

A re-analysis of the molecular phylogeny of Lacertidae with currently available data

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The Lacertidae is one of the most diverse and widespread lizard families throughout Eurasia and Africa. Several studies so far have attempted to unravel the phylogeny of Lacertidae using morphological and molecular data. However, the intra-family relationships remain unclear. In an effort to explore the phylogenetic relationships within the family Lacertidae, a concatenated dataset of 5727 bp from six genes (two nuclear and four mitochondrial) and 40 genera was assembled based on GenBank database. Phylogenetic inference analyses were conducted using Maximum Parsimony (MP), Bayesian inference (BI) and Maximum Likelihood (ML), revealing that even a combined dataset of both mitochondrial and nuclear genes is not able to resolve the phylogenetic relationships of the Lacertidae family under the tribe level.

Key words: GenBank; Lacertidae; phylogeny.

Reanálisis de la filogenia molecular de los Lacertidae usando los datos disponibles en la actualidad. La familia Lacertidae es una de las más diversas y ampliamente distribuidas en Eurasia y África. Varios estudios han intentado hasta ahora aclarar la filogenia de los Lacertidae usando datos morfológicos y moleculares. Sin embargo, las relaciones dentro del grupo permanecen poco claras. En un esfuerzo por explorar las relaciones filogenéticas dentro de la familia Lacertidae, se analizó una base de datos de 5727 pares de bases para 40 géneros diferentes de lacértidos obtenidos por la concatenación de seis genes (dos nucleares y cuatro mitocondriales), todos ellos disponibles en la base de datos de GenBank. Los análisis filogenéticos realizados usando métodos de máxima parsimonia (MP), inferencia bayesiana (BI) y máxima verosimilitud (ML), revelaron que el conjunto combinado de genes mitocondriales y nucleares utilizados no es capaz de resolver las relaciones filogenéticas de los lacértidos a un nivel taxonómico inferior al de tribu.

Key words: filogenia; GenBank; Lacertidae.

Lacertidae is a family of small body sized lizards distributed throughout Eurasia and Africa. In recent years this family has been the subject of several taxonomical studies, considering both molecular and morphological characters. ARNOLD (1989), based on morphological characters, constructed the phylogeny of this family and proposed the division of the Lacertidae into two subgroups, the “Primitive

Palaearctic and Oriental Lacertids” and the “Ethiopian and Advanced Saharo-Eurasian forms”. A series of studies (LUTZ & MAYER, 1984, 1985; MAYER & BENYR, 1995) based on albumin-immunology, resulted in the establishment of two subfamilies, Gallotiinae, which includes two genera, *Gallotia* and *Psammodromus*, and Lacertinae including the rest of the Lacertidae family. HARRIS *et al.* (1998) and FU

(1998, 2000) used mitochondrial DNA sequences to explore the relationships of lacertid lizards. However, in all cases, the datasets were insufficient to reconstruct the intra-family phylogeny of Lacertidae. In the same study, HARRIS *et al.* (1998) combined morphological characters along with molecular data and led to the division of the family into three subfamilies: 1) Gallotiinae, 2) Lacertinae and 3) Eremiainae. The first two correspond to ARNOLD's (1989) "Primitive Palearctic and Oriental Lacertids", while Eremiainae is equivalent to the "Ethiopian and Advanced Saharo-Eurasian forms" and to ARNOLD's (1973) "armatured" clade. A recent work of MAYER & PAVLICEV (2007) confirmed the division in subfamilies proposed by HARRIS *et al.* (1998) and indicated the division of the subfamily Eremiainae into two clades, "Ethiopian" and "Saharo-Eurasian". ARNOLD *et al.* (2007) downgraded Lacertinae and Eremiainae into tribes (i.e. Lacertini and Eremiadini, respectively) of the subfamily Lacertinae. PAVLICEV & MAYER (2009) argued that the use of the tribe as a taxonomic entity causes confusion while they concluded that the polytomy of Lacertini is more likely to be attributed to multiple cladogenesis in a geologically short time than to the poor resolution of the markers used. Despite PAVLICEV & MAYER (2009) opinion, in the current study we follow the systematics proposed by ARNOLD *et al.* (2007) since it is more descriptive of the phylogeny of the family.

The aim of this study was to reassess the phylogeny of the family using all currently available data from GenBank. Primarily the data used here were produced by the molecular studies mentioned before and concluded in a dataset of four mitochondrial and two nuclear genes from 40 genera.

MATERIALS AND METHODS

Published sequences were retrieved from GenBank (four mitochondrial genes: 16S rRNA, 12S rRNA, cyt b, and COI, and two nuclear genes: c-mos and RAG-1). We built a concatenated dataset in which each genus is represented by one chimerical sequence of the six genes (all accession numbers are given in Table 1).

All genes were identified and the corresponding sequences were saved to individual FASTA-formatted files for each gene. The poorly aligned positions for the genes 12S rRNA and 16S rRNA were removed using the online version of Gblocks (V. 0.91b, CASTRESANA, 2000) under the less stringent options (412 bp out of 1455 bp and 168 bp out of 1010 bp were removed for 16S rRNA and 12S rRNA, respectively). The final dataset was comprised of 5727 bp for 40 Lacertidae genera. Representative sequences from the genus *Eumeces* (chimerical sequence out of the species *E. anthracinus*, *E. ergegius* and *E. inexpectatus*) were added to each data set as outgroup.

Phylogenetic analyses

The Bayesian Information Criterion (BIC) as implemented by jModeltest (v.0.1.1; POSADA, 2008), was used to choose the best-fit model of DNA substitution. The best fit models (among 88 available) for 12S rRNA, 16S rRNA, COI, cyt b, rag1 and c-mos were: TPM1uf +I +G, TPM2uf +G, TIM2 +I +G, TPM2uf +G, Trn +I +G and K80 +G, respectively. For the BI analysis in the cases where the model selected by jModeltest could not be implemented the closest more complica-

Table 1: List of sequences used in the analyses. Genera name and GenBank accession numbers for each gene included in the analyses are provided (12S, 16S, cyt b, CO1, rag1 and c-mos). Sequences that were not available in GenBank are indicated as n/a.

Genera	GenBank accession numbers					
	12S	16s	Cyt b	CO1	c-mos	Rag1
<i>Gallotia</i>	AF206587	AF206587	AF101224	AF206562	EF632260	EF632215
<i>Psammodromus</i>	AF206588	AF206588	AF206535	AF206567	EF632284	n/a
<i>Acanthodactylus</i>	AF206607	AF206607	AF206536	AF206568	EF632252	EF632207
<i>Adolfus</i>	AF206615	AF206615	AF206539	AF206578	EF632253	EF632208
<i>Algyroides</i>	AF206598	AF206598	AF206529	AF206557	EF632255	EF632210
<i>Anatololacerta</i>	AJ238188	GQ142107	DQ461765	n/a	DQ461743	EF632224
<i>Dalmatolacerta</i>	AF440601	AF440616	AY278199	n/a	EF632271	EF632228
<i>Darevskia</i>	AF206597	AF206193	U88611.3	AF206552	EF632257	EF632212
<i>Dinarolacerta</i>	AF440600	AF440615	GQ142141	n/a	EF632270	EF632227
<i>Eremias</i>	AF206604	AF206604	AF206549	AF206576	EF632259	EF632214
<i>Heliobolus</i>	AF206608	AF206608	AF206544	AF206583	EF632262	EF632217
<i>Hellenolacerta</i>	AF440602	AF440617	GQ142128	n/a	EF632269	EF632225
<i>Iberolacerta</i>	AF440598	AF440612	AY267242	AF206571	EF632264	EF632219
<i>Latastia</i>	AF206609	AF206609	AF206545	AF206563	EF632272	EF632229
<i>Meroles</i>	AF206610	AF206611	AF206540	AF206581	EF632273	EF632230
<i>Mesalina</i>	AY035832	AF206606	FJ416173	AF206580	EF632274	EF632232
<i>Nucras</i>	AF206612	AF206612	AF206550	AF206565	EF632276	EF632233
<i>Atlantolacerta</i>	AF206603	AF149945	AF206537	AF206579	GQ142144	GQ142154
<i>Ophisops</i>	AF206605	AF206605	AF206532	AF206556	EF632278	EF632235
<i>Parvilacerta</i>	AJ238187	GQ142106	GQ142135	n/a	EF632279	EF632236
<i>Pedioplanis</i>	AF206613	AF206613	AF206546	AF206566	EF632280	EF632237
<i>Poromera</i>	AF080368	AF080370	AF080369	n/a	EF632283	EF632240
<i>Tropidosauria</i>	AF206616	AF206616	AF206541	AF206582	EF632291	EF632248
<i>Ichnotropis</i>	AF080365	DQ871149	AF080366	n/a	EF632266	EF632221
<i>Phoenicolacerta</i>	NC_011606	NC_011606	DQ461762	NC_011606	DQ461740	EF632226
<i>Takydromus</i>	AB080237	AB080237	AB080237	AF206558	EF632288	EF632245
<i>Teira</i>	AJ004884	GQ142096	GQ142121	AF372052	EF632289	EF632246
<i>Timon</i>	AF206595	AF206595	DQ902142	AF206569	EF632290	EF632247
<i>Zootoca</i>	AF206594	AF206594	AY714929	AF206554	EF632292	EF632249
<i>Apathya</i>	AF145444	AF149946	GQ142127	unpublished	EF632268	EF632223
<i>Scelarcis</i>	AF206602	AF206602	AF206538	AF206570	GQ142145	GQ142155
<i>Omanosauria</i>	AF080347	AF080352	AF080351	n/a	EF632277	EF632234
<i>Lacerta</i>	AM176577	AM176577	AM176577	AF206551	EF632267	EF632222
<i>Podarcis</i>	AF206601	AF206601	AY234154	AF206575	EF632282	EF632239
<i>Australolacerta</i>	FR751396	FR751396	FR751398	n/a	n/a	DQ871208
<i>Holaspis</i>	n/a	n/a	n/a	n/a	EF632263	EF632218
<i>Iranolacerta</i>	GQ142088	GQ142111	GQ142140	n/a	GQ142152	GQ142162
<i>Philochortus</i>	n/a	n/a	n/a	n/a	EF632281	EF632238
<i>Pseudemias</i>	n/a	n/a	n/a	n/a	EF632286	EF632243
<i>Archaeolacerta</i>	AF206592	AF206592	GQ142126	n/a	EF632256	EF632211
<i>Scincidae</i>	NC_000888	NC_000888	NC_000888	NC_000888	AY217888	AY662634

ted model was used (RONQUIST & HUELSENBECK, 2003).

Phylogenetic inference analyses were conducted using Bayesian Inference (BI), Maximum Parsimony (MP) and Maximum Likelihood (ML). Nucleotides were used as discrete, unordered characters. BI analysis was performed in MrBayes (v3.1; RONQUIST & HUELSENBECK, 2003), with partitioned dataset by genes, using the models discussed above. The analysis was run four times with eight chains for 10^7 generations and the current tree was saved to file every 10^2 generations. This generated an output of 10^5 trees for every run. The $-\ln L$ stabilized after approximately 10^4 generations, thus the first 10^4 trees (10% “burn-in” in Bayesian terms) of every run were discarded as a conservative measure to avoid the possibility of including random, suboptimal trees. The percentage of samples recovering any particular clade in a BI analysis represents that clade’s posterior probability (HUELSENBECK & RONQUIST, 2001). A majority rule consensus tree (‘Bayesian’ tree) was then calculated from the posterior distribution of trees, and the posterior probabilities calculated as the percentage of samples recovering any particular clade (HUELSENBECK & RONQUIST, 2001), where probabilities $\geq 95\%$ indicate significant support.

MP analysis was performed with PAUP* v.4.0b10 (SWOFFORD, 2002). This analysis was carried out (heuristic searches) using stepwise addition and performing tree bisection-reconnection (TBR) branch swapping (SWOFFORD *et al.*, 1996). Confidence in the nodes of MP trees was assessed by 1000 bootstrap replicates (FELSENSTEIN, 1985). The analysis was run twice with the gap considered as missing and as a fifth character.

Finally ML analysis was performed in the online version of RaxML (STAMATAKIS *et al.*, 2008) using a mixed partitioned model and the following parameters: α -shape parameters, GTR-rates, and base frequencies estimated and optimized for each partition (gen). Furthermore, gamma model of rate heterogeneity was assumed for all partitions while the invariable sites were estimated by the analyses. Confidence in the nodes of ML trees was assessed by 100 bootstrap replicates.

Soft vs. hard molecular polytomies

Unresolved evolutionary relationships are considered soft polytomies in that they are multiple dichotomous branching events occurring in rapid succession. To differentiate between poorly supported clades (soft polytomies) vs. zero-length branches (hard polytomies), we used the likelihood ratio test [$-2(\ln L_{\text{Ha}} - \ln L_{\text{Ho}})$], proposed by SLOWINSKI (2001), where L_{Ha} is the likelihood under the alternate hypothesis (the length of branch in question is nonnegative) and L_{Ho} is the likelihood under the null hypothesis (branch has zero-length). Using the ‘describe trees’ command following our ML run (with ‘Perform likelihood-ratio test for zero branch lengths’ selected in the likelihood settings menu), PAUP* calculated the probability for each likelihood ratio under the χ^2 distribution with one degree of freedom. Significance for the likelihood ratio test for each branch in the phylogeny was determined using the percentage point values under the GOLDMAN & WHELAN (2000) mixed model. We used $\alpha = 0.05$ as significance level to account for possible Type I error.

RESULTS

Of the 5727 sites examined, 2557 were variable, 1860 of which were parsimony informative (2690 and 1945 respectively, when the outgroup was included). The MP analysis when the gap was considered as missing produced one tree with a length of 13 941 steps, while three equally parsimonious trees with a length of 14 361 steps were produced when the gap was treated as a fifth character. The topologies recovered were the same for most of the clades (differences are discussed below).

BI resulted in the topology shown in Fig.1 (identical topologies were recovered for each of the four runs). All analyses recognized the division of the family in two main clades (Gallotiinae and Lacertinae) and the division of the later in two subclades (Lacertini and Eremiadini) with strong statistical support. In the case of Lacertini no analyses managed to resolve the relationships among the 20 genera under study. One monophyletic group within Lacertini was recognized by all analyses (*Teira* and *Scelarcis*) while *Algyroides* and *Dinarolacerta* formed a monophyletic clade for BI and ML, whereas for MP only when the gap was considered as a fifth character. ML supported a sister relationship of the later with *Iberolacerta* (62% bootstrap support) while for MP when the gap was treated as a fifth character another monophyletic group (*Lacerta* and *Timon*) was supported (51% bootstrap support). In the case of Eremiadini, MP (either when the gap was treated as missing or as a fifth character) could not resolve adequately the relationships of the taxa under study. On the contrary BI and ML resulted in the same topology with the exception that ML supported a basal position of *Pedioplanis*

for the clade including *Meroles*, *Ichnotropis*, *Tropidosaura* and *Australolacerta*. Finally the unresolved relationships of the family, according to the likelihood ratio test (SLOWINSKI, 2001), could be considered as soft polytomies with the exception of the branch length separating *Poromera* ($\alpha > 0.05$, Fig. 1).

DISCUSSION

The genera comprised in the Eremiadini tribe (ARNOLD *et al.*, 2007) form a subclade within Lacertinae in all analyses. This clade showed significant internal structure into

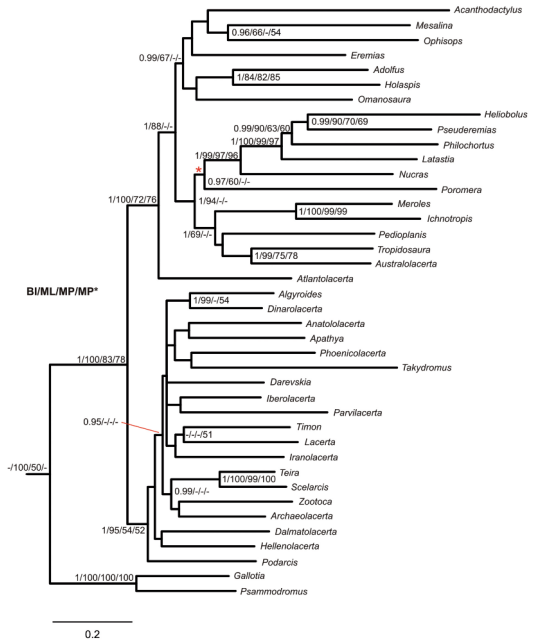


Figure 1: Phylogenetic relationships among the 40 genera included in the analyses as inferred by BI. Bayesian posterior probability values (> 0.95) are presented on the nodes followed by bootstrap values (> 50%) for ML, MP and MP* (considering the gap as a fifth character). Branch lengths statistically not significantly greater than zero are indicated with an asterisk (*). *Eumeces anthracinus*, *E. ergegius* and *E. inexpectatus* (Scincidae) were used as outgroup (not shown).

three groups, two of which correspond to the division of MAYER & PAVLICEV's (2007) 'Ethiopian' and 'Saharo-Eurasian', while *Atlantolacerta* appears as basal in accordance with ARNOLD *et al.* (2007). Surprisingly the Ethiopian group, which includes most of the least studied genera of Lacertidae (SALVI *et al.*, 2011), appeared as the best resolved clade of the family. It is worth noticing that the sister relationship of *Australolacerta* and *Tropidosaura* proposed recently by SALVI *et al.* (2011) is also confirmed by this study.

The genera that belong to the Lacertini tribe appear as a monophyletic clade in all analyses with strong statistical support, although relationships within the clade have low resolution. It is interesting to note that two monophyletic clades were recognized by all analyses, *Scelarcis* with *Teira* and *Dinarolacerta* with *Algyroides* (Fig. 1). For the first case PAVLICEV & MAYER (2009) propose that these two genera should be united in one, while the case of *Dinarolacerta* with *Algyroides* remains puzzling until further data for the taxa will be available. The problem of reconstructing the phylogenetic relationships within Lacertini arose in all previous studies (HARRIS *et al.*, 1998; FU, 2000; ARNOLD *et al.*, 2007; MAYER & PAVLICEV, 2007, PAVLICEV & MAYER, 2009) with different datasets. Here we show that neither a dataset of 5727 bp (two nuclear and four mitochondrial genes) with a wide genera sampling is able to shed light in the relationships between Lacertini. According to the most recent molecular phylogeny of the taxon (PAVLICEV & MAYER, 2009) it was assumed that the poor resolution was more likely to reflect a rapid radiation resulting in a polytomy than considering the markers

used inappropriate. The results of the likelihood ratio test, conducted for the current dataset, suggest that the unresolved relationships of both Ereimiadini and Lacertini are more likely to be seen as a case of a soft polytomy. Hence before the rejection or acceptance of PAVLICEV & MAYER's (2009) view, two aspects could be further researched: 1) taxa sampling and 2) quantity/quality of genetic markers. As far as the first is concerned, even though Lacertidae has been the subject of several studies, the intra genera variation in most of the cases remains unexplored. Taking this under consideration it could be assumed that the unresolved relationships of the family could be attributed to inadequate data sampling. For the near future, analyses with more species per genus could show improvement in case we are facing a 'soft' and not a 'hard' polytomy of Lacertini. Furthermore it should be taken into account that if we assume 'hard' polytomy for Lacertini the same hypothesis should be extended, to some degree, to Ereimiadini since the relationships among some of its genera remain unresolved.

Regarding the amount and the combination of the particular genetic markers there should not be a problem of too much or too little variation, as stated before (PAVLICEV & MAYER, 2009). Nevertheless the combination of multiple genes phylogeny (i.e. complete mtDNA genome) or the application of restriction site-associated DNA tags (RAD tags, BAIRD *et al.*, 2008, EMERSON *et al.*, 2010) could be interesting approaches that could provide much more detailed and extensive information. Also the investigation for RGCs (Rare Genomic Changes) that have become increasingly important in systematics and

complement phylogenetic analyses of primary sequence data, as noted by SPRINGER *et al.* (2004), could ultimately provide the most convincing resolution of intra-Eremiadini and -Lacertini phylogeny.

Summarizing the above we can assume that analyses based on the current available data are able to resolve the phylogenetic relationships on the level of subfamily and tribe. However, resolution of the relationships below the tribe level in Eremiadini and Lacertini necessitates a more sophisticated analysis and better knowledge on the intra genera variation.

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