Lacerta kulzeri – ITS PHYLOGENETIC RELATIONSHIPS AS INDICATED BY DNA SEQUENCES

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In order to resolve the relationships of *Lacerta kulzeri*, parts of mitochondrial genes for 12S rRNA and 16S rRNA of samples of the following species were sequenced: *Gallotia stehlini* (as an outgroup), *Lacerta trilineata, L. cappadocica, L. fraasii, L. danfordi, L. rudis, Podarcis peloponnesiaca*, as well as representatives of different populations of *L. laevis* and *L. kulzeri*. Sequence analysis clearly shows a close relation between *L. laevis* and *L. kulzeri* and no close relation of these species to any other representative of the lizards examined. The other species also show themselves to be representatives of very distinct lacertid groups. The resulting sequence differences within *L. kulzeri* and *L. laevis*, respectively, are very high in relation to those between the two species, and they are higher than one would expect for species with small areas.

Key words: Lacertidae, *Lacerta kulzeri, Lacerta laevis*, phylogenetics, mitochondrial DNA, sequencing.

Beyerlein, P. & Mayer, W.: *Lacerta kulzeri* – **filogenetički odnosi indicirani DNA sekvencijama. Nat. Croat., Vol. 8, No. 3., 181–187, 1999, Zagreb.**

Da bi se razriješili odnosi *Lacerta kulzeri*, sekvencionirani su dijelovi mitohondrijskih gena za 12S rRNA i 16S rRNA uzoraka sljedećih vrsta: *Gallotia stehlini* (kao *outgroup*), *Lacerta trilineata, L. cappadocica, L. fraasii, L. danfordi, L. rudis, Podarcis peloponnesiaca*, kao i predstavnici različitih populacija *L. laevis* i *L. kulzeri*. Analiza sekvenci jasno pokazuje blisku vezu *L. laevis* i *L. kulzeri*, a nikakvu blisku vezu tih vrsta s ostalim ispitanim vrstama guštera. Ostale vrste također su se pokazale kao predstavnici vrlo različitih lacertidnih grupa. Konačne razlike u sekvencijama unutar *L. kulzeri*, odnosno, *L. laevis* su vrlo visoke u odnosu na one između dviju vrsta, i više su nego što bi se očekivalo za vrste s malim arealima.

Ključne riječi: Lacertidae, *Lacerta kulzeri, Lacerta laevis*, filogenetika, mitohondrijalna DNA, sekvencioniranje.

INTRODUCTION

The phylogeny of the Lacertidae have been the subject of much discussion. Even the relationships within the collective genus *Lacerta* are still unclear. From the sys-

tematic point of view, the »typical members of *Lacerta* part II« (sensu ARNOLD, 1973), are the most difficult group. As shown by albumin-immunology its different branches are extremely widely separated and often consist of an extremely low number of species (MAYER & BENYR, 1994). Particularly, the Anatolian – Near East species like the *L. danfordi* group, *L. laevis* and *L. kulzeri*, referred as »Archaeolacertas« by ARNOLD 1989, as well as *L. cappadocica* and the *L. parva* group are hard to categorize. The external features of *L. laevis, L. kulzeri,* and *L. danfordi,* as well as to some extent *L. cappadocica* are very similar and they show few derived features. Therefore they are often misinterpreted and confused.

Lacerta laevis has for a long time been considered to be closely related to *L. danfordi*. On the other hand, that species has been confused with *L. cappadocica* by many authors to date (e.g. CLARK & CLARK, 1973; BAŞOĞLU & BARAN, 1977**).**

Lacerta kulzeri has been regarded as a subspecies of *danfordi* by MÜLLER & WETTSTEIN (1933). EISELT & SCHMIDTLER (1986) consider it to be closer related to *L. laevis* than to *L. danfordi* and have thus decided *L. kulzeri* it is a subspecies of *L. laevis*. BISCHOFF & SCHMIDTLER (1994) found both, *laevis* and *kulzeri*, sympatric near Bloudan (Syria), proving them to be different species, which, however, seem to be closely related.

To elucidate the phylogenetic relationships between *L. laevis* and *L. kulzeri* and between these two species and other representatives of the »Archaeolacertas« we investigated mitochondrial DNA features.

MATERIALS AND METHODS

Parts of the mitochondrial 12S and 16S rRNA genes of the following species were sequenced:

Lacerta kulzeri from Ma'alula, Antilebanon Mts., Syria; *Lacerta kulzeri*, from Djebel Barouk, southern Lebanon Mts., Lebanon; *Lacerta l. laevis*, from Djebel Barouk, southern Lebanon Mts., Lebanon, *Lacerta l. laevis* from Yarpuz, vil. Adana, Turkey; *Lacerta laevis troodica* from western Cyprus, *Lacerta danfordi* from Yarpuz, vil. Adana, Turkey; *Lacerta cappadocica wolteri* from Al Barah, Syria; *Lacerta fraasii* from Bcharré (terra typica), Lebanon Mountains, Lebanon; *Lacerta rudis bischoffi* from Georgia, *Lacerta horvathi* from Carinthia, Austria, *Podarcis peloponnesiaca lais* from Feneos, pref. Corinthia, Greece; *Lacerta trilineata* from pref. Evros, Greece; *Gallotia stehlini* from Gran Canaria, Canary Islands, Spain (as an outgroup according to MAYER & BENYR, 1994 and FU, 1998).

The DNA was purified from blood and tissue samples according to the phenol-chloroform standard protocol. The 12S rRNA sequences were amplified with the primers L1091 (5'-AAACTGGGATTAGATACCCCACTAT–3') and H1557 (5'-GTA-CACTTACCTTGTTACGACTT–3') (KNIGHT & MINDELL, 1993), the 16S rRNA sequences with the primers L2190 (5'-GTGGGCCTAAAAGCAGCCAC–3') and H3056 (5'-CCGGTCTGAACTCAGATCACG–3'), modified after REEDER (1995). The 12S fragment was about 460 bp and the 16S fragment about 900 bp long. The PCR

products were separated in agarose, cut out from the gel, purified by QIAquick-spin columns (Qiagen) and reamplified. Cycle-sequencing was done with biotin-marked didesoxynucleotides using the nested primers LE1318 (5'-ACGTCAGGT-CAAGGTGTAGC–3') (TITUS & FROST, 1996), H1478 (5'-AGGGATGACGGGCGGTG-TGT–3') (modified after KOCHER *et al.*, 1989), and HE1298 (complementary to LE1318) for the 12S fragment, and L2510 (5'-CGCCTGTTTACCAAAAACAT–3') (modified after KNIGHT & MINDELL, 1993) and L2738 (5'-CGAGAAGACCCTATGGAGCTT–3') (KNIGHT & MINDELL, 1993) for the 16S fragment, respectively. Subsequently, the sequences were blotted on a GATC direct blotting apparatus and read by eye.

We were able to read about 460 bp of the 12S and about 410 bp of the 16S fragment for most investigated samples. Unfortunately, it was not possible to read the 16S sequences of both samples of *L. kulzeri*, due obviously to sequence interference. Therefore we decided to clone the relevant fragment of *L. kulzeri* from Dj. Barouk.
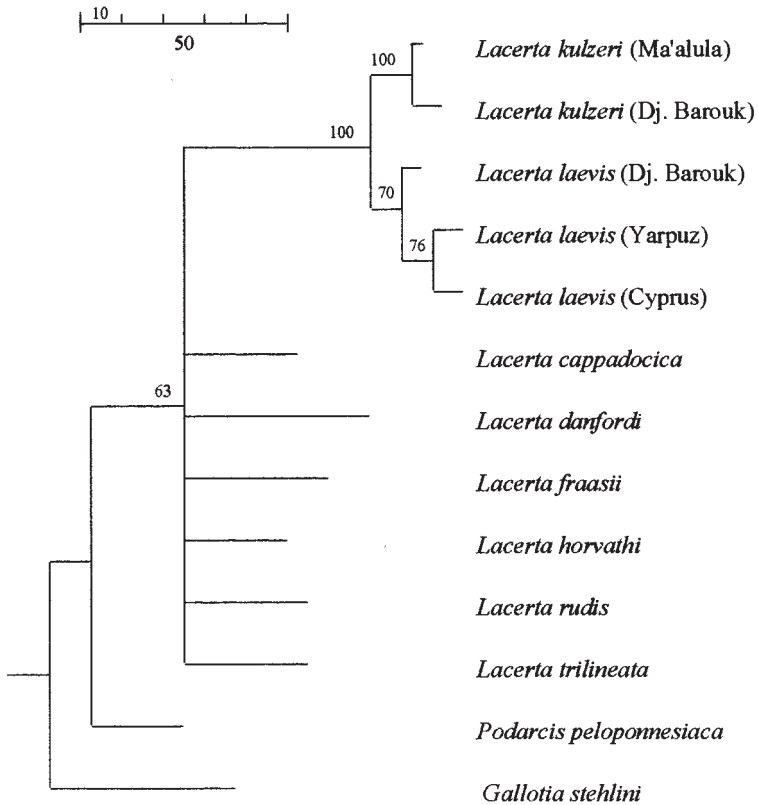

Fig. 1. Fifty percent bootstrap consensus tree (1000 replicates) of phylogenies derived from Maximum Parsimony analyses using the 12S rRNA gene region. Numbers above branches indicate bootstrap support. Numbers above and below scale indicate numbers of substitutions in the most parsimonious tree.

Cloning was performed with the TOPO TA cloning kit (INVITROGENE) according to the manufacturer's instructions and sequencing of five clones was carried out.

The alignments were performed by Clustal X (THOMPSON *et al.*, 1997) software and corrected by eye. Dendrograms were calculated by the PAUP 4.0 (SWOFFORD, 1997) program package, for the two segments, each separately and for all 870 bp together.

RESULTS

While we obtained 12S rRNA sequences for all our taxa without any difficulty, sequencing of the 16S fragment of both the *L. kulzeri* samples did not provide any satisfactory result because of sequence interference. After cloning the 16S part of *L. kulzeri* from Dj. Barouk we obtained different results for all five clones sequenced
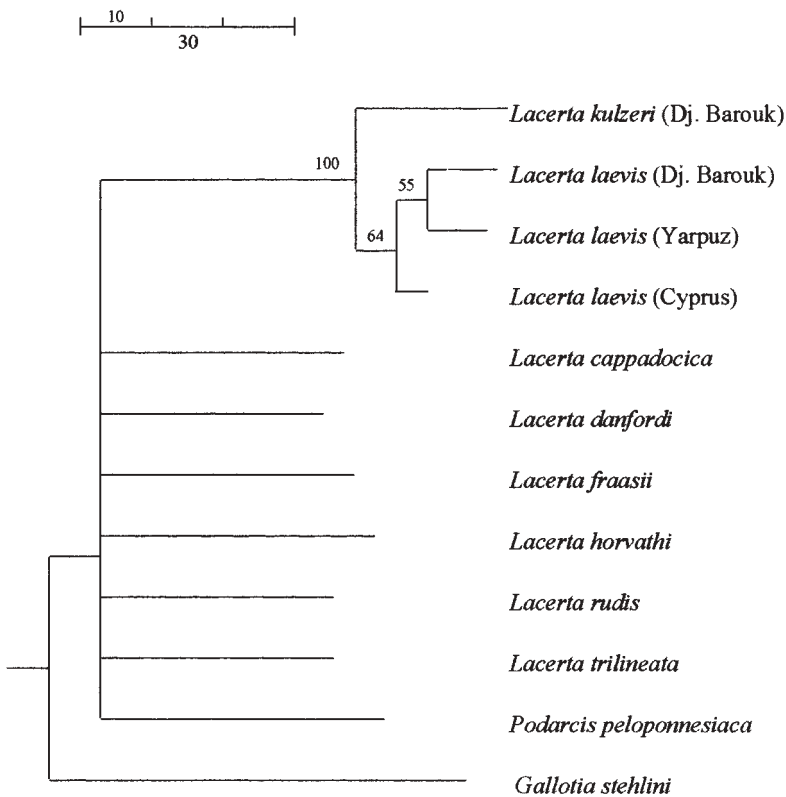

Fig. 2. Fifty percent bootstrap consensus tree (1000 replicates) of phylogenies derived from Maximum Parsimony analyses using the 16S rRNA gene region. Numbers above branches indicate bootstrap support. Numbers above and below scale indicate numbers of substitutions in the most parsimonious tree.

(0.2 – 6.1% sequence difference). Nevertheless, all these sequences form a cluster which is clearly distinct from *L. laevis*. Since we did not have any 16S sequences from *L. kulzeri* from Ma'alula we took one of those sequences generated via cloning for the Fig. 2 (16S tree) and Fig. 3 (12S/16S combined tree) dendrograms. The MP analysis of the 12S+16S data only result in one single very parsimonious tree with a length of 723 steps, the number of parsimony-informative characters is 199. As bootstrap analyses only show weak support for many nodes we only present 50% bootstrap consensus trees in the figures. All our calculated dendrograms show *L. kulzeri* and *L. laevis* to belong close together (bootstrap support of 100%), with few relations to all other taxa investigated. In most cases all other taxa show no closer interrelationships (bootstrap support below 50%).

All sequences are deposited at EMBL with the numbers AF145444, AF149935-AF149946, and AJ238177-AJ238188.
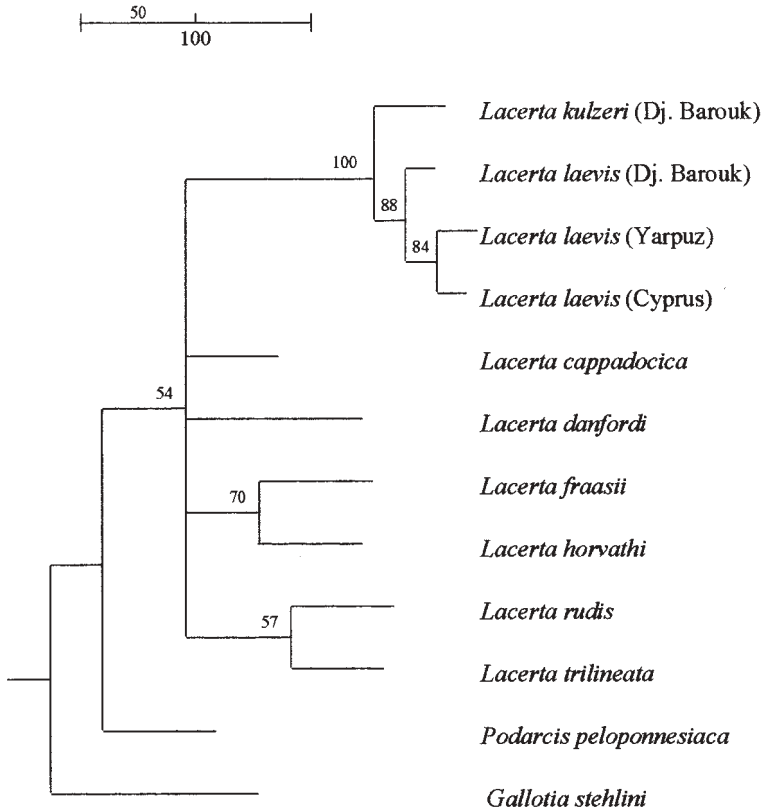

Fig. 3. Fifty percent bootstrap consensus tree (1000 replicates) of phylogenies derived from Maximum Parsimony analyses using both 12S and 16S rRNA gene regions combined. Numbers above branches indicate bootstrap support. Numbers above and below scale indicate numbers of substitutions in the most parsimonious tree.

DISCUSSION

We cannot provide any certain interpretation of there being five different 16S clone sequences of one investigated *L. kulzeri* specimen. The concept of multiple nuclear copies (»numts«) seems to be the most probable interpretation of this phenomenon. As also our Ma'alula 16S sequence shows sequence interference, »numts« could have occurred very early in the species' radiation. So we think that the most basal clone represents the true mitochondrial sequence and therefore selected this sequence for our calculations. As all the clones form one cluster, being a sister group to our *L. laevis* sequences, and as a *L. kulzeri* sequence published by other authors (GenBank no. AF112296) lies within this cluster, we can exclude the possibility of the clones being the result of a contamination. So also the use of one of the other sequences would not change the tree's topology. *Lacerta laevis* and *L. kulzeri* are clearly separated from each other (bootstrap support of 64–88%), and both species' samples from the sympatric distribution at Dj. Barouk show no closer connection with each other. If one considers the different ecological preferences (BISCHOFF & SCHMIDTLER, 1994) both taxa prove themselves to be distinct species.

The large intraspecific differences within *L. kulzeri* and *L. laevis*, respectively, are remarkable (sequence differences between 3 and 5%). These distances are larger than we had expected for species with small areas. Particularly, in *L. laevis* this phenomenon is very surprising, considering that up to now only one subspecies has been described for this long-known lizard – and even this Cyprian *L. laevis troodica* is doubted nowadays (BÖHME & WIEDL, 1994).

It is remarkable that in the combined tree we found some groups with relatively high bootstrap support (see Fig. 3). One would think that a bootstrap value of 70, like that between *L. fraasii* and *L. horvathi*, proves there is a close relationship between them. But as the same taxa are also investigated in the HARRIS *et al*. (1998) study and as there are only minor differences between their sequences and our it is very surprising that we find these clusters quoted in the HARRIS paper. We believe that the alignment is the decisive factor for this problem, and that, depending on the alignment, different results may be generated. Thus, we doubt these clusters and we think that based on sequences of only about 1000 bp of mitochondrial genes a decision on the phylogeny of the collective genus *Lacerta* is hardly possible.

Therefore, we cannot take any decision about the position of the *L. laevis/kulzeri* complex with respect to one of the other lacertid lizards – but it obviously is not very closely related to any of the other species investigated, not even to the genus *Podarcis*, as has been supposed according to albumin-immunological results (MAYER & BENYR, 1994).

Branches between nodes are very insecure and often short and nodes are weakly supported. Therefore, our tree collapses into a »bush« (see the corresponding results of FU, 1998 and HARRIS *et al*., 1998 as well as the intensive discussion of this phenomenon by FU, 1998).

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