Accepted on 10 November 2008
J Zool Syst Evol Res doi: 10.1111/j.1439-0469.2008.00515.x

[1]*Molecular Systematics, 1st Zoological Department, Museum of Natural History Vienna, Vienna, Austria;* [2]*Department of Zoology, Croatian Natural History Museum, Zagreb, Croatia*

Complete mitochondrial genomes of three lizard species and the systematic position of the Lacertidae (Squamata)*

M. Podnar[1,2], W. Pinsker[1] and W. Mayer[1]

Abstract

Complete mitochondrial (mt) genomes were sequenced from representatives of three lacertid lizards: *Podarcis siculus*, *Podarcis muralis* and *Phoenicolacerta kulzeri*. In all three genomes the arrangement of the 22 tRNAs, the two *rRNA*s and the 13 protein-coding genes conforms to the common vertebrate arrangement. The phylogenetic position of Lacertidae within the order Squamata was determined through sequence analyses based on large sections of complete mt genomes. The number of nucleotide sites used for tree construction was 9234 when outgroup taxa were included, and 10 499 when only Squamata were compared. The phylogenetic analyses confirmed the sister group relationship between Lacertidae and Amphisbaenia as previously proposed on the basis of molecular data. Additionally, Bayesian analysis revealed a well supported clade comprising (Gekkonidae (Lacertidae + Amphisbaenia)), which is not in accordance with the traditional morphological view and most of the previous molecular studies. It confirms, however, the close relationship between Gekkonidae and Amphisbaenia as revealed in a recent study based on complete mt genomes from a smaller number of taxa. Intra- and intergeneric sequence comparisons of six commonly used marker genes showed rather high levels of divergence within the Lacertidae. In the intrageneric comparison the control region proved to be considerably more conserved than the protein coding genes.

Key words: Lacertidae – complete mitochondrial genomes – control region – phylogeny – substitution rate – Squamata – lizards

Introduction

The phylogenetic information contained in complete sequences of mitochondrial (mt) genomes has been repeatedly used for the reconstruction of higher-order phylogenetic relationships between vertebrate taxa. The analysis of a great number of nucleotide or amino acid sites is expected to give rise to more reliable phylogenetic conclusions (Kumazawa and Endo 2004). On the other hand, information about structural rearrangements may serve as additional markers for phylogenetic inferences (e.g. Haring et al. 2001). Compared with mammals (244) and birds (83), not many mt genomes of Squamata have been completely sequenced. Complete mt sequences have been reported for only 45 species (15 of them being snakes). Nevertheless, reconstructions of higher-order squamate phylogeny based on complete mt genomes have been presented in several recent molecular studies (Kumazawa 2004, 2007; Dong and Kumazawa 2005; Zhou et al. 2006; Böhme et al. 2007; Albert et al. 2008).

The lizard family Lacertidae encompasses about 280 species inhabiting a wide distribution area in Africa and Eurasia. As model organisms, lacertids were investigated in a variety of morphological, behavioural, ecological, biochemical and life-history studies. However, their phylogenetic position within the Squamata remained still controversial. The systematics of Squamata based on morphology (Estes et al. 1988), which has been widely accepted for a long time, is characterized by a basal dichotomy separating the suborders Iguania (Iguanidae and Acrodonta) and Scleroglossa (all other taxa). The Scleroglossa are divided into three major groups: (1) the first group '*incertae sedis*' encompassing Dibamidae, Amphisbaenia, and Serpentes, (2) the Gekkota, and (3) the Autarchoglossa, which are further divided into Scincomorpha and Anguimorpha. According to this classification the Lacertidae were included into the Scincomorpha, together with the families Scincidae, Cordylidae, Xantusiidae, Teiidae and Gymnophtalamidae. More recent morphological analyses by Caldwell (1999) confirmed the close relationships among these taxa.

In several molecular studies (Harris et al. 1999; Townsend et al. 2004; Vidal and Hedges 2005; Kumazawa 2007), however, the Scincomorpha turned out to be paraphyletic: Lacertids and amphisbaenians form a monophyletic group which is only distantly related to a clade comprising Scincidae, Cordylidae and Xantusiidae. In contrast, two recent molecular studies based on mtDNA sequence data suggest close relationship between Scincidae and Lacertidae (Böhme et al. 2007; Albert et al. 2008).

In this work we report the complete mtDNA sequences of the three lacertid species *Podarcis siculus*, *Podarcis muralis* and *Phoenicolacerta kulzeri* (former name: *Lacerta kulzeri*), thereby increasing the total number of complete mt genomes of lizards (or Squamata) available for phylogenetic analyses. The sequences were characterized regarding their length, gene content and organization. These features were compared with complete mt sequences from other lizards. With this set of mtDNA data we investigated the phylogenetic position of the family Lacertidae within the lizards. The results of the phylogenetic analyses are compared with those obtained from nuclear genes and whole mt genomes published so far. Since it has been shown that the substitution rate is significantly faster in snakes compared with lizards (Kumazawa et al. 1998; Kumazawa 2004; Dong and Kumazawa 2005), we did not include snake taxa in the phylogenetic analysis and concentrated on the phylogenetic position of Lacertidae within the rest of the order Squamata. Finally, the fact that the sequences were obtained from two species belonging to the same genus (*Podarcis*) and one from a distantly related member of another genus (*Phoenicolacerta*) enabled us to compare the intra- and intergeneric substitution rate and thereby the potential phylogenetic usefulness of different mt genes as molecular markers within the family Lacertidae.

*This paper is dedicated to Prof. Dr Dr h. c. Diether Sperlich on the occasion of his 80th birthday.

Materials and Methods

Sequencing of the lacertid mt genomes

MtDNA of *Ph. kulzeri* (Lebanon, Djebel Barouk), *P. muralis* (Austria, Baden), and *P. siculus* (Italy, Mti. Peloritani) was purified from fresh liver tissue following the procedure described by Jones et al. (1988). The sequences of the mt genomes were determined using two different approaches: the cloning (*Ph. kulzeri*) or PCR approach (*P. siculus* and *P. muralis*).

Phoenicolacerta kulzeri: The purified mtDNA was digested with *Hin*dIII and *Sac*I restriction enzymes (separately or in combination) and ligated into pUC 19 cloning vectors using standard procedures (Sambrook et al. 1989). The total mt genome was finally contained in four clones: *Hin*dIII (484) – *Hin*dIII (4750), *Hin*dIII (4750) – *Sac*I (7036), *Sac*I (7036) – *Sac*I (11059), and *Hin*dIII (10832) – *Hin*dIII (484), which were sequenced using standard M13 and specific internal primers (electronic supplement: Fig. S1a and Table S1). In addition, the bridges between clones were examined by means of PCR amplification with specific primers: L-nd2-2/H-cox1-1, L-Ser/H-cox2-1, L-nd4L/H-nd4-2 and L-12S-1/H-12S-3 (Fig. S1a and Table S1).

Podarcis siculus and *P. muralis*: The purified mtDNA served as PCR template. Primers used for PCR amplification are listed in Table S1. The 14 overlapping fragments covered the whole mt genome of both *P. siculus* and *P. muralis* (Figs S1b and S1c). For *P. siculus* we used the following primer combinations: L-cr-4/H-12S-4 (1018 bp), L-Phe/H-12S-6 (590 bp), 12S-5/H-12S-7 (462 bp), L-12S-2/H-16S-7 (1688), L-16S-3/H-Ile-1 (1547), L-16S-4/H-cox1-1 (2840), L-Trp/H-cox1-4 (1686), L-cox1-3/H-cox2-1 (1229), L-Ser/H-Gly (2489), L-cox3-1/H-His (2339), L-nd4L/H-nd5-3 (2124), L-Leu/H-cytb-1 (2817), L-cytb-3/H-Thr (1044), L-cytb-4/H-cr-5 (1231). Primer combinations used for *P. muralis* were as follows: L-cr-4/H-12S-4 (1018 bp), L-Phe/H-12S-6 (590 bp), L-12S-5/H-12S-7 (465 bp), L-12S-2/H-16S-7 (1692 bp), L-16S-3/H-Ile-1 (1544 bp), L-nd1-2/H-Ala-2 (1439 bp), L-Trp/H-cox1-4 (1683), L-cox1-5/H-Lys-2 (1225 bp), L-cox2-2/H-Gly (1861 bp), L-cox3-1/H-nd4-5 (1679 bp), L-nd4L/H-nd5-6 (2124 bp), L-Leu/H-cytb-6 (2904 bp), L-cytb-5/H-Thr (1044 bp), L-cytb-8/H-cr-5 (1259 bp).

The PCR products were purified using PCR products purification kit (Roche, Mannheim, Germany). Direct sequencing of PCR products was carried out by MWG-BIOTECH (Ebersberg, Germany). Since it proved difficult to obtain the sequence of the 5′-end of the control region (CR) in both *P. siculus and P. muralis*, this fragment of the mt genome was additionally amplified with the primer combinations L-cytb-4/H-cr-2 or L-cytb-7/H-cr-2 in *P. siculus* and *P. muralis*, respectively, and subsequently cloned (TOPO TA Cloning Kit; Invitrogen, Karlsruhe, Germany).

Sequence alignment and gene identification

Sequence alignment was performed by eye. The individual gene boundaries were determined by sequence comparison with their counterparts in the published reptilian mt genomes. Moreover, the boundaries of the tRNA genes were determined based on the secondary structures of their sequences. The 5′ repeat sections of the CR sequences were folded using the program MFOLD (Zuker 2003).

Phylogenetic analysis

To asses the phylogenetic position of the family Lacertidae within Squamata, complete mt sequences of 16 taxa were retrieved from GenBank. Two of them, *Chelonia mydas* (NC_000886) and *Sphenodon punctatus* (NC_004815), were used as outgroups. The ingroup taxa included five representatives of the suborder Amphisbaenia (*Amphisbaena schmidti* NC_006284, *Bipes biporus* NC_006287, *Diplometopon zarudnyi* NC_006283, *Geocalamus acutus* NC_006285, and *Rhineura floridana* NC_006282), and 12 representatives of lizards belonging to nine families: *Abronia graminea* NC_005958 (Anguimorpha, Anguidae), *Cordylus warreni* NC_005962 (Scincomorpha, Cordylidae), *Eumeces egregius* NC_000888 (Scincomorpha, Scincidae), *Iguana iguana* NC_002793 and *Sceloporus occidentalis* NC_005960 (Iguania, Iguanidae), *Pogona vitticeps* NC_006922 (Acrodonta, Agamidae), *Shinisaurus crocodilurus* NC_005959 (Anguimorpha, Shinisauridae), *Teratoscincus keyserlingii* NC_007008 (Gekkota, Gekkonidae), *Varanus komodoensis* AB080275 and AB080276 (Anguimorpha,Varanidae) and finally the three newly sequenced genomes of Lacertidae: *Ph. Kulzeri* FJ460596, *P. muralis* FJ460597, and *P. siculus* FJ460598.

Phylogenetic analyses were performed on two data sets. The first one contained all above mentioned Squamata sequences as well as the two outgroup taxa *Ch. mydas* and *Sp. punctatus*. The total length of the alignment was 9234 sites and contained concatenated nucleotide sequences of 12 protein coding genes, two rRNA as well as 20 tRNA genes. Because the *nd5* as well as the $tRNA^{His}$ and $tRNA^{Thr}$ genes are lacking in the mt genome of *Sp. punctatus* (Rest et al. 2003), these sequences were omitted from data set 1. Since it has been shown that the internal phylogenetic relationships of Scincomorpha are sensitive to changes in outgroup selection (Albert et al. 2008), our second data set comprised only Squamata taxa. The alignment contained 10 499 nucleotides and included the sequences of all protein coding genes, both rRNA genes as well as all tRNAs. All unalignable sites were excluded from the two data sets. For the protein coding genes the third codon positions were removed because saturation effects are expected at least for the deeper nodes (Brown et al. 1982; Kumazawa and Nishida 1993).

Phylogenetic analyses were conducted using two different methods of phylogenetic inference: maximum parsimony (MP) as implemented in PAUP (v 4.0b10, Swofford 2002) and Bayesian inference (BI) as implemented in MRBAYES (version 3.1.2, Ronquist and Huelsenbeck 2003). For BI both data sets were divided in seven different partititions (ATP86: for two ATPsynthase subunits, CO123: for the cytochrome c oxidase subunits, CytB: for cytochrome b (*cyt b*), ND123: for the first three NADH dehydrogenase subunits, ND4L46: for the rest of the NADH dehydrogenase subunits, 1216 S: for 12 S and 16 S rRNA, tRNA: for all tRNA genes). The best-fitted model of sequence evolution for each partition was selected by hierarchical likelihood ratio tests using the MODELTEST SOFTWARE (version 3.06, Posada and Crandall 1998). Bayesian analyses consisted of two simultaneous runs. For each of them, four Markov Chain Monte Carlo chains were run for three million generations with trees being sampled every 100 generations. The first 20% of sampled trees were discarded and Bayesian posterior probabilities (BPP) were estimated from the 50% majority-rule consensus tree of the retained trees.

Unweighted MP analyses were conducted using the heuristic search mode with 100 replicates, randomized input orders of taxa, and tree bisection-reconnection branch swapping. Branch support was assessed by bootstrap (BS) analysis (1000 BS replications, 10 addition-sequence replicates). Gaps in the alignment were treated in two separate analyses either as missing data or as a 5th state character.

Intra- and intergeneric substitution rates

To estimate the sequence variability and thus their utility for phylogenetic analyses in the family Lacertidae, we compared the sequences of six frequently used mt marker genes (CR, *12 S* and *16 S rRNA*, *cyt b*, *co1* and *nd4*) of our three species with corresponding sequences of two other lacertid lizards for which complete mt genomes are available (*Lacerta viridis* NC_008328 and *Takydromus tachydromoides* NC_008773). For comparison of the CR we used also the sequence of *Teira dugesii* (AY147879; former name: *Lacerta dugesii*). Pairwise comparisons of uncorrected sequence divergence (p-distances) were obtained using MEGA version 3.1 (Kumar et al. 2004) with the pairwise deletion option for treating gaps in the sequence alignments.

Results and Discussion

Genome organization

Although both gene content and gene order are mostly conservative within mt genomes of vertebrates, novel gene arrangements as well as gene contents were often reported for different species and also for a number of Squamata (i.e. Rest et al. 2003; Kumazawa 2004; Kumazawa and Endo 2004; Macey et al. 2004; Amer and Kumazawa 2005). However, the

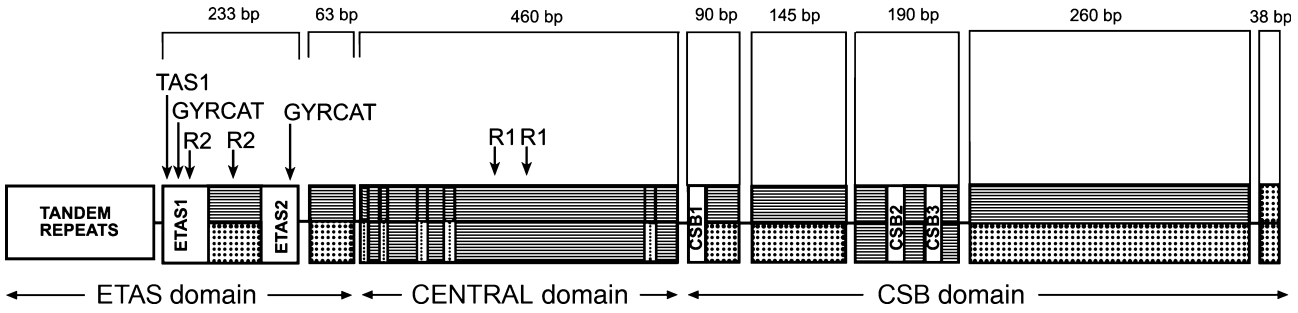

Fig. 1. General organization and sequence variation of the lacertid mt control region. Conserved regions (striped) and variable regions (dotted) are indicated for intrageneric (upper part) and intergeneric (lower part) comparisons

arrangement of the 22 tRNAs, the two rRNAs, the 13 protein-coding genes as well as the non-coding CR in the mt genomes of *Ph. kulzeri, P. siculus* and *P. muralis* (Fig. S1a–c) conforms to the common vertebrate arrangement and is identical to that reported for *L. viridis* (Böhme et al. 2007). Similarly, as reported for above species, an overlap in two adjacent genes encoded by the same strand was found for several protein coding and tRNA genes. Spacer sequences between distinct genes (1–9 bp in length) were also found in all three genomes. Genome size, gene order as well as gene borders are depicted in Tables S2, S3 and S4 for all three species. With a total length of 17 199 bp (*Ph. kulzeri*), 17 297 bp (*P. siculus*) and 17 311 bp (*P. muralis*), our three lacertid genomes are similar in size to that of *L. viridis* (17 156 bp). The nucleotide composition of mt genomes was 31.3% A, 26.2% C, 13.3% G and 29.2% T for *Ph. kulzeri*, 31.6% A, 26.4% C, 12.8% G and 29.2% T for *P. siculus*, and 31.7% A, 25.7% C, 12.9% G and 29.7% T for *P. muralis*. The overall AT content of the sequenced lacertid mitochondrial genomes (60.5 for *Ph. kulzeri*, 60.8 for *P. siculus* and 61.5% for *P. muralis*) appears to be somewhat higher than the average value for lizards (range: 51.5–63.0; mean value: 58.1%, Table S5) but it is very similar to the AT content of the two other currently available lacertid mt genomes *L. viridis* (59.8%, Böhme et al. 2007) and *Ta. tachydromoides* (60.3%, Kumazawa 2007).

Protein coding genes

Start and stop codons for all protein coding genes are presented in Tables S2, S3 and S4. For all three species, codon usage for start codons is identical to that described for *L. viridis*. In contrast to a number of other reptiles no aberrant start codons are found in the three lacertids. ATG is used as initiation codon for 11 genes, while GTG is used as a start codon for the *cox1* and *cox2* genes. In the *cox3* and *nd4* protein coding genes of *Ph. kulzeri* as well as in the *cox3*, *nd3* and *nd4* genes of *P. siculus* and *P. muralis* the coding sequences end with a T and stop codons are probably created by polyadenylation.

Control region

The complete mt CR measures 1844 bp in *P. siculus*, 1872 bp in *P. muralis*, and 1810 bp in *Ph. kulzeri*. The general organization of the lacertid CR with three major domains, i.e., (conserved central domain, variable ETAS (extended termination associated sequences) and CSB (conserved sequence block) domains) as described for mammals (Sbisà et al. 1997) is depicted in Fig. 1. The ETAS domain begins with an array of tandem repeats that can fold in thermodynamically stable stem-loop secondary structures (Fig. S2). The CR of *P. muralis* is characterized by 10 perfect tandem repeats of 34 bp followed by two imperfect repeats (one with four substitutions, the other with six substitutions and one 1 bp deletion) and two repeat rudiments of 13 and 16 bp, respectively. The CR of *P. siculus* contains 11 complete repeats of 36 bp and one incomplete repeat of 19 bp. Three slightly diffent (1–3 bp positions) types of repeat units are found, which are arranged in different arrays depending on the geographic origin of the sample (M. Podnar and W. Mayer, unpublished data). In both *Podarcis* species the stem-loop secondary structure is homogenous suggesting some functional relevance. In contrast, in *Ph. kulzeri* the 12 tandem repeats are quite heterogeneous in sequence and length (34–36 bp and two rudiments of 8 bp). Nevertheless, five of the six positions in the core sequence involved in stem formation are conserved among all repeats. Within the repeat cluster of *Ph. kulzeri* there is a perfect duplication of an array comprising three different repeat units plus the 8 bp rudiment. Although a stable secondary structure can be formed, it appears rather irregular. Interestingly, a similar repeat structure is also found in the other two CR sequences from lacertine species available for comparisons. In *L. viridis* (Böhme et al. 2007) the repeat section consists of a variable number (6–10) of perfect 35 bp repeats, and in *Te. dugesii* Brehm et al. (2003) recorded 4–9 perfect 37 bp repeats. In another lacertid species, *Ta. tachydromoides* (Kumazawa 2007), there are 15 repeats of 65 bp and two modified repeat derivatives of 72 bp in an arrangement which includes also a large 332 bp duplication of five repeat units. In spite of the differences in length and sequence, the core sequence responsible for stem formation is conserved in all these species, indicating that the secondary structure may be a characteristic trait of lacertid CRs. No comparable repeat section exists in the CRs from the suborder Amphisbaenia (representatives of the genera *Amphisbaena*, *Geocalamus*, *Bipes* and *Diplometopon*), but repeats at the 5′-end occur in CR sequences from the genera *Rhineura*, *Teratoscincus*, *Sceloporus*, *Varanus*, *Shinisaurus*, *Abronia* and *Eumeces*. However, these sequences do not contain the core sequence conserved in lacertids and do not show any sequence similarities in the rest of the sequence.

The ETAS1 and ETAS2 boxes were identified by alignment with mammalian consensus sequences and are located downstream of the repeat section (Fig. 1). Regulative 'GYRCAT' motifs as well as TAS (termination associated sequences) like motifs (Doda et al. 1981; Foran et al. 1988) are contained within this segment in all three species. The TAS1 sequence is identical to that identified by Böhme et al. (2007) for *L. viridis* and is contained within the ETAS1 region in all three species.

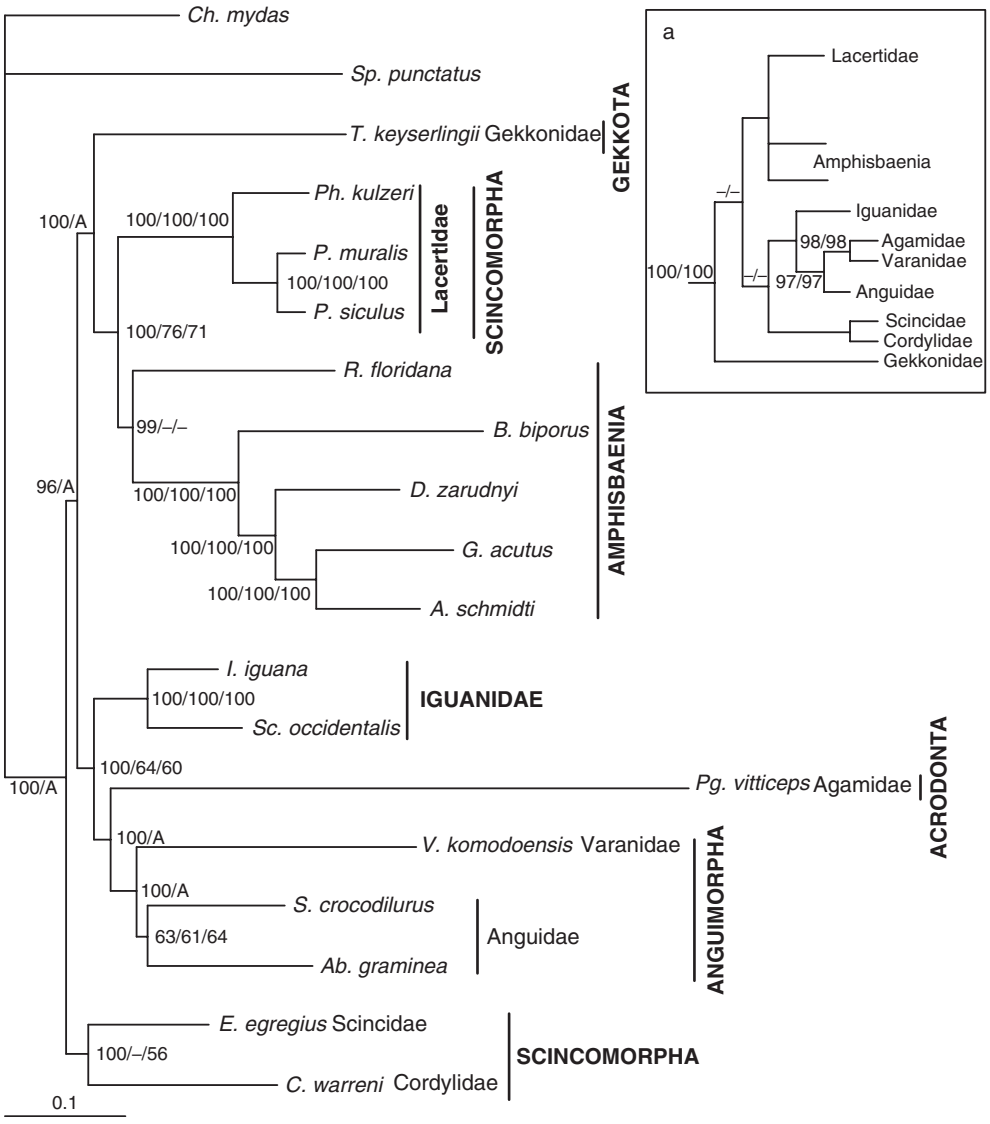

Fig. 2. Phylogram derived from BI analysis of data set 1. Numbers at the nodes represent Bayesian posterior probabilities and MP bootstrap values (gaps 5th state/gaps missing). BS values < 50 are not indicated. The differences in topology obtained by the MP method are depicted in square A. Numbers at the nodes represent MP bootstrap values (gaps 5th state/gaps missing)

Two 'GYRCAT' motifs, although not identical, were found at the same position (the first within the ETAS2 box and the second between ETAS2 and the central domain) in all three species investigated and correspond to those of *Te. dugesii* and *L. viridis*. The perfect repeats R2 reported for *Te. dugesii* and *L. viridis* (Brehm et al. 2003; Böhme et al. 2007) are also present and perfectly conserved in *P. muralis*, and almost perfect in *P. siculus* and *Ph. kulzeri*. The first of them is placed within the ETAS1 box and the other in the section between the two ETAS boxes. The central domain, extending from about 60 bp upstream of the ETAS2 down to the CSB-1 block, is highly conserved with the exception of five short variable stretches. The 34-bp perfect repeats (R1) reported by Brehm et al. (2003) for *Te. dugesii* and Böhme et al. (2007) for *L. viridis* are present and almost perfectly conserved in all three here investigated species. However, while the repeats are perfect in *P. muralis* and *Ph. kulzeri*, they differ by 2 bp in *P. siculus*. The CSB domain is characterized by a great number of AT-rich inverted repeats included in stem-loop secondary structures. All three CSB sequences as delimited by Brehm et al. (2003) for *Te. dugesii* are present and are almost perfectly conserved.

tRNAs

For all three species, the anticodon triplets of all tRNAs are identical to those of other vertebrates. They all assumed the clover-leaf structure allowing for G-U wobble pairings and occasional mismatches (for secondary structures see Fig. S2). The spacer sequence (49 bp in the mt genomes of *P. siculus* and *P. muralis* and 29 bp in *Ph. kulzeri*) separating the tRNA-Asp and tRNA-Cys genes (WANCY cluster) contains the 28 bp sequence motif 5′-cttcccccgtt(t/a)(g/a)(g/a)aaaaaaaacggggg-3′, considered as the putative origin of replication for the light strand (O_L).

Phylogenetic relationships

The results of phylogenetic analyses performed on data set 1 are depicted in Fig. 2. The Bayesian as well as the two different MP analyses (regarding gap treating) of data set 1 revealed the sister group relationship of Lacertidae and Amphisbaenia. This relationship is strongly supported by BPP (100) and moderately by bootstrap values in the two MP analyses (71 gaps missing, 76 gaps 5th state). The same relationship was

recovered in most of the recent molecular investigations based on the analysis of some nuclear protein coding genes and some shorter mtDNA sequences (Townsend et al. 2004; Vidal and Hedges 2004, 2005) as well as complete mtDNA sequences (Kumazawa 2007). However, this placement of the lacertids is not in agreement with two recent phylogenetic studies, which were also based on mtDNA data (Böhme et al. 2007; Albert et al. 2008), postulating sister group relationship between Lacertidae and Scincidae. Yet, in Böhme et al. (2007) this grouping was only weakly supported by bootstrap values. Moreover, our data set differs substantially from the data set used in that study, where only sequences of protein coding genes were included and all three codon positions were used for phylogenetic reconstruction. In the study of Albert et al. (2008) the sister group relationship of Lacertidae and Scincidae was well supported in the BI but not in the ML analysis, and it was shown not to be robust to changes in taxon sampling of the outgroup. The basal position of Gekkota to all other squamate groups is confirmed in most molecular studies. The same was true also for our MP trees (Fig. 2). However, in our Bayesian tree, the Scincidae + Cordylidae (Scincomorpha) clade splits off from the basal node within the Squamata clade, and the Gekkonidae appeared as a sister group of the Lacertidae + Amphisbaenia clade. The obtained intriguing position of Gekkonidae is similar as proposed by Zhou et al. (2006) who revealed a sister group relationship between Gekkota and Amphisbaenia, but no representatives of the Lacertidae were included in their data set. The same sister group relationship between Gekkota and Amphisbaenia was also recovered with MP and NJ methods by Böhme et al. (2007). However, this clade showed no close relationship to the Lacertidae in those trees. Several morphological traits could be considered as support for a Gekkota + Lacertidae + Amphisbaenia clade. Barbadillo and Martínez-Solano (2002) revealed the presence of thoracolumbar intercentra in Lacertidae, a condition previously reported within Squamata only for the families Xantusiidae and Gekkonidae. In the study of Caldwell (1999) based on the analysis of 95 osteological characters the Gekkonoidae turned out to cluster with Amphisbaenia. Furthermore, this clade appeared as the sister clade of the group traditionally comprised in the infraorder Scincomorpha. Sister group relationship of Iguanidae and Anguimorpha was recovered in both MP and NJ with high BPP and moderate BS support. This relationship was also obtained by Zhou et al. (2006) and Kumazawa (2007).

The obtained placement of *Pg. vitticeps* (Acrodonta, Agamidae) within Anguimorpha (MP) or as a sister group of the Anguimorpha seems not reliable as it is probably affected by long branch attraction effects and inadequate taxon coverage. In previous molecular studies the Acrodonta appeared as sister group of the Iguanidae (Townsend et al. 2004; Kumazawa 2007) or as sister group of serpentes (Douglas et al. 2006; Böhme et al. 2007) which are not included in our data set.

Unrooted networks were calculated from data set two comprising Squamata only. The branching pattern in the network obtained from the BI analysis (Fig. 3) is identical to that in Fig. 2, indicating that the outgroup taxa had no influence on the relationships within the Squamata. In the MP analyses the obtained network (not shown) was almost identical, only *Pg. vitticeps* changed its position appearing close to *V. komodoensis*.

Intra- and intergeneric substitution rates

The percentages of uncorrected pairwise sequence divergences (p-distances) found between the lacertid species in mt

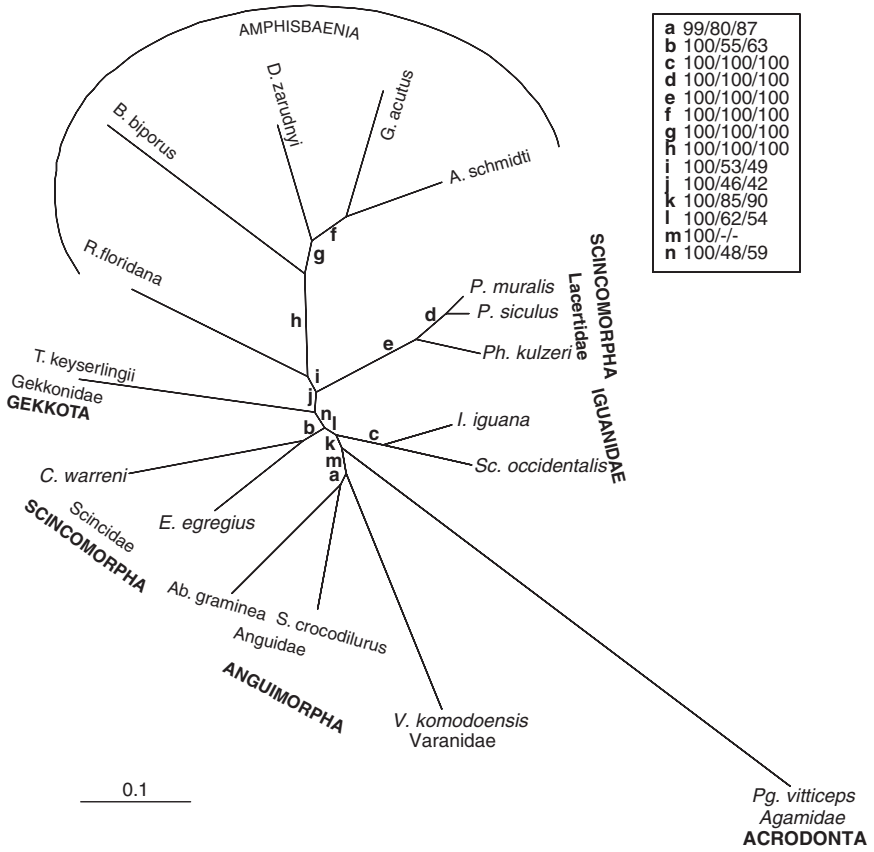

Fig. 3. Unrooted tree derived from BI analysis of data set 2. Letters at the nodes refer to the small table indicating Bayesian posterior probabilities and MP bootstrap values (gaps 5th state/gaps missing). Node m was obtained only in the BI analysis, in both MP analyses Varanidae clustered with Anguidae (BS 85/91)

genes commonly used as phylogenetic markers are shown in Table S6. For the protein coding genes *co1*, *cyt b* and *nd4* the intrageneric distances found between the two *Podarcis* species are remarkably high indicating an early split of these taxa. In *Podarcis* the two rRNA genes are clearly more conserved than the protein genes which are not the case in the intergeneric comparisons. Surprisingly, the non-coding CR is the most conserved sequence in *Podarcis* whereas in the intergeneric comparisons the values are in the range of the *cyt b* and rRNA genes. Two sections of the CR, 460 bp of the central domain and 190 bp of the CSB domain, are highly conserved at the intergeneric level. The rest of the sequence is more divergent with most variable segments found between CSB1 and CSB2 sequence blocks and downstream of the CSB3 block. According to these comparisons the *cyt b*, which is frequently employed as a molecular clock, as well as the *nd4* gene appear too variable to be used as phylogenetic markers for studies on lacertids.

Acknowledgements

We want to express our thanks to E. Haring (NHMW, Vienna) for valuable suggestions and critical discussion and to W. Bischoff (Museum Alexander Koenig, Bonn) for providing a sample of *Ph. kulzeri*. The work was partially supported by the Research Fund of the Republic of Croatia, project ID: 183-1193080-0831.

Zusammenfassung

Die kompletten mitochondrialen Genome von drei Eidechsenarten und die systematische Position der Lacertidae (Squamata)

Komplette mitochondriale (mt) Genome von drei Vertretern der Lacertidae wurden sequenziert: *Podarcis siculus*, *Podarcis muralis* und *Phoenicolacerta kulzeri*. In allen drei Genomen stimmte die Anordnung der 22 tRNAs, zwei rRNAs und der 13 Protein kodierenden Gene mit jener der meisten Vertebraten überein. Die phylogenetische Position der Lacertidae innerhalb der Squamata wurde durch Analyse der Sequenzen von großen Abschnitten kompletter mt Genome ermittelt. Die Zahl der Nukleotidpositionen, die für die Stammbaumkonstruktion verwendet wurden, betrug 9234, wenn Außengruppen-Taxa mit einbezogen wurden, und 10499, wenn nur Squamata verglichen wurden. Die phylogenetische Analyse bestätigte die Schwestergruppen-Verwandtschaft zwischen Lacertidae and Amphisbaenia, wie sie schon früher auf Basis molekularer Daten postuliert wurde. Dieses Monophylum ist eng verwandt zu den Gekkonidae, was mit einer neueren Studie an kompletten mt Genomen einer geringeren Anzahl von Taxa übereinstimmt. Diese Platzierung der Gekkonidae nahe den Lacertiden unf Amphisbaenia widerspricht jedoch der traditionellen morphologischen Sichtweise und früheren molekularen Untersuchungen. Sequenzvergleiche innerhalb und zwischen den Gattungen für sechs häufig eingesetzte Markergene zeigten einen hohen Divergenzgrad innerhalb der Lacertidae. Die Kontrollregion erwies sich im intragenerischen Vergleich als deutlich stärker konserviert als die Protein kodierenden Gene.

References

Albert EM, San Mauro D, García-París M, Rüber L, Zardoya R (2008) Effect of taxon sampling on recovering the phylogeny of squamate reptiles based on complete mitochondrial genome and nuclear gene sequence data. Gene, in press, doi:10.1016/j.gene.2008.05.014

Amer SA, Kumazawa Y (2005) Mitochondrial genome of *Pogona vitticepes* (Reptilia; Agamidae): control region duplication and the origin of Australasian agamids. Gene **346:**249–256.

Barbadillo LJ, Martínez-Solano I (2002) Vertebral intercentra in Lacertidae: variation and phylogenetic implications. Copeia **2002:**208–212.

Böhme MU, Fritzsch G, Tippmann A, Schlegel M, Berendonk TU (2007) The complete mitochondrial genome of the green lizard *Lacerta viridis viridis* (Reptilia: Lacertidae) and its phylogenetic position within squamate reptiles. Gene **394:**69–77.

Brehm A, Harris J, Alves C, Jesus J, Thomarat F, Vicente L (2003) Structure and evolution of the mitochondrial DNA complete control region in the lizard *Lacerta dugesii* (Lacertidae, Sauria). J Mol Evol **56:**46–53.

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol **18:**225–239.

Caldwell MW (1999) Squamate phylogeny and the relationships of snakes and mosasauroids. Zool J Linn Soc **125:**115–147.

Doda JN, Wright CT, Clayton DA (1981) Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. Proc Natl Acad Sci U S A **78:**6116–6120.

Dong S, Kumazawa Y (2005) Complete mitochondrial DNA sequences of six snakes: phylogenetic relationships and molecular evolution of genomic features. J Mol Evol **61:**12–22.

Douglas D, Janke A, Arnason U (2006) A mitogenetic study on the phylogenetic position of snakes. Zool Scr **35:**545–558.

Estes R, de Queiros K, Gauthier J (1988) Phylogenetic relationships within Squamata. In: Estes R, Pregill GK (eds), Phylogenetic Relationships of the Lizard Families. Stanford University Press, CA, USA, pp 119–282.

Foran DR, Hixson JE, Brown WM (1988) Comparisons of ape and human sequences that regulate mitochondrial DNA transcription and D-loop DNA synthesis. Nucleic Acids Res **16:**5841–5861.

Haring E, Kruckenhauser L, Gamauf A, Riesing MJ, Pinsker W (2001) The complete sequence of the mitochondrial genome of *Buteo buteo* (Aves, Accipitridae) indicates an early split in the phylogeny of raptors. Mol Biol Evol **18:**1892–1904.

Harris DJ, Sinclair EA, Mercader NL, Marshall JC, Crandall KA (1999) Squamate relationships based on *c-mos* nuclear DNA sequences. Herpetol J **9:**147–151.

Jones CS, Tegelström H, Latchman DS, Berry RJ (1988) An improved rapid method for mitochondrial DNA isolation suitable for use in the study of closely related populations. Biochem Genet **26:**83–88.

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform **5:**150–163.

Kumazawa Y (2004) Mitochondrial DNA sequences of five squamates: phylogenetic affiliation of snakes. DNA Res **11:**137–144.

Kumazawa Y (2007) Mitochondrial genomes from major lizard families suggest their phylogenetic relationships and ancient radiations. Gene **388:**19–26.

Kumazawa Y, Endo H (2004) Mitochondrial genome of the Komodo dragon: efficient sequencing method with reptile-oriented primers and novel gene rearrangements. DNA Res **11:**115–125.

Kumazawa Y, Nishida M (1993) Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. J Mol Evol **37:**380–398.

Kumazawa Y, Ota H, Nishida M, Ozawa T (1998) The complete nucleotide sequence of a snake (*Dinodon semicarinatus*) mitochondrial genome with two identical control regions. Genetics **150:**313–329.

Macey JR, Papenfuss TJ, Kuehl JV, Fourcade HM, Boore JL (2004) Phylogenetic relationships among amphisbaenian reptiles based on complete mitochondrial genomic sequences. Mol Phylogenet Evol **33:**22–31.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818.

Rest JS, Ast JC, Austin CC, Waddell PJ, Tibbetts EA, Hay JM, Mindell DP (2003) Molecular systematics of primary reptilian lineages and the tuatara mitochondrial genome. Mol Phyl Evol **29:**289–297.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:**1572–1574.

Sambrook J, Frisch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor, NY, USA.
Sbisà E, Tanzariello F, Reyes A, Pesole G, Saccone C (1997) Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. Gene **205:**125–140.
Swofford DL (2002) PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), Version 4. Sinauer Associates, Sunderland, MA.
Townsend T, Larson A, Louis E, Macey JR (2004) Molecular phylogenetics of Squamata: the position of snakes, amphisbaenians, and dibamids, and the root of the squamate tree. Syst Biol **53:**735–757.
Vidal N, Hedges SB (2004) Molecular evidence for a terrestrial origin of snakes. Proc R Soc Lond B (Suppl.) **271:**S226–S229.
Vidal N, Hedges SB (2005) The phylogeny of squamate reptiles (lizards, snakes, and amphisbaenians) inferred from nine nuclear protein-coding genes. C R Biol **328:**1000–1008.
Zhou K, Li H, Han D, Bauer AM, Feng J (2006) The complete mitochondrial genome of *Gekko gecko* (Reptilia: Gekkonidae) and support for the monophyly of Sauria including Amphisbaenia. Mol Phylogenet Evol **40:**887–892.
Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res **31:**3406–3415.

Authors' addresses: Martina Podnar, Croatian Natural History Museum, Department of Zoology, Demetrova 1, 10000 Zagreb, Croatia. E-mail: martina.podnar@zg.t-com.hr; Wilhelm Pinsker, Werner Mayer, (for correspondence), Naturhistorisches Museum Wien, 1. Zool. Abt., Molekulare Systematik, Burgring 7, 1010 Wien, Austria. E-mail: wilhelm.pinsker@univie.ac.at, werner.mayer@nhm-wien.ac.at

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The organization of the mitochondrial genomes of *Ph. kulzeri* (a), *P. siculus* (b) and *P. muralis* (c). For *Ph. kulzeri* sequencing primers are depicted as arrows, and 'bridges' between the four *Ph. kulzeri* clones as curved lines (with names of both primers, outer circle). For *P. siculus* and *P. muralis* the overlapping PCR fragments with corresponding primer names are shown. Primers are listed in Table S1. *Hin*dII and *Sac*I restriction sites are indicated. Abbreviations: nd1–6 = NADH dehydrogenase subunits 1–6, cox1–3 = cytochrome c oxidase subunits 1–3, atp6 and atp8 = ATPase subunits 6 and 8, cytb = cytochrome b, cr = control region

Fig. S2. Secondary structure of tandem repeats in the mitochondrial control regions of *Ph. kulzeri*, *P. siculus* and *P. muralis*

Table S1. Primers used for PCR amplifications of mt DNA fragments for each species

Table S2. Organization of the mt genome of *Ph. kulzeri*

Table S3. Organization of the mtl genome of *P. siculus*

Table S4. Organization of the mt genome of *P. muralis*

Table S5. Genome size and nucleotide composition of the available mt genomes of lizards and amphisbaenians

Table S6. Uncorrected pairwise sequence divergence (p-distances in %) in frequently used marker genes. Intrageneric distances are given for *Podarcis* (*P. muralis* versus *P. siculus*), average intergeneric distances refer to comparisons among *Podarcis* (P), *Phoenicolacerta*. (Ph), *Lacerta* (L), and *Takydromus* (Ta). The 5′ tandem repeats of the CR sequences were excluded

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.