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Complete nucleotide sequence of the mitochondrial genome of a salamander, *Mertensiella luschani*

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Abstract

The complete nucleotide sequence (16,650 bp) of the mitochondrial genome of the salamander *Mertensiella luschani* (Caudata, Amphibia) was determined. This molecule conforms to the consensus vertebrate mitochondrial gene order. However, it is characterized by a long non-coding intervening sequence with two 124-bp repeats between the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes. The new sequence data were used to reconstruct a phylogeny of jawed vertebrates. Phylogenetic analyses of all mitochondrial protein-coding genes at the amino acid level recovered a robust vertebrate tree in which lungfishes are the closest living relatives of tetrapods, salamanders and frogs are grouped together to the exclusion of caecilians (the Batrachia hypothesis) in a monophyletic amphibian clade, turtles show diapsid affinities and are placed as sister group of crocodiles+birds, and the marsupials are grouped together with monotremes and basal to placental mammals. The deduced phylogeny was used to characterize the molecular evolution of vertebrate mitochondrial proteins. Amino acid frequencies were analyzed across the main lineages of jawed vertebrates, and leucine and cysteine were found to be the most and least abundant amino acids in mitochondrial proteins, respectively. Patterns of amino acid replacements were conserved among vertebrates. Overall, cartilaginous fishes showed the least variation in amino acid frequencies and replacements. Constancy of rates of evolution among the main lineages of jawed vertebrates was rejected.

Keywords: Mertensiella; Amphibians; Mitochondrial DNA; Molecular evolution

1. Introduction

Salamanders, the tailed amphibians (order Caudata), may represent a living paradigm of the tempo and mode in which the adaptations that are required to live on land were acquired in the evolution of vertebrates. Many of the 400 or so living salamander species (Frost, 1985) display peculiar feeding, breathing, urogenital, and reproductive apparati, which can be interpreted as alternative or sequential morphological adaptations to terrestrial life that were evolved when sarcopterygian ancestors of amphibians colonized land in the Devonian (360 MYA). Moreover, salamanders are also characterized by a wide variety of gametogenetic, mating, developmental, and parental care strategies (Duellman and Trueb, 1994), which can be perceived as an adaptation to permit reproduction on terrestrial conditions.

To trace and better understand the sequence of evolutionary events that produced the diversity in life modes exhibited by salamanders, it is mandatory first to infer the phylogenetic relationships of the group with other living amphibians (i.e. frogs and caecilians) and with other vertebrates (Titus and Larson, 1995). Therefore, we determined the complete sequence of the mitochondrial genome of an old world salamander, *Mertensiella luschani*. The usefulness of these new mitochondrial sequence data in resolving controversies on amphibian phylogenetic relationships was addressed elsewhere (Zardoya and Meyer, 2001). Here, we present a formal description of the molecular and structural features of the salamander mitochondrial genome and compared it with the complete mitochondrial genomes of other relevant jawed vertebrates.

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2. Materials and methods

2.1. Mitochondrial DNA extraction

Mitochondrial DNA (mtDNA) was purified from eggs and liver of six specimens of the salamander species *M. luschani*, as previously described (Zardoya et al., 1995). After homogenization, mitochondria were separated from intact nuclei and cellular debris by a low-speed centrifugation. Mitochondrial DNA was extracted from isolated mitochondria by a standard alkaline lysis procedure, and cleaved with *Eco*RI and *Hin*dIII restriction enzymes. Two *Eco*RI fragments of 1.3 (comprising the complete control region) and 0.7 (partial ND1) kb, and four *Hin*dIII fragments of 2.2 (including the 3′ end of COI and 5′ end of COII), 0.9 (5′ end of COI), 0.9 (partial ND2), and 0.6 (5′ end of ND1 to tRNA-Met) kb were cloned into pUC18.

2.2. PCR amplification, cloning, and sequencing

The remaining portion of the mitochondrial molecule was amplified by PCR using the mtDNA extract as DNA

Table 1
PCR and sequencing primers utilized in the analysis of the salamander mt genome

Primer	Sequence (5′→3′)	Approximate product length (bp)
PCR		
Sal 12S F	TGT GAA AAT GCC CTT TAT TAC CA	400
Sal 12S R	GTG GCT CGT AGT ACT CTG GCG GA	
L1091[a]	AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT	400
H1478[a]	TGA CTG CAG AGG GTG ACG GGC GGT GTG T	
Sal 12S F1	GAA TGA TTC TAT GAA ATA ATT TC	400
Sal 16S R	TTT GAC TTA CTA GAC CAT TAT GC	
16S-1L[b]	AGT ACC GCA AGG GAA ARC TGA AA	800
16S-2H[b]	GAT TRY GCT ACC TTY GCA CGG TCA	
16Sar[c]	CGC CTG TTT ATC AAA AAC AT	500
16Sbr[c]	CCG GTC TGA ACT CAG ATC ACG T	
Sal 16S F	ATG GTG TAG CCG CTA TTA AAG GT	550
Sal ND1 R	AGA AGG GTA GAG GTA GTG GAA TT	
Sal AT8 F	ATC ACA GCT TCA TAC CAA TTG TT	1200
Sal AT8 R	CAG GAG TAG TGT GGT GTC CTT GA	
LATI COIII F[b]	ATA TAT CAA TGA TGA CGA GA	600
LATI COIII R1[b]	ACA TCA ACG AAA TGT CAG TAC CA	
Sal COIII F	GAA GCC CCA TTC ACA ATC GCA GA	700
Sal ND4 R	TGA GCC GAA ATC ANN GGT CTT	
Sal ND3 F	GAA TGA GTC CAA GGA GGC CTT GAA T	1100
Sal ND4R1	ATT CCG TAG CCT CCG AGT TTT	
ND4[d]	CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC	900
Leu[d]	CAT TAC TTT TAC TTG GAT TTG CAC CA	
LATI Leu F[b]	CTA AAG GAT AAT AGC TCA TCC ATT	500
Sal ND5 R	CAT CAC CCA ATA AGT AAG AAG GA	
LATI ND5 F[b]	CAR YTA TTY ATC GGN TGR GAR GG	650
LATI ND5 R1[b]	CCY ATY TTT CKG ATR TCY TGY TC	
Sal ND5 F	TGT TCT GGG TCA ATT ATT CAC AA	1400
Sal Glu R	TTA ATA ATT TTT AGT AGT GGG TGA	
MVZ-15F[e]	GAA CTA ATG GCC CAC ACW WTA CGN AA	400
H1514g[a]	AAA CTG GAG CCC CTC AGA ATG ATA TTT GTC CTC A	
Sal Cytb F1	TAT AAA GAG ACA TGA AAT AT	1200
Sal Pro R	TTT AGA AGT CTA GAA TTT TGG CCT	
Sequencing		
Sal Hin 2.2 F	TTC TTA TTC TTC CAG GAT TTG GA	
Sal Hin 2.2 F1	TTG CTA TTA TAG GGG GAT TTG TT	
Sal Hin 2.2 R1	TTG GTT ATA TAA TTG ACT TGA	
Sal Hin 2.2 R	GGC TTT AAC TGT TAA GTG AGG AT	
Sal AT8 F1	CCC GCT AAT AGG CCT TGC CCT T	
Sal Eco1.3 R	TTT AGA AGT CTA GAA TTT TGG CCT	

IUB code: R (A/G), Y (C/T), W (A/T), K (G/T).
[a] Kocher et al. (1989).
[b] Zardoya and Meyer (1997).
[c] Palumbi et al. (1991).
[d] Arevalo et al. (1994).
[e] Moritz et al. (1992).

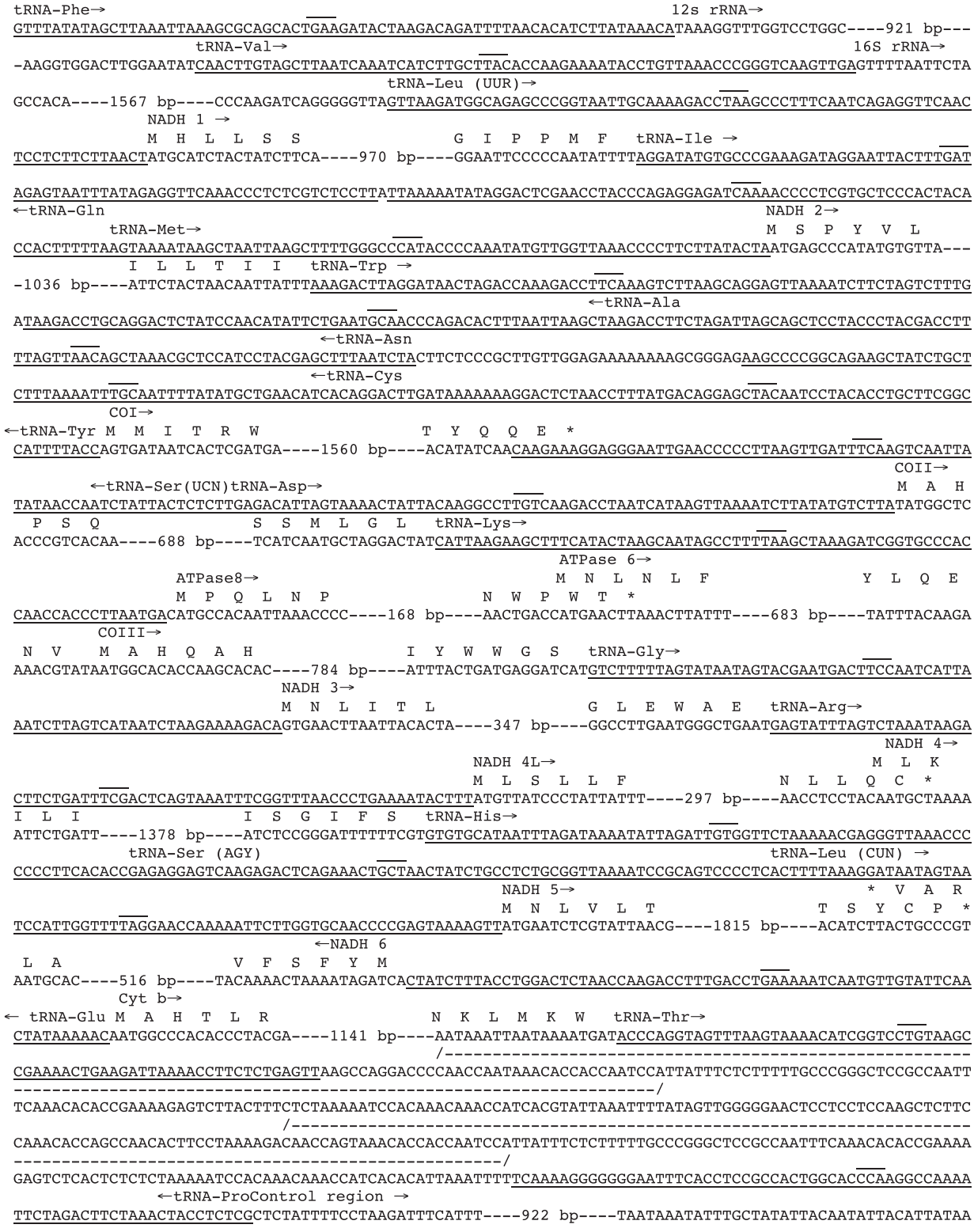


Fig. 1. Schematic representation of the L-strand nucleotide sequence of the salamander mitochondrial genome. Position 1 corresponds to the first nucleotide of the *tRNA-Phe* gene. Direction of transcription for each gene is represented by arrows. The beginning and end of the deduced amino acid sequence for each gene product are shown above the nucleotide sequence (one-letter amino acid abbreviation is placed above the first nucleotide of each codon). Complete termination codons are indicated by an asterisk. Total lengths of each protein-coding gene are shown. tRNA genes are underlined and the corresponding anticodons are overlined. Two repeats are shown between tRNA-*Thr* and tRNA-*Pro* genes.

template source. To this end, highly conserved (Kocher et al., 1989; Palumbi et al., 1991; Moritz et al., 1992; Arevalo et al., 1994; Zardoya and Meyer, 1997), and newly designed PCR primers (Table 1) were used to amplify up to 16 contiguous and overlapping fragments. The PCR amplification was conducted in 25 μl reactions 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, Promega), using the following program: 1 cycle of 2 min at 94 °C, 35 cycles of 60 s at 94 °C, 60 s at 45−50 °C, and 60−105 s at 72 °C, and finally, 1 cycle of 5 min at 72 °C. PCR fragments were cloned into the pGEM-T vector (Promega).

Recombinant plasmids were sequenced using the FS-Taq Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystems) on Applied Biosystems automated DNA sequencers (373A *Stretch* and 377) following manufacturer's protocols. DNA sequences of both strands were obtained using M13 universal (forward and reverse) sequencing primers. The sequences obtained from each clone averaged 450 bp in length and each sequence overlapped the next contig by about 100 bp. In no case were differences in sequence observed between the overlapping regions.

2.3. Molecular and phylogenetic analyses

Sequence data were analyzed with the GCG program package (Devereux et al., 1984), MacClade version 3.06 (Maddison and Maddison, 1992), and PAUP* version 4.0b8 (Swofford, 1998). The average frequency of the different amino acid replacements for the main lineages of vertebrates was estimated with the CHART STATE CHANGES and STASIS option in MacClade based on the species used in the phylogenetic analyses (Appendix A).

To reconstruct a phylogeny of gnathostomes, the deduced amino acid sequences of all salamander mitochondrial protein-coding genes (except ND6) were aligned to homologous sequences of other representative vertebrates (Appendix A) using CLUSTAL W (Thompson et al., 1994). Alignments were refined by eye and gaps were treated as missing data. Ambiguous alignments were determined using the Gblocks v0.73b program (Castresana, 2000) and were excluded from the phylogenetic analyses.

The data set was analyzed with maximum parsimony (MP), minimum evolution (ME), maximum likelihood (ML), and Bayesian methods of phylogenetic inference. MP analyses (PAUP* version 4.0b8; Swofford, 1998) were performed using heuristic searches (TBR branch swapping; MulTrees option in effect) with 10 random stepwise additions of taxa. ME analyses (Rzhetsky and Nei, 1992) were performed using mean distances in PAUP* version 4.0b8 (Swofford, 1998). ML analyses were performed in PUZZLE version 4.0.1 (Strimmer and von Haeseler, 1996) using the mtREV model (Adachi and Hasegawa, 1996). Bayesian analysis was performed in MrBayes version 2.01 (Huelsenbeck and Ronquist, 2001) using the JTT model (Jones et al., 1992). Robustness of the inferred trees was tested by non-parametric bootstrapping (Felsenstein, 1985), quartet puzzling, and Bayesian posterior probability analyses, as implemented in PAUP*, PUZZLE, and MrBayes with 500 pseudoreplications, 1000 quartet puzzling steps, and 100,000 generations, respectively.

Constancy of rates of evolution among vertebrates was estimated with a log likelihood ratio test and the mtREV model (Adachi and Hasegawa, 1996) by using PUZZLE version 4.0.1 (Strimmer and von Haeseler, 1996). To determine which groups were evolving at significantly different rates, a relative ratio test with Poisson-corrected distances was performed by using Phyltest version 2.0 (Kumar, 1996).

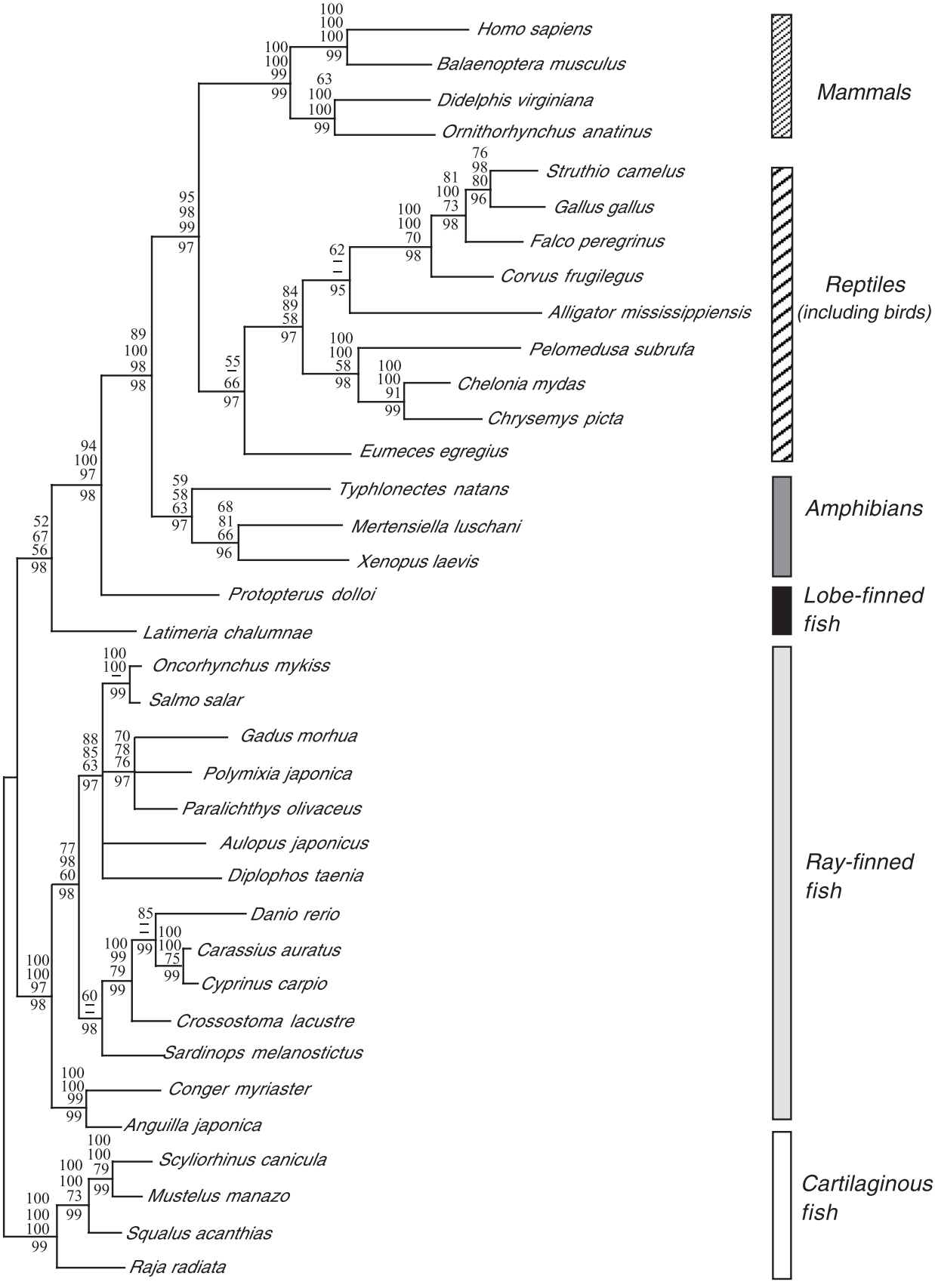
The complete nucleotide sequence of the salamander mt genome was deposited in GenBank under accession number AF154053.

3. Results and discussion

3.1. Organization of the salamander mitochondrial genome

The mitochondrial genome of the salamander *M. luschani* is 16,650 bp long and conforms to the consensus vertebrate mitochondrial gene order (Fig. 1). As in other vertebrates, 2 rRNAs, 22 tRNAs, and 13 proteins are encoded by the salamander mitochondrial genome (Fig. 1). The overall base composition of the L-strand is A: 32%, T: 29%, C: 24%, and G: 15%. There are only nine noncoding intergenic spacer nucleotides. Interestingly, this paucity of intergenic spacers contrasts with the existence of a long non-coding stretch of 320 bp separating $tRNA^{Thr}$ and $tRNA^{Pro}$ genes (Fig. 1). This intervening sequence includes two 124-bp repeats. Both repeats differ in five transitions. No significant matches for the repeat motif were found in a GenBank search, so its evolutionary origin is unknown. Similar cases of intervening noncoding sequences between two tRNAs have been documented in two birds, *Falco peregrinus* and *Smithornis sharpei* (Mindell et al., 1998). In both cases, the stretch is located between $tRNA^{Glu}$ and $tRNA^{Phe}$ genes. In *F. peregrinus*, the intervening sequence also contains repeats. Interestingly, in both bird mitochondrial genomes the

Fig. 2. Majority rule (50%) consensus trees depicting phylogenetic relationships of jawed vertebrates. A data set combining the deduced amino acid sequences of all mitochondrial protein-coding genes (except ND6) and of 36 representative vertebrate taxa was subjected to MP (bootstrap values based on 500 bootstrap pseudoreplications; upper value of each triplet of numbers above the nodes), NJ (bootstrap values based on 500 bootstrap pseudoreplications; middle value of each triplet of numbers above the nodes), ML (quartet puzzling support values based on 1000 puzzling steps; lower value of each triplet of numbers above the nodes), Bayesian (posterior probabilities based on 100,000 generations; numbers below the nodes) analyses. Cartilaginous fishes were used as outgroup taxa.


Homo sapiens
Balaenoptera musculus
Didelphis virginiana
Ornithorhynchus anatinus
Mammals
Struthio camelus
Gallus gallus
Falco peregrinus
Corvus frugilegus
Alligator mississippiensis
Reptiles
(including birds)
Pelomedusa subrufa
Chelonia mydas
Chrysemys picta
Eumeces egregius
Typhlonectes natans
Mertensiella luschani
Xenopus laevis
Amphibians
Protopterus dolloi
Lobe-finned
fish
Latimeria chalumnae
Oncorhynchus mykiss
Salmo salar
Gadus morhua
Polymixia japonica
Paralichthys olivaceus
Aulopus japonicus
Diplophos taenia
Danio rerio
Carassius auratus
Cyprinus carpio
Crossostoma lacustre
Sardinops melanostictus
Conger myriaster
Anguilla japonica
Ray-finned
fish
Scyliorhinus canicula
Mustelus manazo
Squalus acanthias
Raja radiata
Cartilaginous
fish

tRNA^{Thr} and tRNA^{Pro} genes are separated by the control region. The origin of these rearrangements in the bird mitochondrial genomes seems to be related with the translocation capacity of the involved tRNA genes and their close proximity to the origin of the heavy-strand replication within the control region (Mindell et al., 1998). A similar mechanism might be involved in the origin of the intervening sequence that separates salamander mitochondrial tRNA^{Thr} and tRNA^{Pro} genes.

The control region of the salamander mitochondrial genome is 922 bp long. Both conserved sequence blocks (CSB-1, CSB-2, and CSB-3; Walberg and Clayton, 1981) and termination-associated sequences (TAS-1 and TAS-2; Doda et al., 1981) were identified in the salamander mitochondrial control region. Interestingly, the salamander CSB-1 shares high similarity to the vertebrate CSB-1 consensus, and it is not reduced to a truncated pentamotif (5′-GACAT-3′) as in the frog, and the caecilian (Zardoya and Meyer, 2000). As in most vertebrates, the origin of light strand replication (O_L) of the salamander mitochondrial genome, is located in a cluster of five tRNA genes (WANCY region) between tRNA^{Asn} and tRNA^{Cys} (Fig. 1) (but see Seutin et al., 1994). This region is 34 nucleotides in length and has the potential to fold into a stem-loop secondary structure (not shown). The folding of the O_L does not require to use a considerable part of the adjacent tRNA^{Cys} as has been described for the caecilian (Zardoya and Meyer, 2000). The salamander O_L loop, as in other tetrapods, contains a stretch of thymines that is needed for the initiation of L-strand synthesis (Wong and Clayton, 1985).

The salamander *12S* and *16S rRNA* genes are 921 and 1567 nucleotides long, respectively (Fig. 1). Our salamander *12S rRNA* gene sequence showed only minor differences (96% similarity) to that previously reported (Titus and Larson, 1995). The salamander tRNAs ranged in size from 65 to 75 nucleotides, and showed size variability in their DHU and TψC arms when compared to other vertebrate mitochondrial tRNAs. Most salamander mitochondrial protein-coding genes begin with a ATG start codon. However, *COI* and *ND3* start with GTG, and *ND6* with ATC. Most salamander ORFs end with incomplete stop codons, either T (*ND1*, *ND2*, *COII*, *COIII*, *ND3*, *ND4*, and *Cyt b*) or TA

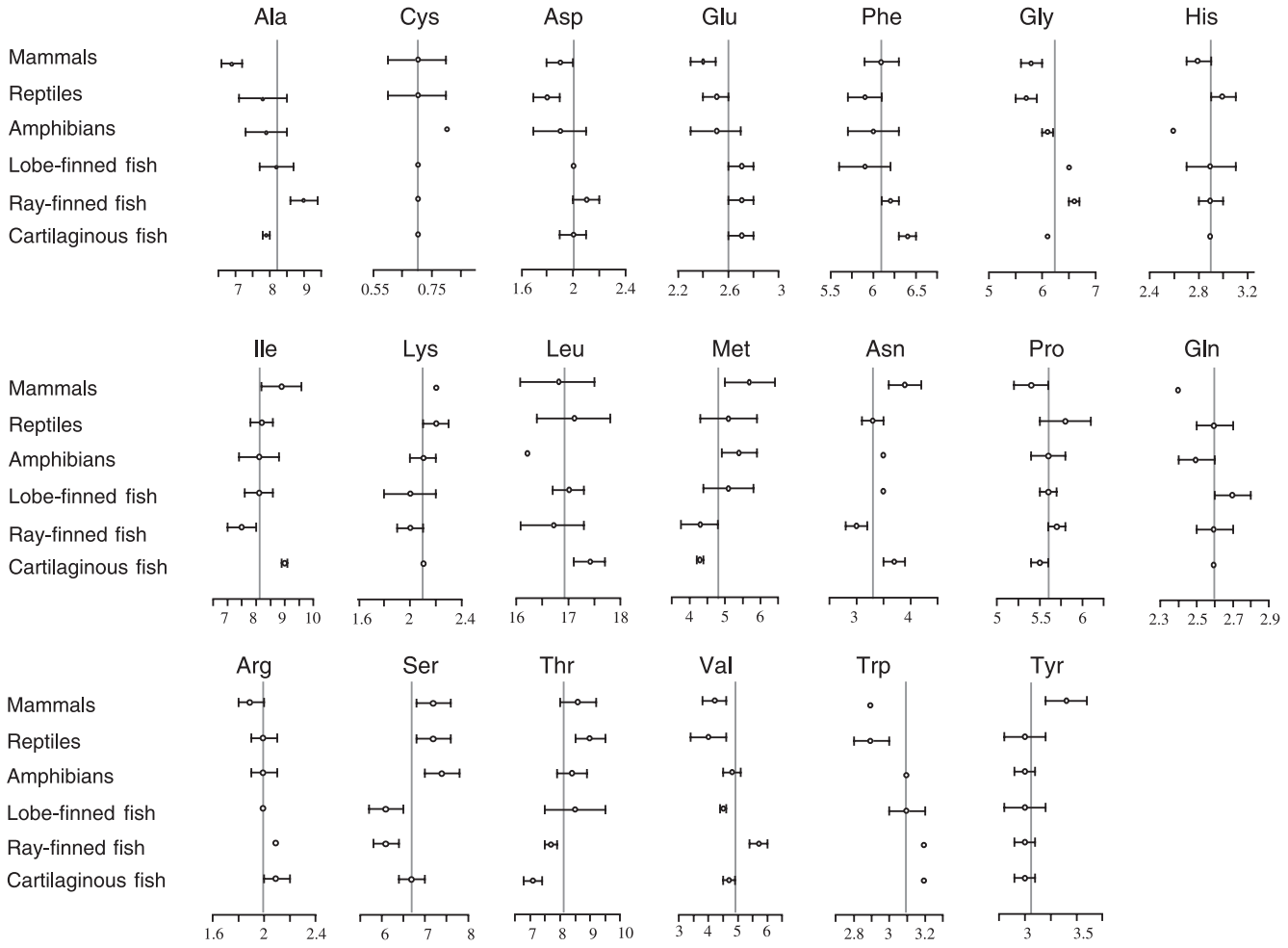


Fig. 3. Mean frequencies (%) and standard deviations of the different amino acids in the main lineages of vertebrates. The grey bars indicate the estimated empirical amino acid frequencies incorporated into the evolutionary model used for the phylogenetic analyses.

(*ATPase 6*). *COI* ends with AGG, *ND6* with AGA, and *ATPase 8*, *ND4L*, and *ND5* with TAA.

3.2. Phylogenetic relationships among jawed vertebrates

The deduced amino acid sequences of all mitochondrial protein-coding genes (except ND6) of 36 representative vertebrate taxa (see Appendix A) were combined into a single data set. We did not include *Rana nigromaculata* (Sumida et al., 2001) in the analyses because this species has a extremely long branch that produces phylogenetic reconstruction artifacts (results not shown). An alignment of 3676 positions was produced. A total of 416 positions were excluded because of ambiguity, 1176 were invariant, and 1609 were parsimony-informative. One single most parsimonious tree of 13,355 steps was recovered when cartilagionous fishes were used as outgroup (Fig. 2). In this tree, eels are the most basal lineage within a monophyletic teleost group (Inoue et al., 2001b; Miya et al., 2001), lungfishes are the sister group of tetrapods (Zardoya et al., 1998), and amphibians are recovered as a monophyletic group at the base of tetrapods (Zardoya and Meyer, 2001). Moreover, turtles show diapsid affinities and are placed as the sister group of archosaurs (crocodiles and birds) (Zardoya and Meyer, 1998; Kumazawa and Nishida, 1999), and marsupials and monotremes form a monophyletic group (Janke et al., 1996) (Fig. 2). Among amphibians, a sistergroup relationship of salamanders and frogs (the Batrachia hypothesis) to the exclusion of caecilians is recovered (Zardoya and Meyer, 2001) (Fig. 2). ME, ML, and Bayesian phylogenetic analyses arrived at similar and congruent topologies (Fig. 2). The robustness of the results was confirmed by high bootstrap (quartet puzzling and Bayesian posterior probability) support of most of the nodes in the tree (Fig. 2).

Most of the phylogenetic relationships recovered in the molecular tree are in agreement with current morphological and paleontological evidence. The basal position of anguilliformes within teleosts is well supported by morphological data (Nelson, 1994). Despite there has been a long-standing controversy on which is the closest living sister group of tetrapods, the latest morphological and paleontological analyses favor lungfishes as the closest relatives of tetrapods (Cloutier and Ahlberg, 1997). Most phylogenetic analyses based on morphological characters support the batrachia hypothesis (Trueb and Cloutier, 1991). Traditionally, turtles have been considered the only survivors of anapsid reptiles. However, recent studies based on reptile morphology strongly support the diapsid affinities of turtles (Rieppel

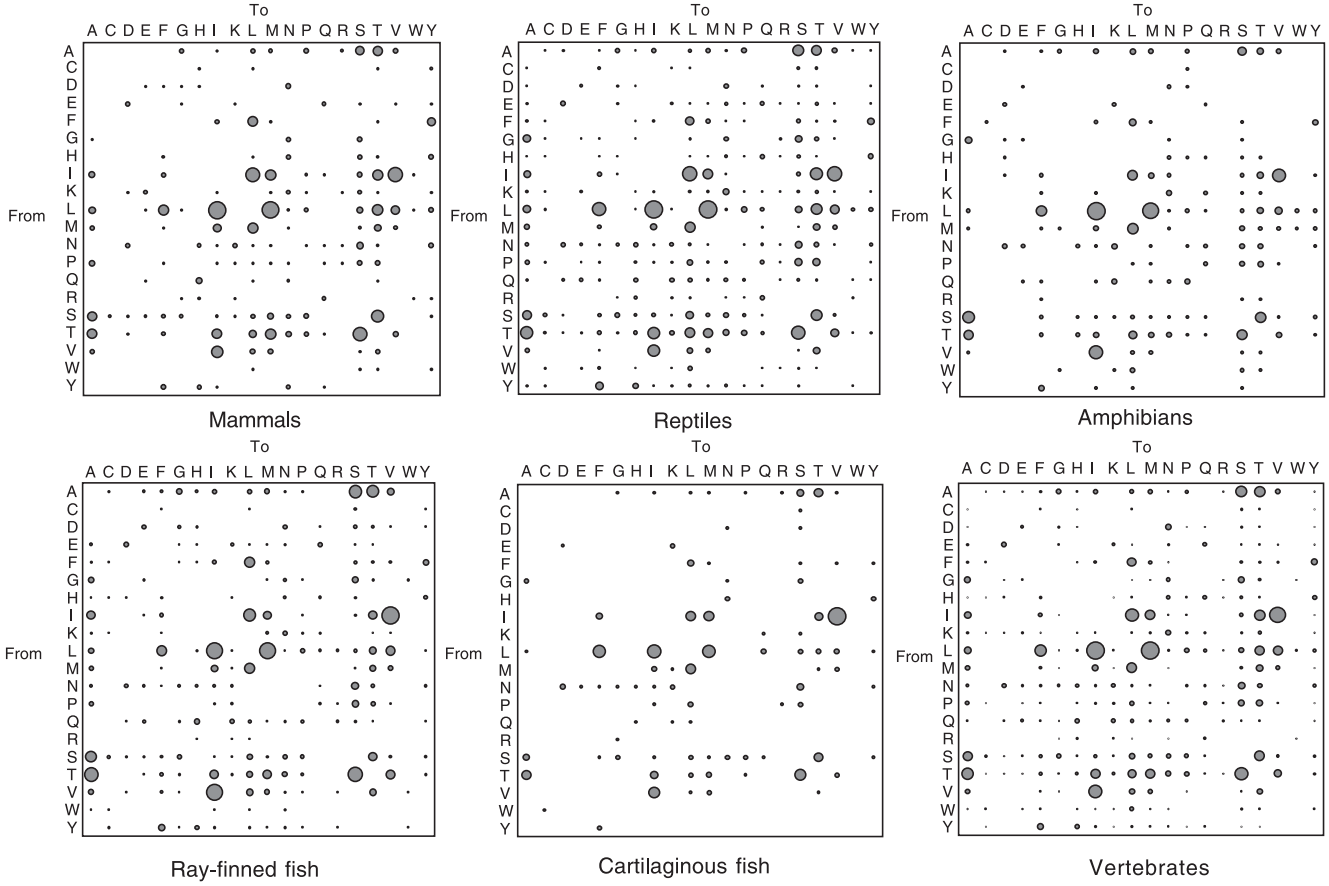


Fig. 4. The average frequency of different amino acid replacements for the main lineages of vertebrates. The diameter of the largest circle equals 485 changes and other circles are scaled in diameter proportionally.

A	Mammals	Reptiles	Amphibians	Lobe-finned	Ray-finned
Mammals	–				
Reptiles	2.29*	–			
Amphibians	9.35*	8.71*	–		
Lobe-finned fish	13.45*	13.38*	5.89*	–	
Ray-finned fish	18.91*	20.47*	13.57*	7.80*	–

B	*Homo*	*Balaenoptera*	*Didelphis*	*Ornithorhynchus*
Homo	–			
Balaenoptera	5.24*	–		
Didelphis	3.69*	1.09	–	
Ornithorhynchus	3.92*	0.84	0.31	–

C	*Struthio*	*Gallus*	*Falco*	*Corvus*	*Alligator*	*Pelomedusa*	*Chelonia*	*Chrysemis*	*Eumeces*
Struthio	–								
Gallus	2.12*	–							
Falco	1.78	1.92	–						
Corvus	0.48	1.38	0.42	–					
Alligator	7.02*	6.00*	7.07*	6.78*	–				
Pelomedusa	7.18*	5.97*	7.07*	6.87*	0.15	–			
Chelonia	3.51*	4.86*	3.51*	3.72*	10.3*	11.22*	–		
Chrysemis	0.61	0.64	0.60	0.30	6.48*	7.22*	5.81*	–	
Eumeces	3.06*	4.26*	2.98*	3.32*	9.40*	9.76*	0.37	3.63*	–

D	*Typhlonectes*	*Mertensiella*	*Xenopus*
Typhlonectes	–		
Mertensiella	3.43*	–	
Xenopus	3.48*	0.09	–

E	*Protopterus*	*Latimeria*
Protopterus	–	
Latimeria	4.01*	–

F	*Oncorhynchus*	*Salmo*	*Gadus*	*Paralichthys*	*Polymixia*	*Aulopus*	*Diplophos*	*Danio*	*Carassius*	*Cyprinus*	*Crossostoma*	*Sardinops*	*Conger*	*Anguilla*
Oncorhynchus	–													
Salmo	2.19*	–												
Gadus	4.61*	5.44*	–											
Paralichthys	4.40*	5.35*	0.22	–										
Polymixia	1.43	2.53*	3.72*	3.49*	–									
Aulopus	1.47	2.57*	3.54*	3.29*	0.62	–								
Diplophos	4.23	5.19*	0.61	0.40	2.91*	2.89*	–							
Danio	5.18*	5.97*	1.12	1.35	4.16	4.15*	1.69	–						
Carassius	1.17	0.20	5.30*	5.19*	2.51*	2.42*	4.79*	7.23*	–					
Cyprinus	0.44	1.40	3.82*	3.72*	0.83		3.29*	5.50*	3.57*	–				
Crossostoma	2.48*	3.48	2.03*	1.81	1.25	1.22	1.44	3.43*	4.56*	2.51*	–			
Sardinops	2.83*	3.88*	1.82	1.66	1.54	1.55	1.27	2.93*	3.97*	2.32*	0.24	–		
Conger	5.26*	6.13*	1.06	1.26	4.25*	4.18*	1.65	0.09	6.21*	4.74*	3.09*	2.78*	–	
Anguilla	0.93	1.91	3.52*	3.27*	0.32	0.31	2.91*	4.50*	2.09*	0.50	1.54	1.74	5.23*	–

Fig. 5. Evolutionary rates of the main lineages of vertebrates and their corresponding members. The *Z* statistics of the relative rate tests performed among (A) and within main lineages of vertebrates (B, mammals; C, reptiles; D, amphibians; E, lobe-finned fish; F, ray-finned fish) are shown. An asterisk denotes those cases in which rate constancy was rejected at the 5% significance level.

and deBraga, 1996). The only important conflict between molecules and morphology is on the phylogenetic relationships of mammals. Mitochondrial data support a close sister group relationship of marsupials and monotremes, the marsupionta hypothesis (Gregory, 1947), whereas most paleontological and morphological data favor a close relationships of marsupials and placental mammals to the exclusion of monotremes (Carroll, 1988). Recently, a large nuclear gene, the mannose 6-phosphate/insulin-like growth factor II receptor, was isolated and sequenced from various representatives of monotremes, marsupials, and placental mammals (Killian et al., 2001). Nuclear gene sequence data support marsupials as the sister group of placentals to the exclusion of monotremes (Killian et al., 2001).

Our results reject earlier phylogenetic studies based on mitochondrial sequence data that used lamprey or hagfish as outgroup taxa, and recovered rather unorthodox vertebrate phylogenies (Rasmussen and Arnason, 1999a,b). Strong support of the vertebrate tree both from morphology and molecules provides a phylogenetic framework that will be helpful in interpreting the results of many comparative studies of living vertebrates.

3.3. Comparative vertebrate mitogenomics

The average frequencies of different amino acids for each of the main lineages of jawed vertebrates are shown in Fig. 3. Leucine is the most abundant amino acid in mitochondrial proteins, whereas cysteine is the rarest. The relative frequency of amino acids in mitochondrial proteins is in agreement with their hydrophobic properties and membrane location (Naylor et al., 1995). The relative proportion of Ile, Lys, Met, Ser, and Thr in mitochondrial proteins increases from fish to mammals. In contrast the relative frequency of Ala, Glu, Gly, Arg, and Trp in mitochondrial proteins apparently decreases from fish to mammals. The remaining amino acids maintain stable frequencies among vertebrates. Cartilaginous fishes showed the least variation in amino acid frequencies.

Amino acid replacements were found to be relatively constant among main vertebrate lineages (Fig. 4) and might indicate functional and structural constraints of mitochondrial proteins. The most frequent amino acid changes were Leu to Ile, leu to Met, Ile to Val, Val to Ile, Ala to Ser, and Thr to Ser. Cys and Arg are barely replaced in vertebrates. Almost no amino acid replacement yields Trp. Amino acid replacements were less frequent in cartilaginous fishes compared to other vertebrates. This phenomenon may be directly related to the observed slow rates of evolution of shark mitochondrial proteins (Martin et al., 1992).

Evolutionary rate constancy among the main vertebrate lineages was tested. A log likelihood ratio test between the most likely trees with and without the assumption of a molecular clock was performed. The clock-like tree was rejected on a significant level of 5%. None of the main lineages of vertebrates evolve at the same rate (Fig. 5A). Moreover, most of the vertebrate taxa analyzed show heterogeneous rates of evolution (Fig. 5B–E). Hence, it was not possible to estimate divergence dates for the main lineages of vertebrates under the assumption of a molecular clock.

In conclusion, we have determined the complete sequence of the mitochondrial genome of a salamander and used it to infer phylogenetic relationships among the main lineages of vertebrates and to characterize general trends in the molecular evolution of vertebarte mitochondrial proteins. Our results show slight differences in amino acid frequencies of mitochondrial proteins among vertebrate main lineages but a fairly constant amino replacement pattern across all vertebrate groups. Moreover, vertebrate taxa are characterized by significantly different rates of evolution with few exceptions. Despite these putative biases, phylogenetic inference based on different methods and a combined mitochondrial amino acid sequence data set recovered a strongly supported vertebrate phylogeny that is in agreement with morphological and paleontological evidence.

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Appendix A

Phylogenetic analyses of jawed vertebrate relationships included the following 36 complete vertebrate mitochondrial DNA genomes: Raja, *Raja radiata* (AF106038, Rasmussen and Arnason, 1999b); spiny dogfish, *Squalus acanthias* (Y18134, Rasmussen and Arnason, 1999a); gummy shark, *Mustelus manazo* (AB015962, Cao et al., 1998); dogfish, *Scyliorhinus canicula* (Y16067, Delarbre et al., 1998); Japanese eel, *Anguilla japonica* (AB038556, Inoue et al., 2001a); conger eel, *Conger myriaster* (AB038381, Inoue et al., 2001b); Japanese sardine, *Sardinops melanostictus* (AB032554, Inoue et al., 2000); rainbow trout, *Oncorhynchus mykiss* (L29771, Zardoya et al., 1995); Atlantic salmon, *Salmo salar* (U12143, Hurst et al., 1999); codfish, *Gadus morhua* (X99772, Johansen and Bakke, 1996); carp, *Cyprinus carpio* (X61010, Chang et al., 1994); loach, *Crossostoma lacustre* (M91245, Tzeng et al., 1992); gold-

fish, *Carassius auratus* (AB006953, Murakami et al., 1998); zebrafish, *Danio rerio* (AC024175, J.E. Milam, R.E. Broughton, and B.A. Roe, unpublished); Japanese flounder, *Paralichthys olivaceus* (AB028664, Saitoh et al., 2000); Japanese thread-sail fish, *Aulopus japonicus* (AB047821, Kawaguchi et al., 2001); Pacific portholefish, *Diplophos taenia* (AB034825, Miya and Nishida, 2000); silvereye fish, *Polymixia japonica* (AB034826, Miya and Nishida, 2000); African lungfish, *Protopterus dolloi* (L42813, Zardoya and Meyer, 1996); coelacanth, *Latimeria chalumnae* (U82228, Zardoya and Meyer, 1997); clawed frog, *Xenopus laevis* (M10217, Roe et al., 1985); caecilian, *Typhlonectes natans* (AF154051, Zardoya and Meyer, 2000); salamander, *M. luschani* (AF154053, Zardoya and Meyer, 2001 and this work); skink, *Eumeces egregius* (AB016606, Kumazawa and Nishida, 1999); side-necked turtle, *Pelomedusa subrufa* (AF039066, Zardoya and Meyer, 1998); green turtle, *Chelonia mydas* (AB012104, Kumazawa and Nishida, 1999); painted turtle, *Chrysemys picta* (AF069423, Mindell et al., 1999); alligator, *Alligator mississippiensis* (Y13113, Janke and Arnason, 1997); ostrich, *Struthio camelus* (Y12025, Härlid et al., 1997); Falcon, *F. peregrinus* (AF090338, Mindell et al., 1999); Rook, *Corvus frugilegus* (Y18522, Härlid and Arnason, 1999); chicken, *Gallus gallus* (X52392, Desjardins and Morais, 1990); platypus, *Ornithorhynchus anatinus* (X83427, Janke et al., 1996); opossum, *Didelphis virginiana* (Z29573, Janke et al., 1994); blue whale, *Balaenoptera musculus* (X72204, Arnason and Gullberg, 1993); and human, *Homo sapiens* (D38112, Horai et al., 1995).

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