 0.22
		CR	4	0.49 ± 0.15	7.8	1.00 ± 0.18
L. v. guentherpetersi IV	2	total	2	0.50 ± 0.25	13.0	1.00 ± 0.50
		cyt *b*	2	0.12 ± 0.05	1.0	1.00 ± 0.50
		CR	2	0.69 ± 0.34	11.0	1.00 ± 0.50
L. b. bilineata s.s. V	6	total	5	0.17 ± 0.07	4.2	0.93 ± 0.12
		cyt *b*	3	0.19 ± 0.10	1.7	0.60 ± 0.22
		CR	4	0.17 ± 0.06	2.5	0.80 ± 0.17
L. b. bilineata s.l. VI	8	total	7	0.34 ± 0.10	8.4	0.96 ± 0.08
		cyt *b*	5	0.46 ± 0.20	3.9	0.79 ± 0.15
		CR	6	0.26 ± 0.06	3.8	0.89 ± 0.11
L. bilineata ssp. West Balkan VII	16	total	12	0.71 ± 0.13	17.8	0.97 ± 0.03
		cyt *b*	7	0.92 ± 0.20	7.7	0.75 ± 0.11
		CR	10	0.55 ± 0.08	8.3	0.94 ± 0.04

We assume that the southern Balkans (Greece) is the refugial area of *L. v. viridis*, despite the fact that mtDNA could not resolve a deeper phylogeographical structure or migration routes within this subspecies. A refuge further north in the Carpathian Basin, as demonstrated for other species, e.g. small mammals (Bilton *et al*. 1998; Stewart & Lister 2001), *Microtus agrestis* (Jaarola & Searle 2002) and *Rana arvalis* (Babik *et al*. 2004), seems not likely because these species are actually distributed far further to the north than *L. v. viridis*, indicating that the above-mentioned species are better adapted to colder climates than *L. v. viridis*.

The distribution of haplotypes of the *L. bilineata* clade resembled the pattern of *L. viridis*, as a much higher diversity was found in the southern compared to the northern parts of the range (Figs 2 and 3, Table 3). However, the existence of the west Balkan lineage suggests two highly distinct refugial areas, one in the Apennine peninsula and a second in the southern Balkan peninsula (Fig. 1). The distinctiveness of lineages V (corresponding to *L. b. bilineata s.l.*) and VI (*L. b. chloronota*), also found by Brückner *et al*. (2001) and Godinho *et al*. (2005), argues for two distinct Apennine microrefuges. A similar pattern was detected for other European reptile species in southern Italy (Steinfartz *et al*. 2000; Fritz *et al*. 2005; Podnar *et al*. 2005; Ursenbacher *et al*. 2006). We suggest that one major postglacial migration event of lineage V resulted in the current wide distribution of *L. b. bilineata*, which is reflected in the low genetic differentiation between the geographically distant regions.

The second major refugial area in the south-western Balkans, west of the Pindos and Dinaric Mountains, harboured lineage VII. We suggest that this area is inhabited by an early offshoot of *L. bilineata*, which was isolated during a previous Pleistocene glaciation from the western *L. bilineata* populations. A similar phylogeographical pattern was discovered for *Emys orbicularis hellenica* (Fritz *et al*. 2005). Separated from the east European lineage I (*L. v. viridis*) by the Dinaric Mountains, this west Balkan lineage spread over the east Adriatic region northwards, until the Alps hindered further distribution. According to our data, *L. b. bilineata*, the west Balkan lineage and *L. v. viridis* occur today in close geographical proximity in the northern Adriatic region (Cres, Slovenia). By analysing allozyme data, Amann *et al*. (1997) and Joger *et al*. (2001) hypothesized a hybrid zone of *L. viridis* and *L. bilineata* in the western part of Slovenia and the region of Friuli (I4). Within this zone they postulated an introgression of *L. viridis* genes into *L. bilineata*, reaching the area of Trieste or even Udine. Our data do not correspond well with this result, as the eastern Slovenian (SLO1) and the central Slovenian populations (SLO2) displayed mitochondrial characteristics of the *L. bilineata* clade and the most southern population of the *L. viridis* clade in Slovenia was located in the area of Maribor (Fig. 1, SLO3). However, such discordances

between morphological, nuclear and mitochondrial DNA, especially within hybrid zones, were also observed in other studies on vertebrates (Mallet 2005; Godinho *et al.* 2006; Vörös *et al.* 2006). Further investigations using nuclear genomic markers are needed to reveal whether gene flow exists between the different mitochondrial haplotype lineages occurring in that area.

Implications for taxonomy

A morphological distinction of *Lacerta viridis* and *L. bilineata* is very difficult as most colour patterns and other morphological characters have no strong geographical restriction (Nettmann 2001).

Probably due to those difficulties, the species range of *L. viridis* was not correctly defined so far (Brückner *et al.* 2001; Godinho *et al.* 2005). Our data provide evidence that the newly discovered west Balkan lineage is more closely related to *L. bilineata* than to *L. viridis*. Nevertheless, our data support the existence of three currently recognized subspecies of *L. viridis*: *L. v. viridis* (lineage I), *L. v. meridionalis* (II) and *L. v. guentherpetersi* (IV).

Despite its well supported morphological distinctiveness, *L. viridis meridionalis* seems to be closely related to *L. v. viridis*; this was supported by all phylogenetic analyses (Figs 2 and 3) and the very low levels of nucleotide differentiation between these clades (Table 2). The high degree of differentiation of *L. v. guentherpetersi* (lineage IV) was also supported by quantitative morphological data (Rykena 2001). The occurrence of lineage III was unexpected. According to their collection sites, the sequence from the region of Thessaloniki should represent *L. v. meridionalis*, while the south-western Bulgarian sequences should represent *L. v. viridis*. The phylogenetic distinctness of these sequences suggests the existence of a further, hitherto undiscovered haplotype lineage or even subspecies in the south-eastern Balkans. Obviously, a zone of secondary contact exists between this undescribed subspecies and *L. v. viridis*, as we found a fourth sequence from Kresna (B5) belonging to lineage I (*L. v. viridis*).

Within *L. bilineata*, the weak differentiation of *L. b. bilineata*, *L. b. fejervaryi* and *L. b. chlorosecunda* (Figs 2 and 3, Table 2) raises doubts concerning the validity of these subspecies. In contrast, our data supported the distinctiveness of *L. b. chloronota* from Calabria (Aspromonte).

The hitherto unrecognized green lizard lineage of the west Balkan region clearly represents a further taxon. According to our mitochondrial data, its range extends from north-eastern Italy (Friuli) through Slovenia along the Adriatic and Ionic coasts to the Epirus region (Figs 2 and 3). We refrain from assigning the available name *Lacerta viridis intermedia* (Mehely, 1905) to this taxon, because its type locality (Ogulin, Croatia) is in a region where all three mitochondrial lineages (corresponding to *L. v. viridis*, *L. b. bilineata* and the west Balkan taxon) could be expected.

Our finding that green lizards of Arta (western Greece) belong to the newly discovered west Balkan taxon seems to contradict the previous allocation of green lizards of this region to *L. viridis* (Brückner *et al.* 2001). The above-mentioned authors used 400 bp of the 5′-end of the mitochondrial cyt *b* gene for their phylogeographical investigations. We used a longer, phylogenetically more informative fragment of the adjacent mitochondrial fragment, resulting in a clear distinction between these samples and *L. viridis* and their sister-group relationship to *L. bilineata*. Our finding is corroborated by the results of Mayer & Beyerlein (2001), using 12S rDNA and 16S rDNA sequences.

Based on mtDNA (16S, 12S) and nDNA (β-fibint7, C-mos) gene data, Godinho *et al.* (2005) assumed that along the eastern Adriatic coast gene flow occurs between *L. viridis* and *L. bilineata*. Moreover, their phylogeny supported a paraphyly of *L. viridis* with respect to *L. bilineata*. However, their conclusions were based on the assumption that green lizards from Arta and from the western slope of the Dinaric Mountains (Bosnia, Croatia) represent *L. viridis*. If those samples were not labelled as *L. viridis* in their 16S/12S phylogeny, *L. viridis* would not be paraphyletic with respect to *L. bilineata*. Moreover, the close relationship and position of their samples from Bosnia, Croatia and Arta within the β-fibint7 phylogenetic tree would correspond well to our findings of a distinct west Balkan taxon.

Conclusions

The phylogenetic pattern of the *L. viridis* clade suggests that there was one refugial area, highly structured due to rich microgeographical patterns in the mountains, resulting in several highly differentiated and long persisting haplotype lineages, while only one lineage successfully recolonized the northern areas. By contrast, our data suggest two independent refugial areas for the *L. bilineata* clade: the Apennines and the west Balkans. However, the taxonomic level of this west Balkan lineage is difficult to determine, as it showed a high genetic distance to all other lineages within the dataset. Summarizing the picture with regard to the *L. viridis* and *L. bilineata* clades, our data support the current opinion that the refuges comprise the main portion of biodiversity and are a source for speciation processes. We furthermore suggest that a more detailed revision of the populations within the refugial areas would reveal a more complex pattern of subspecies division or hybridization events between the subspecies or even between different species of Lacertidae inhabiting these areas.

Although our study supports a division of *L. viridis* and *L. bilineata*, the taxonomic conclusions remain difficult. Further molecular analyses using nuclear markers and detailed morphological analyses of the phylogenetic lineages revealed in our study are essential to obtain more information about

their taxonomic level. However, our results clearly document that the general pattern of recolonization and possible speciation events in *Lacerta* is comparable to the those of other vertebrates.

Acknowledgements

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