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Phylogeny and evolution of the green lizards, *Lacerta* spp. (Squamata: Lacertidae) based on mitochondrial and nuclear DNA sequences

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Abstract. Partial DNA sequences from three mitochondrial (cytochrome *b*, 12S rRNA and 16S rRNA) and two nuclear (β-fibrinogen intron 7 and C-*mos*) genes were used to estimate the phylogenetic relationships among all eight extant species of green lizards, *Lacerta sensu stricto*, and many currently recognized subspecies. All eight species form a monophyletic group. *L. agilis*, *L. schreiberi* and *L. strigata* are genetically well differentiated species. *L. trilineata* and *L. pamphylica* are not monophyletic units based on analyses of the β-fibrinogen intron 7. *Lacerta media* is closely related to some *Lacerta trilineata*. *L. bilineata* and *L. viridis* are closely related, and recognition of *L. bilineata* as a distinct species makes *L. viridis* paraphyletic also. For both *L. bilineata* and *L. viridis*, some subspecies appear to remain in their southern glacial refugia, while a single genetic entity shows successfully postglacial expansion. The topology derived from C-*mos* variation is concordant with that derived from mtDNA, with substitutions occurring at a similar rate to that of transversions in the rRNA genes. Although C-*mos* is typically used at deeper taxonomic levels it is also phylogenetically informative within green lizards. β-fibrinogen intron 7, typically used for assessing phylogenetic relationships among bird species, is a useful phylogenetic marker for reptiles also, showing considerable variation between species. There is not complete concordance between estimates of relationships derived from the mtDNA and nuclear markers, probably because rapid diversification led to incomplete lineage sorting in the green lizards. Introgression could also be occuring between some species.

Introduction

Green lizards of the genus *Lacerta sensu stricto* inhabit a large area extending from Western Europe to Central Asia. With the exception of the more widespread *Lacerta agilis*, they are almost restricted to the southern European peninsulas well known for exceptionally richness in biodiversity. They are essentially parapatric, with relatively small areas where two or rarely three species are found and each species substitutes the other in a clinal continuum throughout the Mediterranean belt (fig. 1). They are easily identifiable from other lizards by their relatively large size and often brilliant colour, but some are difficult to discriminate from each other phenotypically.

Peters (1962) recognized five species: *L. agilis*, *L. schreiberi*, *L. strigata*, *L. trilineata* and *L. viridis*. Since then they have been subject to morphological studies which resulted in the splitting of *L. trilineata* into three species, *L. media*, *L. pamphylica* and *L. trilineata* (Schmidtler, 1986a). *L. viridis* was recently split into two species *L. bilineata* and *L. viridis*, based first on the results of hybridization experiments (Rykena, 1991) and later by both protein electrophoretic data (Amann et al., 1997) and mitochondrial DNA (mtDNA) sequences (Brückner et al., 2001). Based on morphological characters Arnold (1973) suggested that green lizards and the *Lacerta lepida* group (*L. lepida*, *L. pater* and *L. princeps*) formed a clade. Later, Rykena and Nettmann (1986) showed that the *L. lepida* group is strongly supported as a monophyletic clade by a chromosomal arrangement unique among lacertids (36 diploid with two biarmed chromosomes instead of the typical 38 diploid number). Subsequently, evidence from

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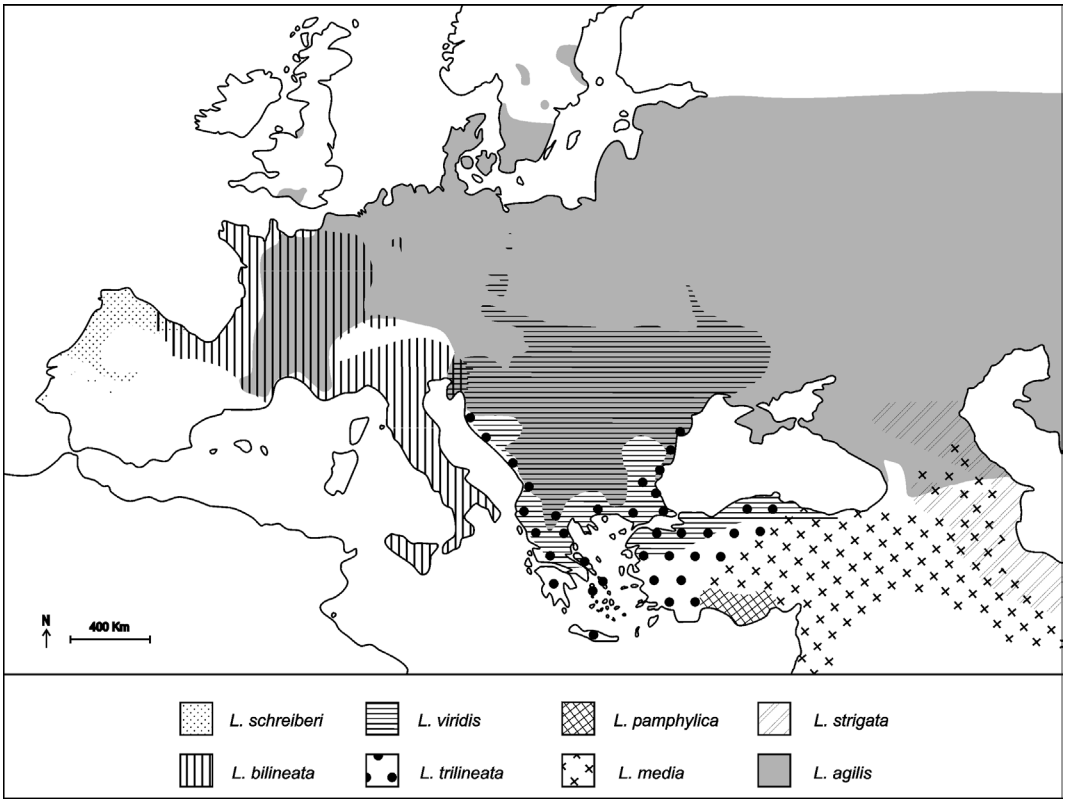

Figure 1. Map showing the distribution of green lizard species in Europe and Western Asia (adapted from Gasc et al., 1998 and Nettmann, 2001).

mtDNA sequences suggested that the *L. lepida* group is the sister clade to the green lizards (Harris et al., 1998).

No cladistic attempt has previously been made to reconstruct the phylogeny of the green lizards group. Using the micro-complement fixation technique, Lutz and Mayer (1985) indicated that *L. trilineata* and *L. viridis* are more closely related to each other than to *L. agilis*, but did not examine other species. Later, Rykena (1996) suggested a distant relationship of *L. schreiberi* to the other green lizards based on hybridization experiments. Similarly, Brückner et al. (2001) described a close association of *L. viridis* with *L. bilineata* relative to *L. trilineata* using mtDNA sequences and Mayer and Beyerlein (2001) described the paraphyly of *L. trilineata* cluster in relation to *L. pamphylica*. However, no other green lizards were included in these studies. Therefore, at present, the phylogeny of the group is unresolved. Furthermore, it is unclear whether species and subspecies defined on the basis of morphological and ecological features are well supported by molecular data. To address this question we have used new and published sequences from three mtDNA (cytochrome *b*, 12S rRNA and 16S rRNA) and two nuclear (β-fibrinogen intron 7 (*β-fibint7*) and C-*mos*) genes to assess the phylogeny of all eight species of green lizards, including many of the distinct subspecies.

Materials and methods

Laboratory procedures

Samples consisted of tail tips kept in 100% ethanol, frozen tissues from the lacertids tissue bank (Vienna Natural History Museum) or were the same as those used in previous studies (table 1). Total genomic DNA was extracted using standard methods. Polymerase Chain Reaction (PCR)

Table 1. List of samples analysed, GenBank Accession Nos. for the four loci used and respective references. 1. New for this study; 2. Fu et al. (1997); 3. Harris et al. (1998); 4. Fu (2000); 5. Brüchner et al. (2001); 6. Godinho et al. (2001); 7. Harris et al. (2001); 8. Mayer and Beyerlein (2001); 9. Paulo et al. (2001); 10. Kalyabina-Hauf and Deichsel (2002).

	Location	Ref.	12S+16S	Cyt-*b*	C-*mos*	*β-fibint7*
Lacerta agilis agilis	Holland	1, 3, 7	DQ097096 DQ097100 AF080298 AF080300	AF080299	AF315397	DQ097109
	Austria	1, 8	AF149947 AF149963	DQ097090	DQ097136	DQ097108
	Germany	9	–	AF373032	–	–
Lacerta bilineata	Croatia	5	–	AF233422	–	–
	Italy	1, 5	–	AF233415 AF233416 AF233417 AF233420	DQ097133	DQ097115
	Introduced, USA	10	–	AY099282 AY099283 AY099284	–	–
Lacerta bilineata bilineata	Spain	1, 8	AF149955 AF149971	–	DQ097134	DQ097116
	Italy	5	–	AF233414	–	–
	France	9	–	AF373033	–	–
Lacerta bilineata chloronota	Italy	1, 5, 8	AF149956 AF149972	DQ097087 AF233421	DQ097135	–
Lacerta bilineata chlorosecunda	Italy	5, 8	AF149957 AF149973	AF233419	–	–
Lacerta bilineata fejervaryi	Italy	5	–	AF233418	–	–
Lacerta lepida	Portugal	1, 3	AF042551 AF042561	AF080296	DQ097145	DQ097124
Lacerta media media	Armenia	4, 5	AF206590	U88603	–	–
Lacerta media wolterstorfii	Lebanon	1	–	–	DQ097144	DQ097105 DQ097106
Lacerta pamphylica	Turkey	1, 8	AF149954 AF149970	DQ097089	DQ097142 DQ097143	DQ097103 DQ097104
Lacerta pater	Marocco	3	AF080293 AF080295	AF080294	–	–
Lacerta princeps	–	3	AF080382 AF080384	AF080383	–	–
Lacerta schreiberi	Portugal	1	DQ097093 DQ097097	AF386784	DQ097126	DQ097101 DQ097102
	Spain	1, 3, 4, 6	AF206591	AF386785 AF080301	DQ097127	–
Lacerta strigata	Georgia	1	DQ097094 DQ097098	DQ097091	DQ097137	DQ097107
	–	2	–	U88602	–	–
Lacerta trilineata dobrogica	Greece	8	AJ238177 AF149935	–	–	–
Lacerta trilineata hansschweizeri	Greece	1, 8	AF149952 AF149968	–	DQ097140	DQ097114
Lacerta trilineata major	Bosnia	1	–	–	DQ097139	DQ097110
	Croatia	5	–	AF233427	–	–

Table 1. Continued

	Location	Ref.	12S+16S	Cyt-*b*	C-*mos*	*β-fibint7*
	Greece	8	AF149949 AF149950 AF149965 AF149966	–	–	–
Lacerta trilineata polylepidota	Greece	1, 8	AF149948 AF149964	DQ097092	DQ097138	DQ097111 DQ097112
Lacerta trilineata trilineata	Greece	1, 8	AF149951 AF149953 AF149967 AF149969	–	DQ097141	DQ097113
Lacerta viridis guntherpetersi	Greece	1, 5, 8	AF149959 AF149975 AF149961 AF149977	AF233424	DQ097131	DQ097119 DQ097120
Lacerta viridis meridionalis	Greece	1, 8	AF149960 AF149976	–	DQ097130	DQ097123
Lacerta viridis viridis	Austria	8	AF149962 AF149978	–	–	–
	Bosnia	1	–	DQ097088	–	
	Croatia	1	–	–	DQ097129	DQ097117 DQ097118
	Germany	5	–	AF233426	–	–
	Hungary	1, 5, 9	–	AF233425 AF373034	DQ097128	DQ097121
	Greece	1, 5, 8	AF149958 AF149974	AF233423	DQ097132	DQ097122

primers used in both the amplification and the sequencing were *glu*DG and c*b*3, 12Sa and 12Sb, and 16Sar and 16Sbr, for mtDNA (Palumbi, 1996), FIB-B17U and FIB-B17L for *β-fibint7* (Prychitko and Moore, 1997) and Mos-F (5′- CTC TGG KGG CTT TGG KKC TGT STA CAA GG - 3′ [974]) and Mos-R (5′-GGT GAT GGC AAA NGA GTA GAT GTC TGC-3′ [1577]) for C-*mos* (numbers in brackets after the primer refer to the 5′ position of the primer, as localized on the complete sequence of the chicken C-*mos* gene). These five sets of primers amplified regions of approximately 900, 450, 590, 700-1200 and 520 base pairs, respectively. Amplifications were made using annealing temperature of 50°C for mtDNA genes, 58°C for *β-fibint7* and 48°C for C-*mos* gene. Successful PCR bands were purified using a QIAEX II kit (Quiagen) and sequenced on an Applied Biosystems Model 310 DNA Sequencing System, using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit. Centrisep spin columns (Princeton Separations Inc.) were used for excess dye extraction. All sequences generated for this study were deposited in GenBank (Accession numbers DQ097087 to DQ097146).

The presence or absence of an insertion of 380 bp on the *β-fibint7* for *L. viridis* and *L. bilineata* was used as a marker without the need for sequencing, as its detection depends only on a PCR amplification. PCR products were screened in one to three samples for 14 locations, and length variants were identified in 2% agarose gels. Two individuals of *L. viridis* carrying both haplotypes (with and without the 380 bp insertion) were cloned using a T/A cloning kit (Fermentas MBI) and sequenced as described before.

Phylogenetic analyses

Sequences were aligned against those already published using Clustal W (Thompson et al., 1994). Minor adjustments were made by eye in loop regions of the 12S and 16S rRNA sequences and in indel regions for *β-fibint7*. They were then imported into PAUP* 4.0b10 (Swofford, 2003) for phylogenetic analyses. For the phylogenetic analysis of the combined data we used maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference. When estimating phylogenetic relationships among sequences, one assumes a model of evolution. We used the approach outlined by Huelsenbeck and Crandall (1997) to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (Posada and Crandall, 1998). Once a model of evolution was chosen, it was used to estimate a tree using maximum likelihood (Felsenstein, 1981) with random sequence addition (10 replicate heuristic search). Support for nodes was estimated using the bootstrap technique (Felsenstein, 1985). The maximum parsimony analysis was also carried out with random sequence addition (100 replicate heuristic

Table 2. Parsimony informative indels of *β-fibint7*. [a]Indels are listed in 5′-3′ direction of *β-fibint7* and nucleotide sites are given in parenthesis and are numbered in reference to outgroup species; [b]Indel origin is defined in relation to outgroup species; [c]Number of base pairs (bp) involved in the indel.

Indel number and nucleotide sites[a]	Indel origin[b]	bp involved[c]
1 (133-144)	Deletion	12
2 (202-208)	Deletion	7
3 (after 258)	Insertion	2
4 (after 266)	Insertion	380
5 (after 363)	Insertion	2
6 (after 494)	Insertion	5
7 (after 605)	Insertion	6
8 (after 636)	Insertion	1

search), and support for nodes estimated by bootstrapping with 1000 replicates.

The *β-fibint7* sequences contained a number of parsimoniously informative indels of various lengths (table 2). We carried out the MP analysis with these gaps treated as missing data, and separately with indels coded as 1 or 0 for presence/absence and included in the analysis, with all sites weighted equally. The Bayesian analysis was implemented using MrBayes (Huelsenbeck and Ronquist, 2001) which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MCMCMC) sampling approach. Bayesian analyses were conducted with random starting trees, run 1×10^6 generations, and sampled every 10 generations using a general-time-reversible model of evolution with a gamma model of among site rate variation. In both searches stationarity of the Markov Chain was determined as the point when sampled log likelihood values plotted against generation time reached a stable mean equilibrium value; "burn-in" data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny. Two independent replicates were conducted and inspected for consistency to check for local optima (Huelsenbeck and Bollback, 2001). In all mtDNA analyses three members of the *Lacerta lepida* group, *L. lepida*, *L. princeps* and *L. pater* were designated as outgroups. For *β-fibint7* and for C-*mos L. lepida* and *Iberolacerta monticola* were included as outgroups. Sequences from the protein coding gene C-*mos* were aligned against published sequences (Saint et al., 1998; Harris, 2003). There were no indels. Because variation is very low, the sequences were joined in a network using the program TCS (Clement et al., 2000).

Results

Mitochondrial DNA sequence variation

Sequences for partial 12S and 16S rRNA genes totaling 893 base pairs were analyzed for 27 lizards. The most appropriate model of evolution for this data set was the Tamura Nei model with a discrete approximation of a gamma distribution of variable sites. Using this model a ten replicate heuristic search found a single maximum likelihood tree of score −ln 3416 (fig. 2). Using MP, 147 characters were informative. A 100 replicate heuristic search found three trees of 366 steps (fig. 2). For the cytochrome *b* gene region, 35 sequences of 429 base pairs were analysed, which included approximately 50 base pairs of tRNA glu. Some of the sequences included from GenBank did not include the tRNA glu region; unavailable regions were coded as missing data. There were no insertions or deletions. The most appropriate model of evolution for this data set was the general time reversible model with a discrete approximation of a gamma distribution of variable sites and an estimated proportion of invariable sites. Using this model a ten replicate heuristic search found 60 equally likely trees of score − ln 2097 (fig. 3). Using MP, 120 characters were informative. A 100 replicate heuristic search found 198 trees of 358 steps (fig. 3).

Nuclear DNA sequence variation

For *β-fibint7*, 23 individuals generating 26 different sequences were analysed. Base composition in the *β-fibint7* sequences did not vary significantly between taxa using the chi-square test (A = 0.32, C = 0.22, G = 0.18, T = 0.28, χ^2 = 83, P = 0.23). There were 18 indels of lengths between 1 bp and 380 bp, eight of which were parsimoniously informative for the evolution of the group (table 2). The most appropriate model of evolution for this data set was the HKY model with an estimated proportion of invariable sites. Using this model a 100 replicate heuristic search found one most likely tree of score −ln 2856 (fig. 4). Using MP, 64 characters were informative. A 100 replicate heuristic search found one tree of 165 steps (fig. 4).

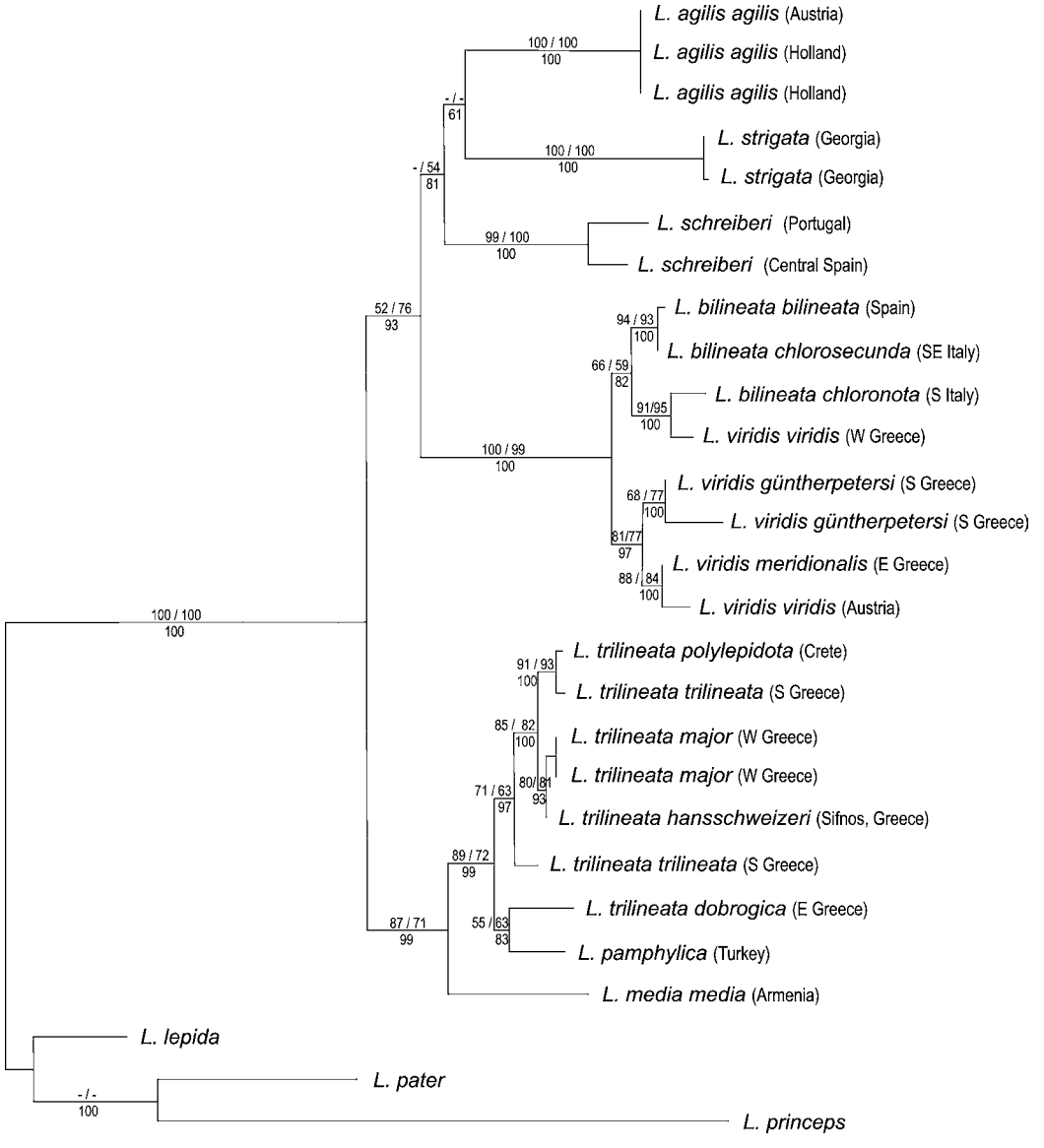

Figure 2. Maximum likelihood tree derived from combined 12S and 16S rRNA sequences. The most appropriate model was the Tamura Nei model (i.e. with different transition rates), including a discrete approximation of the gamma distribution (0.16). The tree was rooted using *L. lepida*, *L. pater* and *L. princeps*. Bootstrap values (MP/ML) are given above nodes. Bayesian posterior probabilities are given below nodes. Dashes indicate less than 50% support levels.

For the nuclear gene C-*mos*, 25 individuals generating 19 different sequences of 522 base pairs were analyzed. Thirty-eight positions were variable, with 17 being parsimoniously informative. There were no insertions or deletions, and no homoplasy in the data set (fig. 5). Two sequences contained two heterozygous positions, thus the haplotypes had to be inferred, following Harding et al. (1997). Base composition in the C-*mos* sequences was typical of other lacertids (Harris, 2003) and did not vary significantly between taxa using the chi-square test (A = 0.29, C = 0.20, G = 0.24, T = 0.27, χ^2 = 1.58, P = 0.99). In passerine birds all C-*mos* nucleotide substitutions accumulate at a rate similar to that

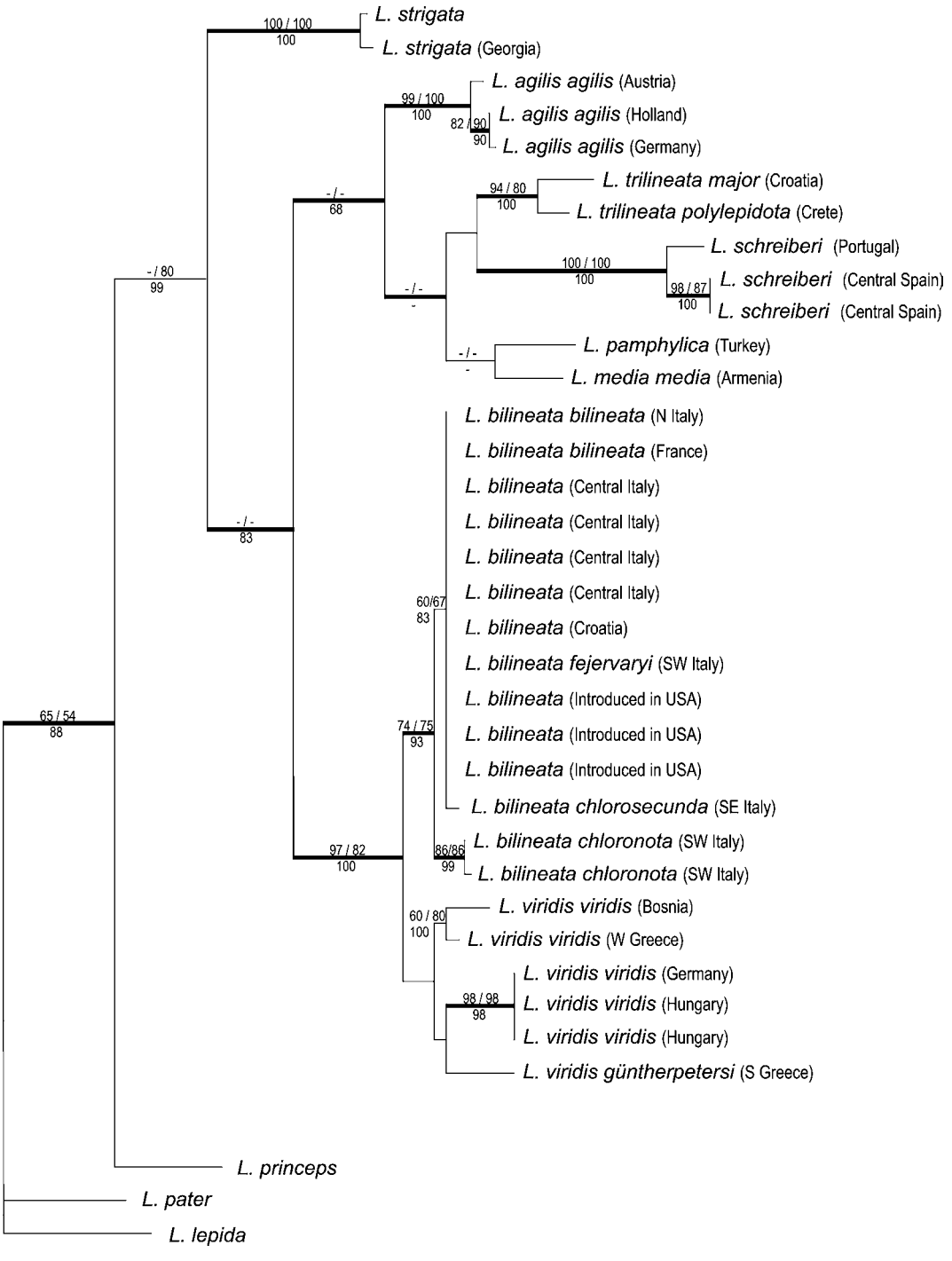

Figure 3. Maximum likelihood tree derived from the cytochrome *b* sequences. The most appropriate model was the GTR model, including a discrete approximation of the gamma distribution (1.49) and an estimated proportion of invariable sites (0.56). The tree was rooted using *L. lepida*, *L. pater* and *L. princeps*. Bootstrap values (MP/ML) are given above nodes. Bayesian posterior probabilities are given below nodes. Dashes indicate less than 50% support levels. Thicker lines indicate support in the MP strict consensus.

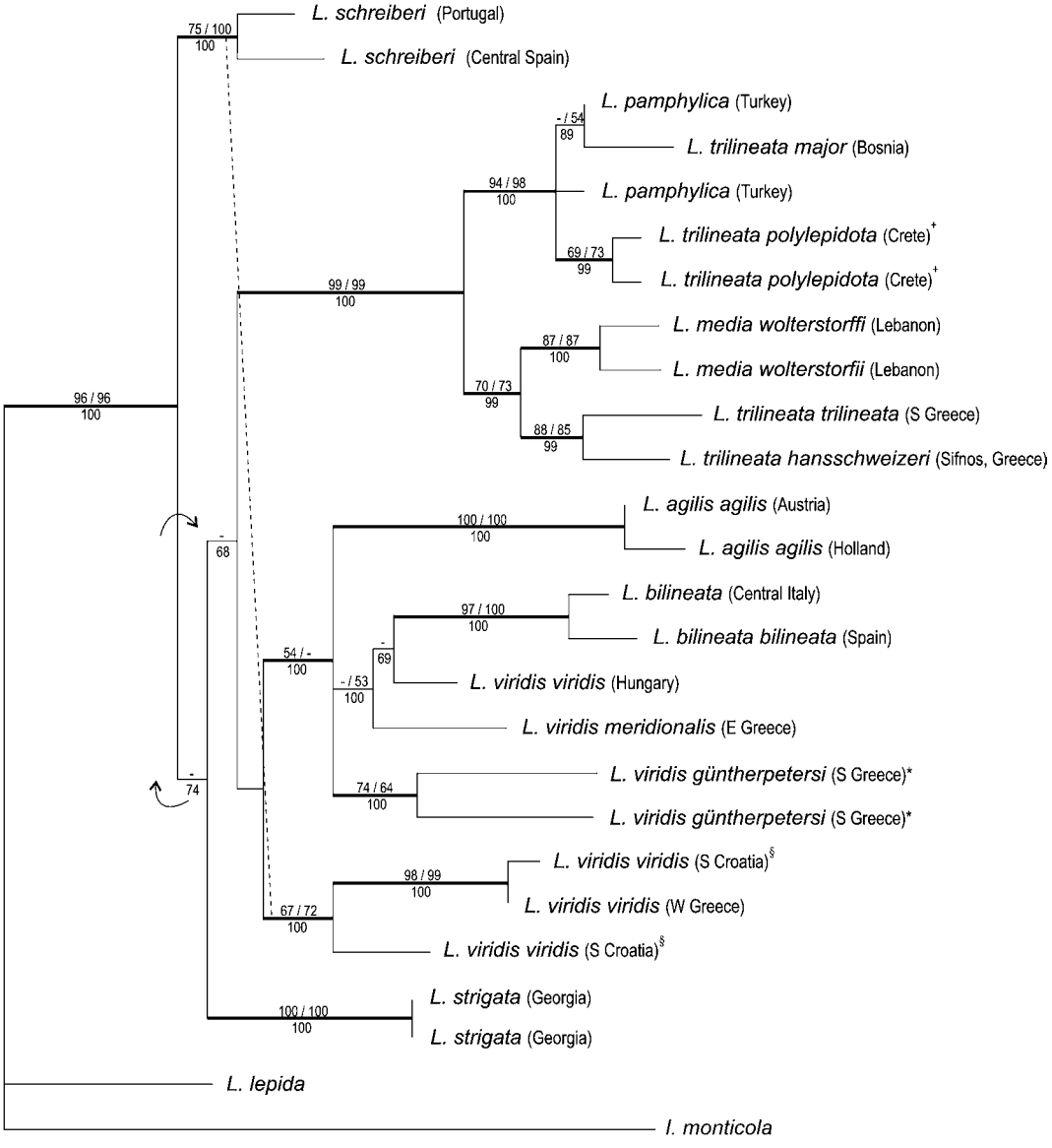

Figure 4. Maximum likelihood tree derived from the *β-fibint7* sequences. The most appropriate model was the HKY model. Bootstrap values (MP/ML) are given above nodes. Bayesian posterior probabilities are given below nodes. Thicker lines indicate support in the MP strict consensus. Arrows indicate alternative arrangement of branches in the Bayesian analysis. Dotted line indicates alternative arrangement of branches in the MP analysis when insertions where coded as a fifth character. +, * and § indicate two haplotypes from the same individual.

of transversion substitutions in the mitochondrial genes examined (Lovette and Bermingham, 2000). In our cytochrome *b* data set, there is high variation in the rate of substitution in the different types of transversions. This is probably due to the low proportion of guanosines at the third positions (0-5%) which is typical in reptiles (Harris, 2002). This means tranversions involving guanosines are virtually nonexistent. However, in the 12S and 16S rRNA data sets, different types of tranversions accumulate at an equal rate, which is similar to that of all substitutions in the C-*mos*, like the passerine birds.

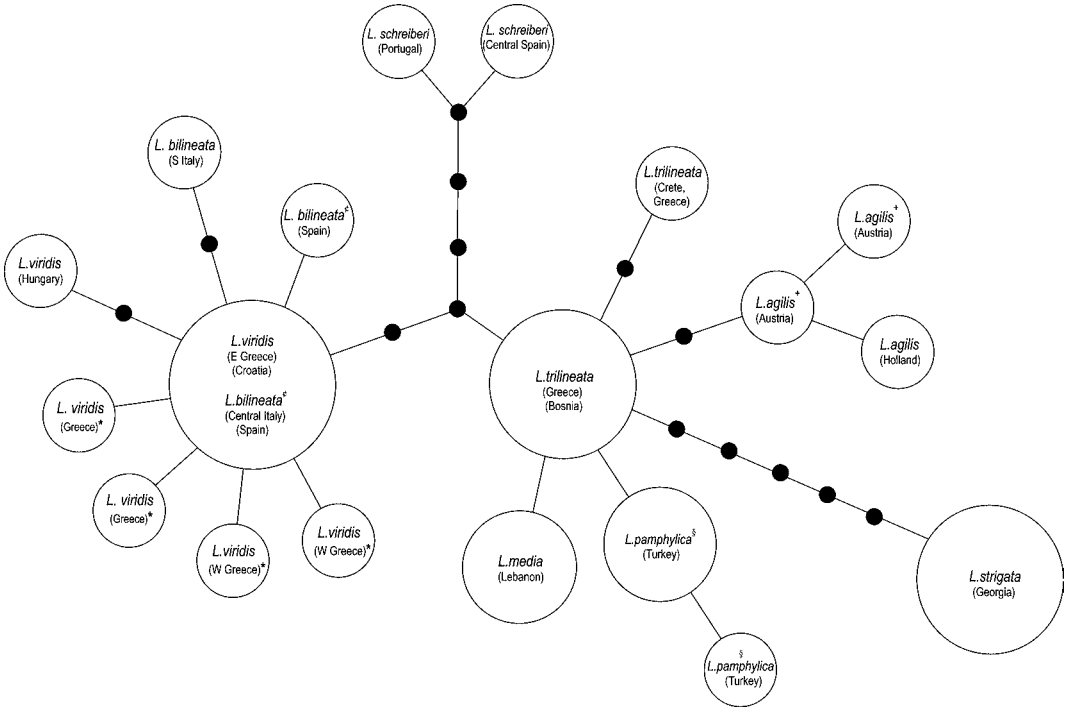

Figure 5. Most parsimonious network of C-*mos* sequences. There were no homoplastic characters. Size of circles is relative to the frequency of haplotypes. Filled circles indicate presumed missing haplotypes. * indicates estimated haplotypes. §, * and + indicate two haplotypes from the same individual.

Discussion

Phylogenetic relationships — mtDNA

As expected estimates of relationships derived from the different mtDNA partial gene regions are very similar, despite the availability of different taxa for the different genes, both support the monophyly of *Lacerta sensu stricto*. *L. agilis*, *L. schreiberi* and *L. strigata* are all strongly supported as distinct genetic units based on the separate mtDNA data sets (bootstrap support between 95-100%) but their relationship relative to the other green lizards is not well supported by the mtDNA sequence data. The species status of *L. strigata* has been questioned by some authors due to its ecological and morphological similarities to *L. viridis* (Schmidtler, 1986b). However, hybridization experiments (Rykena, 1996, 2001) and the high genetic separation based on the DNA sequence data suggest it is a distinct species.

L. bilineata and *L. viridis* are strongly associated as a clade by both mtDNA data sets. Within *L. bilineata*, several subspecies are currently recognized. All of these were sampled, but *L. b. bilineata*, *L. b. chlorosecunda*, *L. b. fejervaryi* and the undetermined subspecies from Central and Northeastern Italy (see Nettmann, 2001) have little or no genetic variation using the cytochrome *b* gene. Only *L. b. chloronota* is distinct (1.6% uncorrected genetic distance from other *L. bilineata* forms). Since these subspecies are difficult to distinguish morphologically, the present data supports the existence of only two, *L. b. chloronota* in Sicily and southwestern Italy and *L. b. bilineata* in all other regions. This pattern is consistent with phylogeographic results obtained by other authors reporting genetically divergent groups of populations in these regions (Pierpaoli et al., 1999; Steinfartz et al., 2000).

Based on 12S and 16S rRNA sequences, one individual of *L. b. chloronota* and one

of *L. v. viridis* are associated with high bootstrap support. Separate examination of these two genes (analysis not shown) indicates that the 16S rRNA sequence for this *L. b. chloronota* is identical to one of *L. viridis viridis*, while for the 12S rRNA gene region alone the positions are equivocal (see also Mayer and Beyerlein, 2001). This sequence could be the result of ancient introgression between *L. viridis* and *L. bilineata*, especially as these species are known to hybridize (Rykena, 1996). Large scale screening of individuals from the two species, possibly using a recently described RFLP technique (Brückner and Düring, 2001), would be needed to test if introgression was widespread.

L. viridis viridis is paraphyletic based on cytochrome *b* sequences, with a large genetic difference between samples (from Greece and Bosnia compared to Hungary and Germany). Genetic variability is high in *L. viridis* — up to 5% uncorrected distances based on the cytochrome *b* sequences, compared to only 1.6% in *L. bilineata*.

Concordant with their overall morphological similarity, *L. trilineata*, *L. media* and *L. pamphylica* as a clade is supported by the 12S and 16S rRNA sequence data. There are some differences in the estimate of relationships between the species derived from the cytochrome *b* compared to the 12S and 16S rRNA data sets, but these are at weakly supported nodes. Of these three species, *L. media* is the sister clade to the other two. Five subspecies of *L. trilineata* were included, of which *L. t. major* is the sister group to *L. t. hansschweizeri*, and *L. t. trilineata* is paraphyletic, as one sequence is more closely related to the subspecies from Crete island, *L. t. polylepidota* (see also Mayer and Beyerlein, 2001). Then *L. t. dobrogica* is associated with *L. pamphylica*. This association is weak but given the low genetic divergence between *L. pamphylica* and *L. trilineata* and its overall morphological similarity its species status could be questioned, in which case it would be referred back to a subspecies of *L. trilineata*. However, using the Shimodaira and Hasegawa (1999) test, the tree where *L. trilineata* is constrained to be monophyletic does not fit our data significantly less well, so this cannot be rejected (SH test using 1000 RELL bootstraps, diff $-\ln L = 2.70$, $P = 0.176$).

Phylogenetic relationships — nuclear DNA sequences

Estimates of relationships derived from C-*mos* are generally similar to those derived from mtDNA. Using a 95% statistical parsimony framework even the closest extant outgroup (*L. lepida*) cannot be used to infer the root of the network of green lizards. However, as with mtDNA, three species — *L. agilis*, *L. strigata* and *L. schreiberi* — are monophyletic units with at least one synapomorphy supporting them as a clade. Analysis of *β-fibint7* also supports these species as distinct genetic lineages, and that the green lizards are a clade relative to *L. lepida* and *I. monticola*. Within *L. schreiberi* there is a consistent existence of two distinct groups in all genes examined which is concordant with that indicated by protein electrophoretic data (Godinho et al., 2003) and mtDNA (Godinho et al., 2001; Paulo et al., 2001). Analysis of both nuclear markers, like mtDNA, suggests a close relationship between *L. bilineata* and *L. viridis*, with the former arising from a paraphyletic *L. viridis* in the analysis of *β-fibint7*, as also seen in the analysis of the mtDNA rRNA genes. For the C-*mos* sequences some *L. viridis* and *L. bilineata* share the same haplotype. An additional group of haplotypes of *L. viridis* for *β-fibint7* are genetically distinct from the remaining *L. bilineata*/*L. viridis* haplotypes. As in the mtDNA analysis estimates of relationships derived from *β-fibint7* infer that *L. trilineata* is paraphyletic relative to *L. pamphylica*. However in the *β-fibint7* analysis *L. trilineata* is also paraphyletic relative to *L. media*.

The short branches separating the species / species groups suggest that green lizards went through a period of rapid diversification. This could have led to incomplete lineage sorting, a possible cause of the incongruencies seen be-

tween the mtDNA and nuclear data sets. Coupled with probable introgression between *L. bilineata* and *L. viridis*, and possibly also within the *L. media/L. pamphylica/L. trilineata* group, it is possible that there is no single estimate of phylogeny for green lizards. This would also explain why there is little consensus of species status or relationships based on morphological characters.

Phylogeographic and evolutionary history of the group

Seven out of the eight species that form the green lizards group are almost restricted to the southern European peninsulas or the Caucasian mountains while a single one, *L. agilis*, occupies nearly all of Europe, including parts of England and southern regions of Scandinavia, but is not present in the most southerly refugia. Kalyabina et al. (2001) produced a phylogeographic analysis of *L. agilis* using mtDNA and suggested that the three subspecies that represent the European genetic lineages of *L. agilis* come from a Balkan-Carpathian refugium, after a radiation from the Caucasian mountains in the Late Pliocene where the species presumably originated (Bischoff, 1988). In our work, this species is represented by samples of Lower Austria and Holland (as well as Germany for cytochrome *b*) that exhibit a low level of differentiation within all five genetic markers used which is congruent with a recent remarkable successful colonization across Central and Western Europe with the exception of the southern regions where most of the diversity of the Green lizards' group is concentrated. Furthermore, this is also in agreement with the low genetic variability of Swedish populations of this lizard in comparison with an Hungarian one (Gullberg et al., 1998, 1999). According to this scenario, the pattern of colonization of the green lizards fits with one of the proposed models of Hewitt (1999, 2000) for the colonization of Europe after the last glacial age, where a single species colonized the central and northern regions of the continent coming from one of the southern refugia. Results from experiments on egg incubation time (Rykena, 1987) suggest that *L. agilis*, *L. viridis* and *L. trilineata* all occur as far north as the climatical conditions for successful incubation allows. However, both *L. schreiberi* and *L. strigata* could have successful egg incubation at much more northerly latitutes than their present ranges. A plausible explanation for this pattern is that their limited ranges are due to historical rather than physiological reasons. Species with larger distribution areas could have a special dispersal ability, allowing faster expansion into available space. This hypothesis is specially supported for *L. agilis* that has wider ecological requirements, in comparison to the others, inhabiting open steppe, hedgerows, woodland, among other habitats, as well as occurring from lowlands up to 2200 m (Korsós and Bischoff, 1997). Additionally, some forms may have been subject to the barrier effect of the Pyrenees and the Alps.

β-fibrinogen intron 7 is a phylogeographically useful marker for *L. bilineata* and *L. viridis* species. The insertion of 380 bp in this locus is a helpful signature for the understanding of the common history of these two species. This insertion is only present in some individuals of *L. viridis* within a well defined geographical area but it was observed in all the *L. bilineata* individuals analysed throughout the distribution range of this species, from southern Italy to Spain (fig. 6). Individuals carrying both haplotypes (with and without the 380 bp insertion) were found in a transect between southern Croatia, western Greece (*L. v. viridis*) and the Aegean Greek islands (*L. v. guntherpetersi*), representing two described subspecies. We believe that this result gives further evidence that *L. bilineata* originated from central and northern population of *L. viridis* and consequently, makes this species younger than the presumed historic event that limited the range of the ancestral green lizards to the southern peninsulas. A possible contact between the two peninsulas could be explained because the Adriatic sea is quite shallow, specially in its northern range

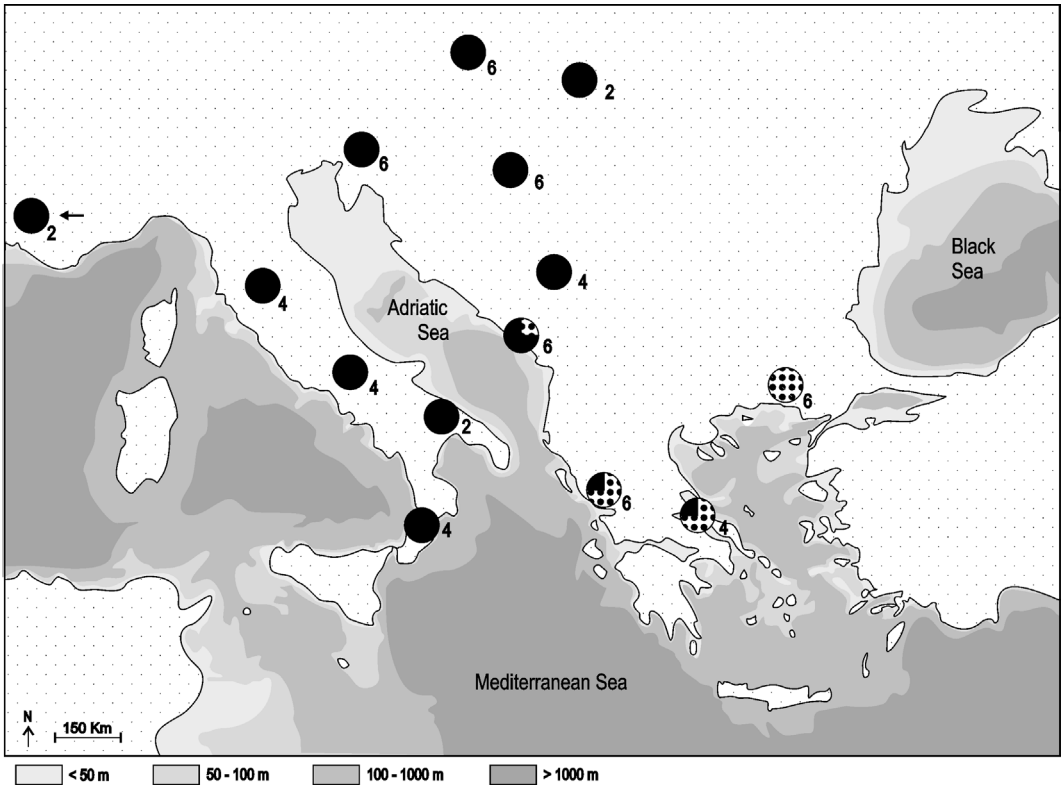

Figure 6. Geographic distribution and frequency of two *β-fibint7* length variants in *L. bilineata* and *L. viridis*. Number of sampled chromosomes in each site is included. Black represents the haplotype with the 380 bp insertion. Dots represent the haplotype without the 380 bp insertion. The black pie on the left with the arrow represents samples from Spain.

(fig. 6), and parts of it have been dry several times over the past three million years when the sea level oscillations reached amplitudes of 130 m (Lambeck et al., 2002) opening windows of opportunity for dispersal. More recently, contraction of populations may have provided separate refugia in the two peninsulas and subsequent divergence forming separate species. Finally, a pattern of well supported intraspecific divergence in combination with a postglacial expansion of a single genetic entity is detected both within *L. bilineata* and *L. viridis*. Concerning the former species, we suggest that *L. b. bilineata* was able to spread northwards to the present day distribution following the climatic warming whereas *L. b. chloronota* may well be restricted to its former glacial refugium (Southwestern Italy and Sicily). The presence of isolated populations of this lizard in Germany is likely a remnant of a warmer period of the present interglacial, when this ectothermic lizard found favourable conditions to spread to the northernmost area of its present range, but further genetic work in these relict populations is needed. Congruent with this scenario is the distribution of *L. v. viridis*, suggesting a successful postglacial expansion while *L. v. guntherspeteri* and *L. v. meridionalis* remained in their Mediterranean refugia. The paraphyly of Balkan haplotypes suggests this region as the origin for the extant lineages of this species. Divergence of *L. viridis* phylogroups probably occurred first, followed by the splitting of *L. viridis* and *L. bilineata*. Other studies are required to verify the degree of genetic distinctiveness of two other described subspecies, *L. v. infrapunctata* and *L. v. paphlagonica*. Similarly, patterns of molecular divergence forming

intraspecific clades were described previously for two other species, *L. schreiberi* (Godinho et al., 2001, 2003; Paulo et al., 2001) and *L. agilis* (Kalyabina et al., 2001).

As well as inferring some of the relationships between presently accepted species of green lizards from our analyses, we can also try to assess whether some of these groups deserve their present taxonomic status. In the case of subspecies within *L. bilineata*, we recommend the acceptance of only two, *L. b. bilineata* across all the range except *L. b. chloronota* in Sicily and Southwestern Italy. Although genetic divergences alone are poor indicators of species status, they can be a source of useful information where there is taxonomic debate based on morphology and ecological criteria. The existence of possible introgression between *L. bilineata* and *L. viridis*, and their known ability to hybridize suggest they are not good biological species. Genetic divergence for the cytochrome *b* gene between these two groups ranges from 4.1% to 5.8%, which is the same level of differentiation found between individuals from different localities of *L. schreiberi*. These numbers can be directly compared to reptiles as a whole (13.6% average uncorrected divergence between species in the same genera, Harris, 2002) and for other lacertids (average 11% divergence, Harris, 2002). Given that adults of *L. bilineata* and *L. viridis* are difficult to distinguish on morphological grounds (Brückner and Düring, 2001), it is not unreasonable to refer them as single species, as some authors do (e.g. Arnold, 2002).

Concluding remarks

Partial sequences of the C-*mos* gene have typically been used to investigate deeper taxonomic level than within genera (e.g. Harris et al., 1999). However, it has been used within *Mabuya* (Brehm et al., 2001) and *Tarentola* (Carranza et al., 2002) and is here informative for the green lizards, even showing variation within species. Here we show that *β-fibint7* is also a useful nuclear marker for assessing relationships within lizards, especially at shallow taxonomic levels where C-*mos* variation is low. Finally we show how an approach including mtDNA and nuclear markers can show a much more complex network of relationships between species than mtDNA alone.

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