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Effect of taxon sampling on recovering the phylogeny of squamate reptiles based on complete mitochondrial genome and nuclear gene sequence data

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ABSTRACT

The complete nucleotide sequences of the mitochondrial (mt) genomes of three species of squamate lizards: *Blanus cinereus* (Amphisbaenidae), *Anguis fragilis* (Anguidae), and *Tarentola mauritanica* (Geckkonidae) were determined anew. The deduced amino acid sequences of all 13 mt protein-coding genes were combined into a single data set and phylogenetic relationships among main squamate lineages were analyzed under maximum likelihood (ML) and Bayesian Inference (BI). Within Squamata, the monophyly of Iguanidae, Anguimorpha, Amphisbaenia, Gekkota, Serpentes, and Acrodonta received high statistical support with both methods. It is particularly striking that this is the first molecular analysis that recovers the monophyly of Scincomorpha (including Scincidae, Xantusiidae, Cordylidae, and Lacertidae), although it is only supported in the Bayesian analysis, and it is sensitive to changes in the outgroup (see below). Phylogenetic relationships among the main squamate lineages could not be resolved with ML but received strong support with BI (above 95%). The newly reconstructed phylogeny of squamates does not support the Iguania–Scleroglossa split. Acrodonta and Serpentes form a clade, which is the sister group of the remaining squamate lineages. Within these, Gekkota were the first branching out, followed by Amphisbaenia, and a clade including Anguimorpha as sister group of Scincomorpha + Iguanidae. The recovered topology differed substantially from previously reported hypotheses on squamate relationships, and the relative effect of using different outgroups, genes, and taxon samplings were explored. The sister group relationship of Serpentes + Acrodonta, and their relative basal position within Squamata could be due to a long-branch attraction artifact. Phylogenetic relationships among Scincomorpha, Amphisbaenia, and Anguimorpha were found to be rather unresolved. Future improving of squamate phylogenetic relationships would rely on finding snake and acrodont species with slower mt evolutionary rates, ensuring thorough taxon coverage of squamate diversity, and incorporating more nuclear genes with appropriate evolutionary rates.

1. Introduction

The molecular phylogeny of land vertebrates is presently among the best documented (Meyer and Zardoya, 2003) owing to newly-compiled large sequence data sets based on mitochondrial (mt) and/or nuclear genes, as well as on rather thorough lineage samplings. This is particularly true for recently reported amphibian (San Mauro et al., 2005; Frost et al., 2006; Roelants et al., 2007), and mammal (Murphy et al., 2001a,b; Springer et al., 2001) molecular phylogenies, which are relatively robust from a statistical point of view, and will be essential as a framework to any future comparative study pertaining these taxa. In contrast, our understanding of phylogenetic relationships within the third main lineage of tetrapods, i.e. sauropsids (reptiles + birds) is still emerging because thus far accumulated molecular data for this group are limited as compared to mammals and amphibians. The classic hypothesis on sauropsid phylogenetic relationships is based on the absence or presence of two skull temporal fenestrae, and considers a basal split into Anapsida (turtles) and Diapsida (other reptiles + birds), respectively (Meyer and Zardoya, 2003). The latter are further divided into Lepidosauria (squamates + the New Zealand living fossil, the tuatara) and Archosauria (crocodiles + birds). The traditional view of turtles as anapsids (Lee, 2001) has been challenged by several morphological studies suggesting diapsid affinities of turtles (Rieppel and deBraga, 1996; Hill, 2005). Molecular phylogenies (Zardoya and Meyer, 1998; Hedges and Poling, 1999; Kumazawa and Nishida, 1999;

Abbreviations: mt, mitochondrial; ML, maximum likelihood; BI, Bayesian inference; bp, base pairs; AIC, Akaike information criterion; ATP, ATP synthase; AU, Approximately Unbiased test; Cox, Cytochrome *c* oxidase; Cytb, Cytochrome *b*; DHU, dihydrouridine; KH, Kishino–Hasegawa test; MCMC, Markov chains Monte Carlo; Mya, million years ago; Myr, Million years; NADH, NADH dehydrogenase; O_L, origin of mitochondrial light strand replication; PL, Penalized likelihood; RTTM, Root to tip mean; SH, Shimodaira–Hasegawa test; TN, truncated Newton algorithm.

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Hugall et al., 2007) place the turtles as derived diapsids related with Archosauria.

With nearly 8000 living species and a worldwide distribution (Zug et al., 2001; Pianka and Vitt, 2003; Pough et al., 2004), squamate reptiles conform a highly diversified clade that includes lizards, snakes and amphisbaenians (Townsend et al., 2004; Estes et al., 1988). The main lineages of squamates exhibit a great variety of specialized morphological, behavioral and ecological forms (Zug et al., 2001; Pianka and Vitt, 2003; Pough et al., 2004), which have seriously hindered establishing higher-level phylogenetic relationships within the group based on morphology (e.g., Estes et al., 1988; Lee, 1998, 2000; Kearney, 2003). Traditionally, squamates have been divided into two major groupings (Iguania and Scleroglossa) based mostly on osteological and soft anatomy characters (Estes et al., 1988; Lee, 1998; Reynoso, 1998; Lee and Caldwell, 2000). This main cladogenetic split has been linked to major differences in tongue structure and associated feeding behavior (Vitt et al., 2003; Vitt and Pianka, 2005). Iguania, which include iguanids, agamids, and chamaeleonids (the latter two grouped together into Acrodonta), use the tongue for prey prehension (as Tuataras) whereas Scleroglossa, which include the remaining squamates, use teeth and jaw for prey prehension, freeing the tongue for chemosensory reception, and seemingly allowing present-day predomination of scleroglossans over iguanians worldwide (Schwenk, 1993; Vitt et al., 2003; Pough et al., 2004; Townsend et al., 2004; Vidal and Hedges, 2005). Scleroglossa is further divided into three infraorders: Gekkota, Scincomorpha, and Anguimorpha, with the latter two grouped into a higher rank, the Autarchoglossa. The limbless groups, i.e. Amphisbaenia, Serpentes and Dibamidae are normally left as "incertae sedis" within the Scleroglossa (Estes et al., 1988) (Fig. 1A).

Several recent papers (Townsend et al., 2004; Vidal and Hedges, 2005; Böhme et al., 2007; Douglas et al., 2006; Kumazawa, 2007) have focused on the molecular phylogeny of squamates deriving at very different conclusions (Fig. 1B, C, and D). Thus far, no molecular phylogeny recovers the basal split of squamates into Iguania and Scleroglossa, against morphological evidence (Townsend et al., 2004; Vidal and Hedges, 2005; Kumazawa, 2007) (Fig. 1). Moreover, molecular phylogenies based on either mt (Böhme et al., 2007; Kumazawa, 2007), nuclear (Vidal and Hedges, 2005) or combined (Townsend et al., 2004; Hugall et al., 2007) sequence data fairly agree in supporting Dibamida and Gekkota as the most basal squamate lineages (but see Harris, 2003; Zhou et al., 2006) (Fig. 1). Scincomorpha are recovered generally as paraphyletic with Scincoidea (Scincidae, Xantusiidae, and Cordylidae) placed as a sister group of Lacertoidea (Lacertidae and Teiidae) + Amphisbaenia, and within a larger clade that also includes Anguimorpha and Iguania (Fig. 1). However, phylogenetic relationships within this larger clade remain largely unresolved (Townsend et al., 2004; Vidal and Hedges, 2005; Böhme et al., 2007; Hugall et al., 2007; Kumazawa, 2007), and the monophyly of Scincomorpha cannot be statistically rejected (Kumazawa, 2007). In addition, the relative phylogenetic position of Serpentes varies among studies, and it is particularly volatile in

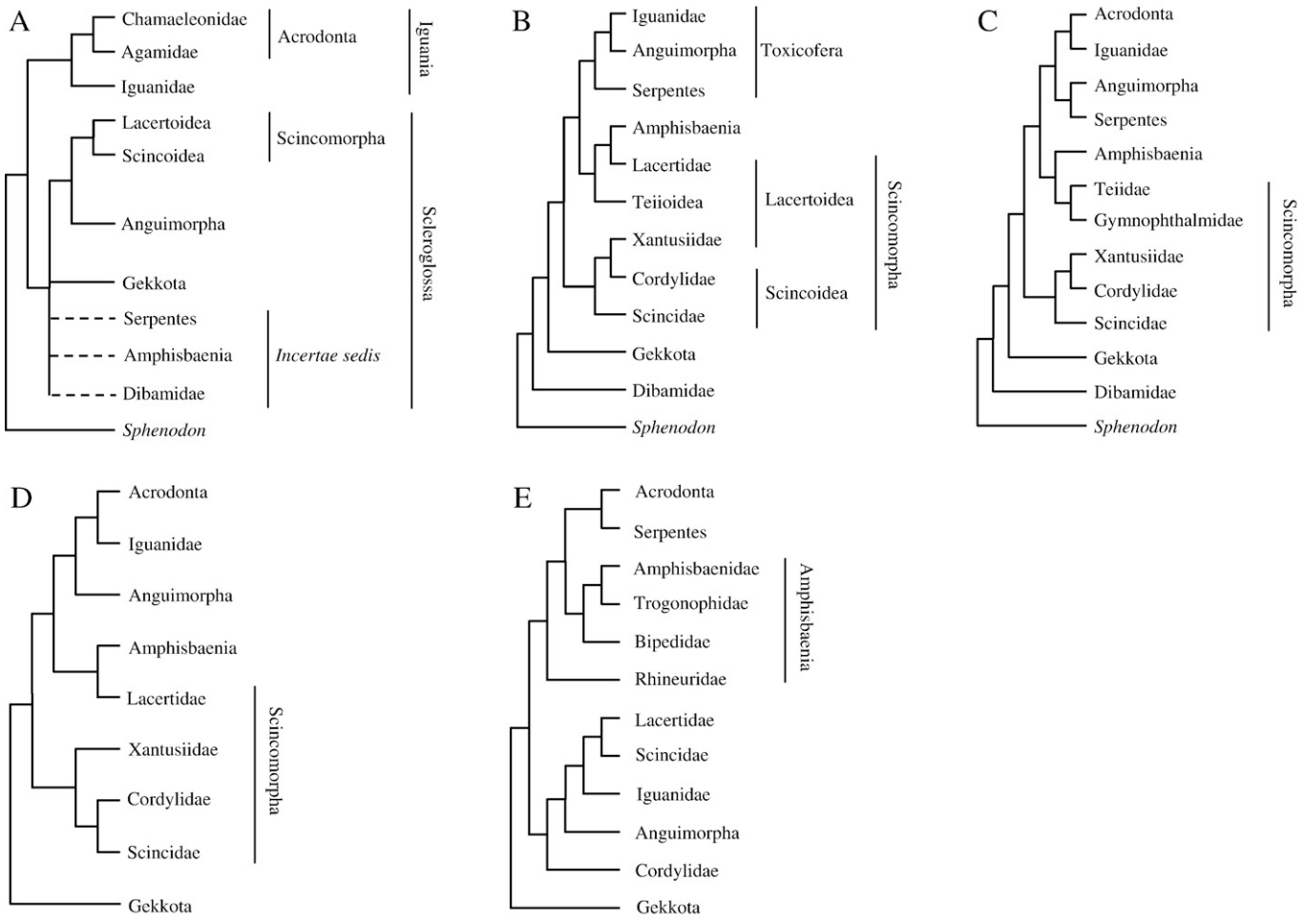

Fig. 1. Alternative hypotheses for squamate phylogenetic relationships. A. Morphology-based hypothesis (Estes et al., 1988); B. Nuclear-based (RAG1, C-mos, RAG2, R35, HOXA13, JUN, alpha-enolase, amelogenin, MAFB genes) hypothesis (Vidal and Hedges, 2005); C. Nuclear-based (RAG1, C-mos genes) hypothesis (Towsend et al., 2004); D. Mt-based (complete mt genomes) hypothesis (Kumazawa, 2007); E. Mt-based (complete mt genomes) hypothesis (Böhme et al., 2007).

those based on mt data, likely due to the relatively long branches of the taxon (Townsend et al., 2004; Böhme et al., 2007; Zhou et al., 2006). Moreover, recent studies (Vidal and Hedges, 2005; Fry et al., 2006) have also reported the existence of a 'venom clade' (= Toxicofera), including all major squamate lineages with species possessing toxin-secreting oral glands (namely Serpentes, Iguania, and Anguimorpha) (Fry et al., 2006). However, venom glands were reported in *Pogona* (Acrodonta; Agamidae) and not in Iguanidae (Fry et al., 2006) whereas Iguanidae (and not Acrodonta) were used to represent Iguania in the phylogenetic analyses (Vidal and Hedges, 2005; Fry et al., 2006). Overall, it seems that main differences among recovered molecular phylogenies of squamates may be related with the use of different outgroup taxa, taxon coverages, and gene data sets, as well as with the observations that terminal branches are rather long with respect to internal nodes in the squamate phylogeny, and that some lineages (e.g. Serpentes; Kumazawa et al., 1998) have consistently higher evolutionary rates than others. The relative contribution of these factors to the instability of the squamate molecular phylogeny awaits further investigation.

In order to further resolve the molecular phylogeny of squamates, we increased the number of analyzed species by sequencing three new complete squamate mt genomes. Our phylogenetic analyses including the new mt genome sequence data recovered a topology that differed substantially from those previously reported (Townsend et al., 2004; Vidal and Hedges, 2005; Kumazawa, 2007). We explored whether topological differences could be correlated with the choice of molecular marker, i.e. mt or nuclear DNA, with using different outgroup taxa, i.e. amphibians (*Xenopus laevis*) versus amniotes, with increasing species sampling of the different squamate lineages, and/ or with incorporating lineages with different evolutionary rates into the phylogenetic analyses.

2. Materials and methods

2.1. Taxon sampling

The nucleotide sequence of the complete mt genome was determined anew from a single individual of three species of squamate lizards: the Iberian worm lizard *Blanus cinereus* (Amphisbaenidae; collected by MGP and Iñigo Martínez-Solano in Santa Maria de Alameda-Madrid, Spain; May 2001; voucher MNCN/ADN 21711) the slow worm *Anguis fragilis* (Anguidae; collected by RZ in Vilarmiel-Lugo, Spain; April 2003; voucher MNCN/ADN 7215) and the Moorish gecko *Tarentola mauritanica* (Geckkonidae; collected by MGP in Las Gaviras-Granada,Spain; March 2003; voucher MNCN/ADN 7216). In addition, 24 complete mt genome sequences of species representing the major lineages of squamates were retrieved from public sequence databases, and included in the phylogenetic analyses (Genbank accession numbers are listed in Supplementary material). The Tuatara (*Sphenodonon punctatus*; Rhynchocephalia), as well as several representatives of more distantly related amniotes (Archosauria, Testudines, and Mammalia), and of amphibians were used as outgroup taxa (Genbank accession numbers of employed outgroups are provided in Supplementary material). Phylogenetic performance of mt sequence data was compared with that of previously published nuclear sequence data (*Rag1*, *c-mos*; Townsend et al., 2004).

2.2. DNA purification, amplification, and sequencing

Total genomic DNA was purified from preserved (ethanol 80–96%) small amounts of tissue using standard proteinase K/ SDS digestion, phenol–chloroform extraction, and ethanol precipitation (Sambrook et al., 1989). Standard PCR reactions (Saiki et al., 1988) were conducted in a total volume of 25 μl containing 67 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 2.5 μM of each primer, template mtDNA (10–100 ng), and Taq DNA polymerase (1 unit, Biotools). The following PCR cycling conditions were used: an initial denaturing step at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 60 s, annealing at 42–52 °C (Supplementary material) for 60 s, and extending at 72 °C for 90 s; and a final extending step of 72 °C for 7 min. A suite of 34 primers was used to amplify by PCR contiguous and overlapping fragments that covered the entire mt genome (Supplementary material). PCR products were purified by ethanol precipitation, and sequenced in an automated DNA sequencer (ABI PRISM 3700) using the BigDye dideoxy Terminator cycle-sequencing kit (Applied Biosystems) following manufacturer's instructions. Short amplicons were sequenced directly using the corresponding PCR primers. Long amplicons were cloned into pGEM-T vectors (Promega), and recombinant plasmids were sequenced using both M13 (forward and reverse) universal primers, and walking primers (available from the authors on request). The obtained sequences averaged 700 base pairs (bp) in length, and each sequence overlapped the next contig by about 150 bp. In no case were differences in sequence observed between the overlapping regions.

The complete mt genome nucleotide sequences reported in this paper have been deposited in the GenBank (EU443255-57).

2.3. Molecular and phylogenetic analyses

The deduced amino acid sequence of each of the 13 mt protein-coding genes were aligned separately using CLUSTAL X version 1.83 (Thompson et al., 1997) with default parameters, and revised by eye in order to maximize homology of position. Ambiguous alignments and gaps were excluded from the analyses using GBLOCKS version 0.91b (Castresana, 2000) with default parameters. The resulting 13 mt amino acid alignments were concatenated into a single data set (henceforth referred to as the allmt data set; 3161 positions). Sequence alignments are available from the authors upon request.

Phylogenetic relationships among squamates were inferred using maximum likelihood (ML; Felsenstein, 1981) and Bayesian inference (BI, Huelsenbeck et al., 2001). Best-fit models of sequence evolution were selected both for the ML analysis and the different BI partitions using PROTTEST version 1.2.6 (Abascal et al., 2005) under the Akaike Information Criterion (AIC, Akaike, 1973). ML analyses were performed with PHYML version 2.4.3 (Guindon and Gascuel, 2003) using a BioNJ as input tree. BI analyses were conducted using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) based on four different partitions (ATP: for the ATP synthase F0 subunits, COX: for the cytochrome *c* oxidase subunits, Cytb: for the cytochrome *b*, NADH: for the NADH dehydrogenase subunits). The rationale behind using these partitions was that subunits of the same mitochondrial enzyme complex are likely subjected to similar evolutionary forces, and therefore they can be grouped together for model parameter estimation. Four Markov chains Monte Carlo (MCMC) were run for one million generations, sampling every 100 generations, and discarding generations before MCMC reached stationarity (100,000) as "burn-in". Two independent BI runs were performed to control for an adequate mixing of the MCMC. Robustness of the resulting ML and BI trees was evaluated by non-parametric bootstrapping with 1000 pseudoreplicates, and Bayesian posterior probabilities, respectively.

In order to further evaluate the effect of taxon sampling on the topology and robustness of squamate phylogeny, four additional data sets were analyzed. The combined mt protein-coding data set was evaluated at the amino acid level using either one single or two (one basal and one derived) representative species per major lineage (henceforth referred to as 1mt and 2mt data sets, respectively). A nuclear data set combining nucleotide sequences of the *c-mos* (311 bp) and RAG-1 (2499 bp) genes (Townsend et al., 2004) was analyzed using either one single or two (one basal and one derived) representative species per major lineage (henceforth referred to as the 1nuc and 2nuc data sets, respectively). The recovered phylogenetic

trees based on these four data sets were compared with those based on a full taxon sampling coverage, and either the combined mt protein-coding (this paper) or nuclear (Townsend et al., 2004) gene data sets.

In order to compare our results based on the combined mt protein-coding gene amino acid data set and those of Kumazawa (2007), the effect of using a more distantly related outgroup was explored using a combined mt protein coding data set, which included the amphibian *X. laevis* (henceforth referred to as the mt*Xenopus* dataset. Furthermore, the effect of incorporating lineages with different evolutionary rates into phylogenetic analyses was evaluated by excluding from the combined mt protein-coding gene amino acid data set either all representatives of the two major lineages (snakes and acrodont lizards) that exhibited long branches, or only excluding representatives of one of them at a time. These datasets were henceforth referred as to the noSA, noS, and noA datasets, respectively.

Finally, seven alternative tree topologies (taken from the literature) were evaluated by the non-parametric tests Approximately Unbiased (AU; Shimodaira, 2002), Shimodaira–Hasegawa (SH; Shimodaira and Hasegawa, 1999), and Kishino–Hasegawa (KH; Kishino and Hasegawa, 1989). All tests were carried out on the allmt dataset using Consel version 0.1i (Shimodaira and Hasegawa, 2001) with site-wise log-likelihoods calculated by PAML version 3.14 (Yang, 1997). A total of one million multiscale bootstrap replicates were used in order to reduce sampling error.

2.4. Dating of divergence times

The main cladogenetic events of the squamate phylogeny were dated using relaxed molecular clock approaches (Welch and Bromham, 2005). A Bayesian estimation of divergence times was performed using Multidivtime (Thorne et al., 1998; Kishino et al., 2001; Thorne and Kishino, 2002). We used the best topology that was inferred from the combined mt protein-coding gene BI analyses (Fig. 2) as the starting phylogeny. Branch lengths of the inferred topology, and divergence times were estimated using PAML and the programs Estbranches and Multidivtime (available at http://statgen.ncsu.edu/thorne/ multidivtime.html). We performed the analysis under a

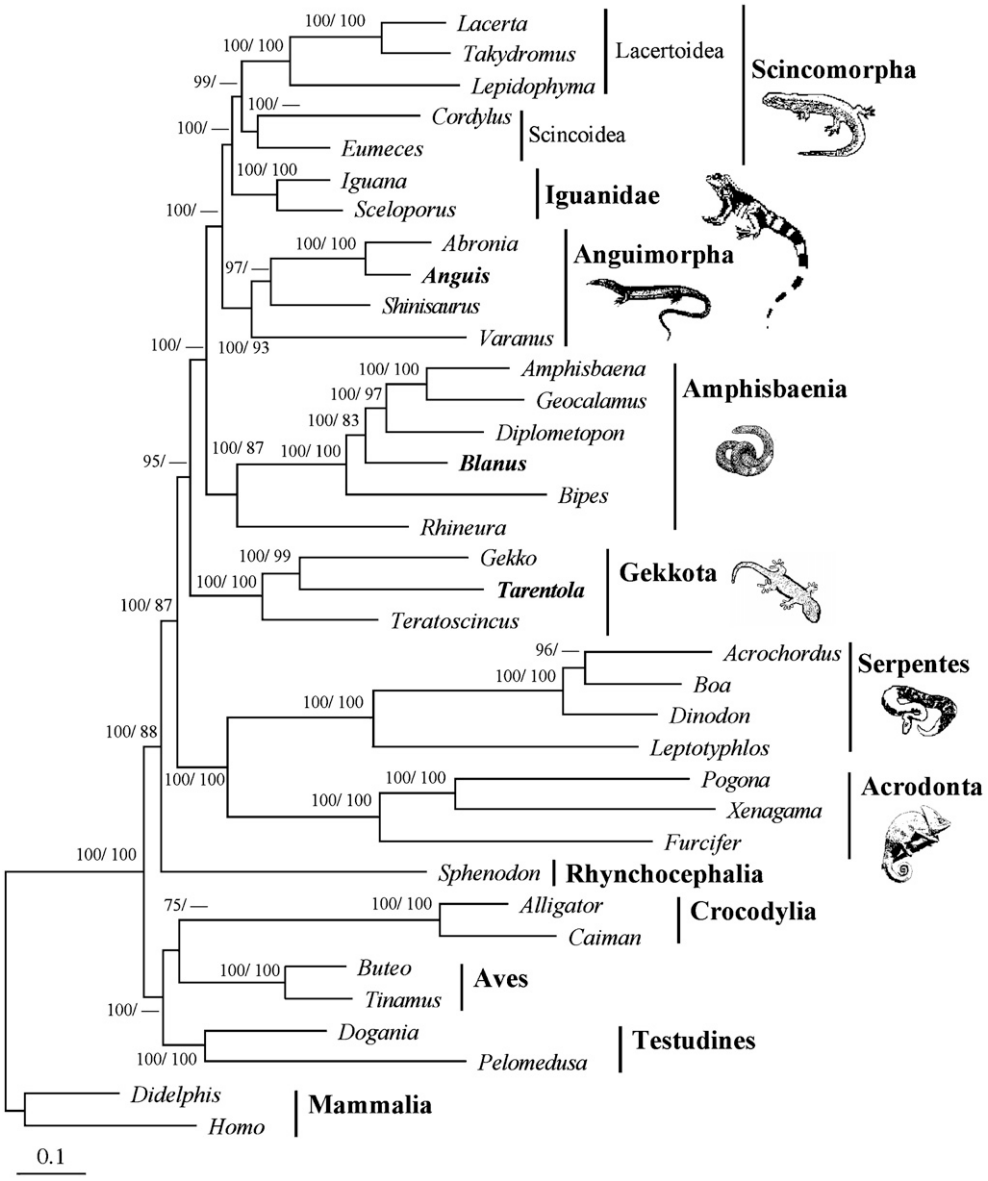

Fig. 2. Squamate phylogenetic relationships based on combined mt protein-coding gene amino acid sequence data (allmt dataset). The BI phylogram is shown. Numbers in the nodes are BI posterior probabilities/ML bootstraps. The main lineages of squamates are indicated. Taxa in bold are the ones sequenced anew for this study.

mtREV (Adachi and Hasegawa, 1996) +Γ model, constructed following the instructions by Yoshinori Kumazawa at the multidivtime website. The MCMC was run for ten million generations, with sampling intervals of 100 generations, and a burn-in period corresponding to the first million generations. The prior assumption for the mean of the time of the ingroup root node (root to tip mean; rttm) was set to 3.123 time units with a standard deviation of 0.20, where 1 time unit in this analysis represents 100 million years (Myr). This value was obtained based on the recommendation by Benton and Donoghue (2007) of the estimated split of synapsids and sauropsids 312.3 million years ago (Mya). We calibrated our time estimates using 12 internal time constraints on nine internal nodes based on fossil evidence: (1) split between Archosauromorpha and Lepidosauromorpha between 299.8 and 259.7 Mya (Benton and Donoghue, 2007), (2) split between Crurotarsi and Ornithodira between 250.4 and 235 Mya (Benton and Donoghue, 2007), (3) split between Palaeognathae and Neognathae between 86.5 and 66 Mya (Benton and Donoghue, 2007), (4) Minimum age for the Pleurodira–Cryptodira split at 210 Mya (fossil record of *Proterochersis*, (Gaffney and Meylan, 1988), (5) Minimum age for the Rhynchocephalia–Squamata split at 227 Mya (Sues and Olsen, 1990), (6) Minimum age for the origin of Anguimorpha at 160 Mya (Evans, 2003), (7) Minimum age for the *Cordylus–Eumeces* split at 65.5 Mya (Krause et al., 2003), (8) Minimum age for the split of Rhineuridae at 60.5 Mya (Sullivan, 1985), and (9) Minimum age for the origin of Colubridae at 33.7 Mya (Rage et al., 1992).

Divergence times were also estimated using a penalized likelihood (PL) approach (Sanderson, 2002) with the program r8s version 1.70 (Sanderson, 2003). We used the best ML topology with branch lengths optimized with PHYML, and the same calibration constraints employed for the Bayesian dating analysis. The truncated Newton (TN) algorithm and the additive penalty function were used for the PL analyses. In order to find the optimal smoothing parameter (λ) for PL, cross-validation was performed over a range of values of λ ranging from 10^0 to $10^{2.8}$ in 15 steps. Confidence intervals were estimated by calculating an age distribution based on chronograms generated from 100 bootstrapped datasets. These datasets were generated with the SEQBOOT module of PHYLIP 3.6 (Felsenstein, 1989), and optimized under a mtREV + Γ + I model of sequence evolution in PHYML.

3. Results and discussion

3.1. Mitochondrial genome organization and structural features

The complete nucleotide sequences of the L-strand of the mt genomes of three squamates were determined anew. The total lengths of the new squamate mt genomes were 17.035 bp for *B. cinereus*, 16.593 bp for *T. mauritanica* and 17.479 bp for *A. fragilis*. These lengths are within the range reported for other squamate mt genomes (Kumazawa, 2007). All three mt genomes encoded for two rRNA, 22 tRNA and 13 protein-coding genes. The gene organization of the three newly determined mt genomes conformed to the vertebrate consensus mt gene arrangement (Boore, 1999; Jameson et al., 2003). However, in the mt genome of *B. cinereus*, the $tRNA^{Pro}$ gene was separated from the $tRNA^{Thr}$ by a tandem repeat.

The differences in length among the three mt genomes determined in this study were mainly due to tandem repetitions located in the control region (as described in other vertebrate mt genomes; see e.g. San Mauro et al., 2004a). The number, length, and motif of tandem repeats in the control region differed across taxa. *B. cinereus* showed seven repeats of 59 bp each at the 5′ end of the control region; *A. fragilis* exhibited 10 repeats of 54 bp each, and five complete (60 bp each) plus one incomplete repeats at the 5′ and 3′ ends of the control region, respectively; *T. mauritanica* showed seven repeats of 75 bp each and 11 complete (66 bp each) plus one incomplete repeats at the 5′ and 3′ ends of the control region, respectively.

The $tRNA^{Cys}$ of *B. cinereus* lacks a DHU stem, and instead shows a D-arm replacement loop, as has been reported in all other amphisbaenians thus far sequenced (Macey et al., 2004). A lack of the DHU stem in the $tRNA^{Cys}$ has been associated with the adjacent presence of a nonfunctional origin of replication of the light strand (O_L) in amphisbaenian bipedids (Macey et al., 2004) and acrodonts (Macey et al., 2000; Amer and Kumazawa, 2005). In contrast to these observations, the O_L folds perfectly into a stem-loop secondary structure in *B. cinereus*, as in amphisbaenian trogonophids and amphisbaenids (Macey et al., 1997, 2004).

3.2. Squamate phylogenetic relationships

Phylogenetic relationships among squamate main lineages were inferred based on the allmt dataset that was analyzed at the amino acid level. The recovered BI tree ($-\ln L = 94{,}708.37$) using best-fit models for the four partitions (mtREV + Γ + I in all cases) is shown in Fig. 2. ML analyses based on the same data set under the mtREV + Γ + I model arrived at the same topology. Mammals were used as outgroup, and turtles were recovered as a sister group to Archosauria (crocodiles + birds; Zardoya and Meyer, 1998; Hedges and Poling, 1999; Kumazawa and Nishida, 1999; Fig. 2). Within Lepidosauria, the tuatara was recovered as a sister group to Squamata (Rest et al., 2003; Townsend et al., 2004; Fig. 2) with high statistical support (ML, 87%; BI, 100%). Within Squamata, the monophyly of Iguanidae, Anguimorpha, Amphisbaenia, Gekkota, Serpentes, and Acrodonta received high statistical support both with BI and ML (Fig. 2). Scincomorpha (including Scincoidea and Lacertoidea) was supported with BI but not with ML. Thus far, the monophyly of Scincomorpha had received support only from morphology (Estes et al., 1988; but see Lee, 1998) whereas no previous molecular phylogenetic analysis (Townsend et al., 2004; Vidal and Hedges, 2005; Kumazawa, 2007) was able to recover it. However, the recovered monophyly of Scincomorpha still remains tentative since it is not robust to changes in the taxon sampling of the outgroup (see below). Phylogenetic relationships among the main squamate lineages could not be resolved with ML but received strong support with BI (above 95%). However, it is important to note that Bayesian posterior probabilities are well known to be significantly higher than corresponding non-parametric bootstrap frequencies leading to overcredibility of the recovered nodes (Suzuki et al., 2002; Erixon et al., 2003). According to the BI tree (Fig. 2), Acrodonta and Serpentes form a clade (see also the NADH2 tree of Townsend et al. (2004), which is the sister group of the remaining squamate lineages. Within these, Gekkota were the first branching out, followed by Amphisbaenia, and a clade including Anguimorpha as sister group of Scincomorpha + Iguanidae (Fig. 2).

As reported in previous molecular studies (Vidal and Hedges, 2005; Böhme et al., 2007; Kumazawa, 2007), the newly reconstructed phylogeny of squamates does not support the Iguania–Scleroglossa split (Estes et al., 1988). According to overall molecular evidence, thus, it is rather arguable that changes in prey capture constitute a major unique evolutionary shift in squamates (Vitt et al., 2003). The sister group relationship of Serpentes + Acrodonta (Böhme et al., 2007; Douglas et al., 2006) and the relative basal position of both taxa within Squamata is in clear disagreement with most previous molecular phylogenetic studies, which placed Acrodonta in a more derived position in the squamate tree as sister group of Iguanidae based on the analyses of both nuclear (Townsend et al., 2004) and mt (but without including Serpentes in the phylogenetic analyses; Kumazawa, 2007) sequence data. Such sister group relationship would be in agreement with the morphology-based Iguania hypothesis (Estes et al., 1988). On the other hand, Serpentes are placed as sister group of either Anguimorpha (Townsend et al., 2004) or Anguimorpha + Iguanidae (Vidal and Hedges, 2005) based on nuclear evidence. Both Acrodonta and Serpentes exhibit relatively long branches in mt-based phylogenies, and their sister group relationship could be due to a long-

Table 1
Log-likelihoods and *p*-values of Approximately Unbiased (AU), Shimodaira–Hasegawa (SH) and Kishino–Hasegawa (KH) tests for each of the seven alternative topologies evaluated

Alternative topologies	−ln *L*	AU	KH	SH
1. Unconstrained tree (Fig. 2)	94,907.216	0.809	0.693	0.978
2. Gekkonidae as most basal squamate lineage	94,911.365	0.439	0.307	0.882
3. Amphisbaenia sister group of Serpentes + Acrodonta	94,919.622	0.251	0.212	0.721
4. Amphisbaenia sister group of Gekkota	94,920.347	0.134	0.105	0.681
5. Amphisbaenia sister group of Scincomorpha	94,931.451	0.031	0.047	0.528
6. Amphisbaenia sister group of Lacertidae	95,015.801	0.001	0.002	0.013
7. Monophyly of Scleroglossa and Iguania	95.086.726	<0.001	<0.001	<0.001
8. Monophyly of Toxicofera	95,153.366	<0.001	<0.001	<0.001

branch attraction artifact (Felsenstein, 1978; see below). Phylogenetic relationships among the remaining squamate lineages as shown in Fig. 2 strongly differed from those recovered by previous studies based on both nuclear (Townsend et al., 2004; Vidal and Hedges, 2005) and mt genome (Böhme et al., 2007; Douglas et al., 2006; Kumazawa, 2007) datasets, particularly regarding the relative phylogenetic position of Lacertidae and Amphisbaenia. In fact, the phylogenetic position of Amphisbaenia is not fully resolved, and varies depending on the study. Morphology-based phylogenetic studies support Amphisbaenia affinities with Dibamidae (Lee, 1998, 2000) or Scincomorpha (Schwenk, 1988). Most molecular analyses recover Lacertidae as the sister group of Amphisbaenia (Townsend et al., 2004; Vidal and Hedges, 2005; Kumazawa, 2007), but in some analyses based on complete mt genome sequence data, Amphisbaenia is grouped with either Gekkota (Zhou et al., 2006) or with Serpentes + Acrodonta (Douglas et al., 2006). More recent analyses based on multiple nuclear loci also failed to conclude firmly on the phylogenetic position of Lacertidae and Amphisbaenians within Squamata (Townsend et al., 2008).

Results of AU, SH, and KH tests of alternative tree topologies (Table 1; Supplementary Material) rejected the Scleroglossa (Estes et al., 1988) and Toxicofera (Vidal and Hedges, 2005; Fry et al., 2006) hypotheses. More generally, any other hypothesis that implied breaking up the Serpentes–Acrodonta clade was strongly rejected (not shown). This may be a consequence of an underlying long-branch attraction effect between Acrodonta and Serpentes (see below), and is in agreement with both the short length of the internodes connecting squamate main lineages, and the generally moderate bootstrap support of basal squamate phylogenetic relationships. The tests also indicated that alternative hypotheses placing Amphisbaenia as a sister group of either Gekkota or Serpentes + Acrodonta were not significantly different from the unconstrained hypothesis whereas putative sister group relationships of Amphisbaenia with either Lacertidae or Scincomorpha were rejected. As indicated by previous studies (San Mauro et al., 2004b), *p*-values from AU and KH are markedly correlated, whereas the SH test is always more conservative.

3.3. Effects of molecular marker choice, outgroup selection, and taxon coverage

Our best phylogenetic hypothesis for squamate relationships (Fig. 2) clearly differed from those previously reported based on different molecular markers, outgroups, and/or taxon samplings. In order to disentangle the relative contribution of these variables to the observed discrepancies, we performed further phylogenetic analyses. Incorporating *X. laevis* into the analyses tested the influence on the recovered topology of using a non-amniote species as outgroup (mt*Xenopus* dataset). The resulting tree was similar to the one shown in Fig. 2, regarding the basal position of Acrodonta + Serpentes, and the subsequent branching out of Gekkota (Fig. 3A). However, the main

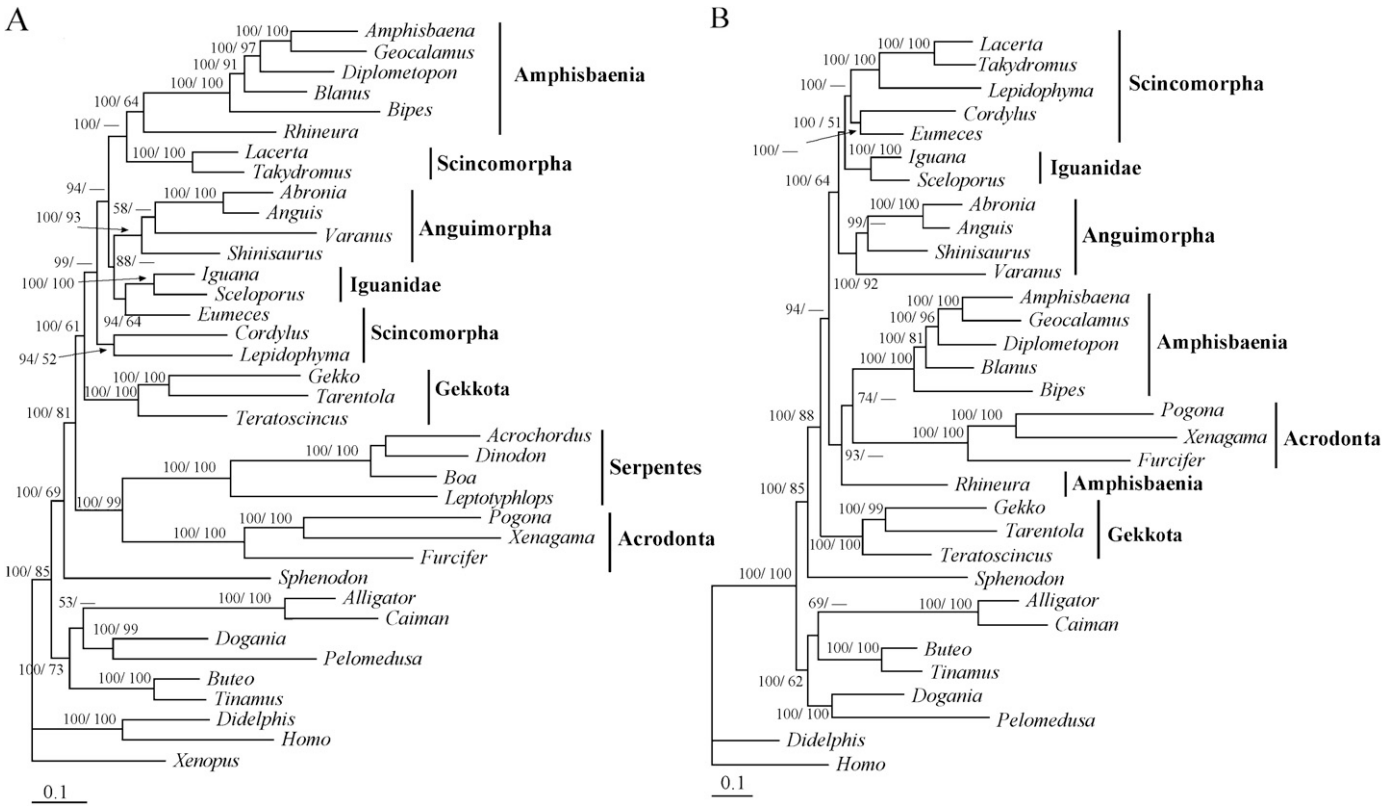

Fig. 3. Testing the effect on the recovered topology of using the amphibian *Xenopus laevis* as outgroup (A) and of removing Serpentes (long branch) from the phylogenetic analyses (B). BI phylograms are shown. Numbers in the nodes are BI posterior probabilities/ML bootstraps. The main lineages of squamates are indicated.

differences affected the monophyly and internal phylogenetic relationships of Scincomorpha since Lacertidae were recovered as a sister group of Amphisbaenia (Kumazawa, 2007), *Eumeces* (Scincidae) as a sister group of Iguanidae, and *Cordylus* (Cordylidae) as a sister group of *Lepidophyma* (Xantusiidae) (Fig. 3A).

As mentioned above, the sister group relationship of Serpentes and Acrodonta supported by the allmt dataset may be related with a long-branch attraction artifact (Townsend et al., 2004; Böhme et al., 2007; Douglas et al., 2006). The effect of removing long-branch taxa from phylogenetic analyses was further explored. If Acrodonta (noA) or both Serpentes and Acrodonta (noSA) were removed from the analyses, no changes in the phylogenetic relationship among the remaining squamate main lineages (as depicted in Fig. 2) were detected (not shown). However, if Serpentes (noS) were removed from the BI analysis, Acrodonta was placed unexpectedly within the Amphisbaenia, breaking up its monophyly (Fig. 3B). The ML analysis of the noS data set rendered a tree with Acrodonta as the most basal squamate lineage, and monophyletic Amphisbaenia (not shown).

The effect of using different taxon samplings (one or two species) per lineage on reconstructing the squamate phylogeny was tested based on either mt (Fig. 4A and B) or nuclear (Fig. 4C and D) sequence data. The mt-based phylogenies recovered Acrodonta + Serpentes as the most basal squamate clade, followed by Gekkota, regardless of whether each squamate lineage was represented by one (mt1), two (mt2) or all available (allmt) species (Figs. 2, 4A and B). However, mt-based phylogenetic relationships among the remaining squamate lineages were highly influenced by taxon sampling (Fig. 4A and B). In contrast to the topology recovered based on the allmt dataset (Fig. 2), Iguanidae was consistently placed as a sister group to Anguimorpha in the phylogenetic analyses based on the mt1 and mt2 datasets (Fig. 4A and B). The relative phylogenetic position of Amphisbaenia was highly dependent not only on including one or two species per lineage in the phylogenetic analyses but also on which species were representing the other squamate lineages (particularly Scincomorpha) (not shown). Similarly, the nuclear dataset recovered Gekkota followed by Scincomorpha as the most basal squamate lineages, as well as the Iguania hypothesis regardless of whether each squamate lineage was represented by one (nuc1), two (nuc2) or all (allnuc) available species (Figs. 2, 4C and D). However, phylogenetic relationships among Anguimorpha, Amphisbaenia, and Serpentes varied dependently on changes in the taxon sampling (Figs. 2, 4C and D).

Our results confirm that long branches exhibited by both Serpentes and Acrodonta constitute the main drawback to accurately reconstruct

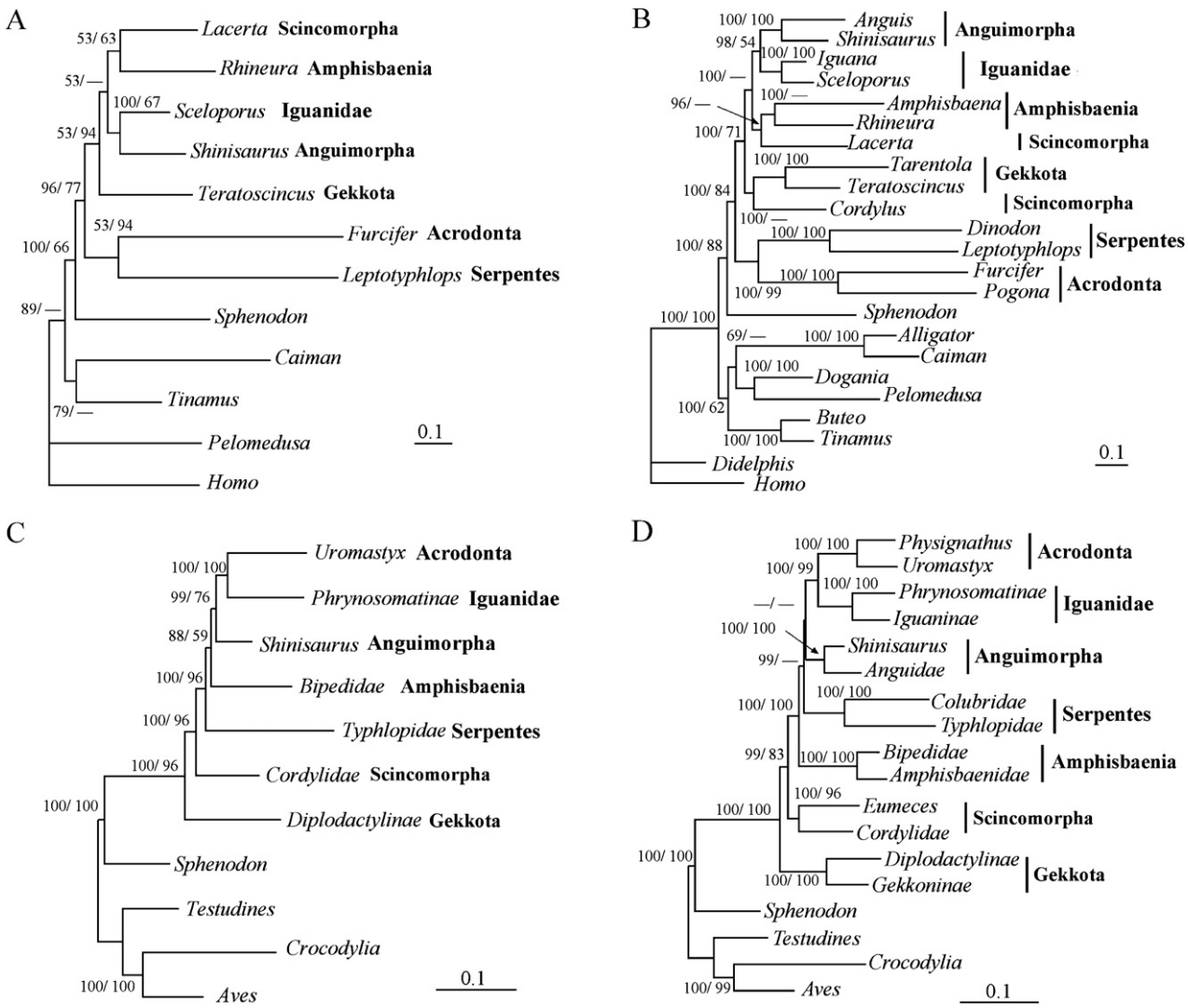

Fig. 4. Testing the effect taxon sampling on the recovered topology. BI phylogenetic analyses were performed either based on mt sequence data using one (A) or two (B) species representing each main squamate lineage or based on nuclear sequence data using one (C) or two (D) species representing each main squamate lineage. BI phylograms are shown. Numbers in the nodes are BI posterior probabilities/ML bootstraps. The main lineages of squamates are indicated.

phylogenetic relationships among squamates based on complete mt genomes. In order to circumvent this shortcoming, it would be desirable to find snake and acrodont species exhibiting slower mt evolutionary rates, in order to incorporate them into the phylogenetic analyses. It would be also important to ensure that all main squamate lineages (e.g. dibamids) are represented in the mitogenomic phylogenetic analyses, and that each lineage encompasses thorough taxon coverage of their diversity (particularly for those lineages such as e.g. Scincomorpha that may not be monophyletic). Given that the phylogeny of Squamata shows relatively short internodes, phylogenetic accuracy could be improved by analyzing more genes (e.g., derived from ongoing nuclear genomic sequencing initiatives; Shedlock et al., 2007) with appropriate evolutionary rates that maximize the number of informative sites.

3.4. Squamate divergence dates

Although no fossil record of Squamata has been found before the early Jurassic, Evans (2003) suggested that the presence of crown-group Rhynchocephalia in the Late Triassic (Sues and Olsen, 1990; Benton and Donoghue, 2007) could provide some evidence for a Triassic (250–206 Mya) origin of Squamata. Molecular clock BI analyses based on mt genome sequence data (four calibration points, Kumazawa, 2007) and nuclear sequence data (five calibration points, Vidal and Hedges, 2005) suggested a Permian origin of Squamata, and dated the radiation of major squamate lineages back to the Triassic–Jurassic times.

Our analyses based on the allmt dataset and nine calibration points rendered similar datings, and estimated that divergence of the main squamate lineages took place in a short window of time of less than 60 Myr around 200 Mya (Fig. 5). This rapid radiation pattern likely causes that internal branches of the Squamata tree are relatively short compared to the (much longer) terminal branches, thus rendering phylogenetic reconstruction of internal relationships particularly challenging. As with most other molecular dating studies (Benton and Ayala, 2003; Reisz and Müller, 2004), our Bayesian time estimates appear to be older than the ages deduced from the fossil record (Evans, 2003). Interestingly, the confidence intervals of the estimates based on PL were significantly shorter than those based on Bayesian analyses (one-way ANOVA $F_{1,64} = 98.81$; $p < 0.001$; Fig. 5), but the mean estimates based on both methods were not significantly different (one-way ANOVA $F_{1,64} = 0.750$; $p = 0.389$; Fig. 5). A strong correlation was detected between PL and Bayesian estimates ($r = 0.99$; see Supplementary material). For most nodes, PL estimates were 10–25 my older than Bayesian estimates. However, for the Acrodonta + Serpentes, Acrodonta, and Serpentes nodes, the PL estimate were

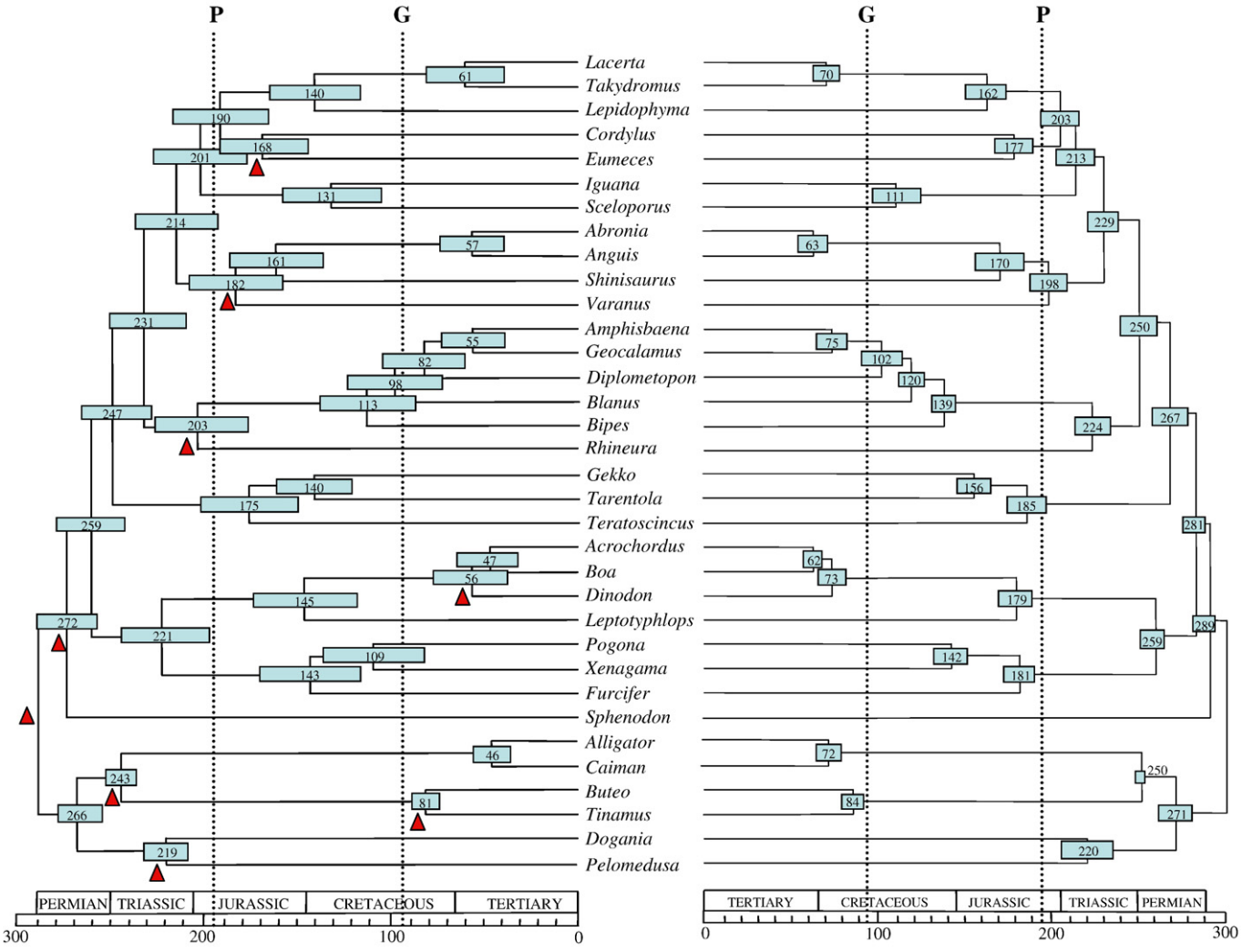

Fig. 5. Estimates of time divergence (mean and confidence interval) for the major lineages of Squamata taxa, estimated based on complete mitochondrial genomes using either a Bayesian (left) or a penalized likelihood (right) approach. Calibration points (triangles) are listed in "Materials and methods". Dotted vertical lines mark the periods of the initial breakup of Pangaea (P) and the breakup of the Gondwana (G). Numbers adjacent to nodes indicate million years ago.

about 40 my older than Bayesian estimates (Fig. 5). Given the use of similar input topologies and calibration constraints, the discrepancy between the two methods may be related to their different assumptions about rate change, and different implementations of models of evolution, branch length and confidence interval estimation, and use of prior information (Welch and Bromham, 2005). The difference in dating between PL and Bayesian methods is exacerbated at the nodes leading to long branches. However, it is not possible based on our results to conclude which, the PL or the Bayesian approach, is better suited to deal with extreme cases of long branches. Moreover, in a recent study (Smith et al., 2006), PL estimates are consistently younger than Bayesian estimates for the same equinoderm dataset. Hence, it is not easy to discern a clear trend on which method provides older or younger ages.

According to the estimated dates, and in agreement with previous studies (Vidal and Hedges, 2005; Kumazawa, 2007), we can conclude that the formation of the major squamate lineages predated the breakup of Pangaea. A similar pattern was found recently for amphibians (San Mauro et al., 2005), and contrasts with the more recent diversification of mammals, which was greatly influenced by continental fragmentation of the Pangea supercontinent during the Cretaceous (Wildman et al., 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2008.05.014.

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