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Complete mitochondrial genome of *Nanorana pleskei* (Amphibia: Anura: Dicroglossidae) and evolutionary characteristics

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Abstract The complete mitochondrial genome of *Nanorana pleskei* from the Qinghai-Tibet Plateau was sequenced. It includes 17,660 base pairs, containing 13 protein-coding genes, two *rRNA*s and 23 *tRNA*s. A tandem duplication of $tRNA^{Met}$ gene was found in this mitochondrial genome, and the similarity between the two $tRNA^{Met}$ genes is 85.8%, being the highest in amphibian mitochondrial genomes sequenced thus far. Based on gene organization, 24 types were found from 145 amphibian mitochondrial genomes. Type 1 was present in 108 species, type 11 in 11 species, types 5, 16, 17, and 20 each in two species, and the others each present in one species. Fifteen types were found in Anura, being the most diversity in three orders of the Lissamphibia. Our phylogenetic results using 11 protein-coding gene sequences of 145 amphibian mitochondrial genomes strongly support the monophyly of the Lissamphibia, as well as its three orders, the Gymnophiona, Caudata, and Anura, among which the relationships were ((Gymnophiona (Caudata, Anura)). Based on the phylogenetic trees, type 1 was recognized as the ancestral type for amphibians, and type 11 was the synapomorphic type for the Neobatrachia. Gene rearrangements among lineages provide meaningful phylogenetic information. The rearrangement of the *LTPF tRNA* gene cluster and the translocation of the *ND5* gene only found in the Neobatrachia support the monophyly of this group; similarly, the tandem duplication of the $tRNA^{Met}$ genes only found in the Dicroglossidae support the monophyly of this family [*Current Zoology* 57 (6): 785–805, 2011].

Keywords *Nanorana pleskei*, Complete mitochondrial genome, Tandem duplication of $tRNA^{Met}$ genes, Mitochondrial genome type, Phylogenetic, Amphibia

The mitochondrial genome of vertebrates is a closed circular molecule that is generally 16 kb in size (varying from 15 kb to 21 kb). Vertebrate mitochondrial genomes usually encode 13 protein-coding genes, two *rRNA* genes, 22 *tRNA* genes, the control region (CR) that contains information for regulating and initiating mtDNA replication and transcription, and a short non-coding sequence referred to as the "light-strand replication origin" (OL) (Pereira, 2000). The arrangement of genes in the mitochondrial genome is conserved among vertebrates, with genes and the CR being organized in relatively the same order (Anderson et al., 1981; Roe et al., 1985; Desjardins and Morais, 1990; Wolstenholme, 1992; Boore, 1999; Tzeng et al., 1992; Inoue et al., 2003). However, gene rearrangement, pseudogenes, duplications and deletions of genes in the mitochondrial genome are increasingly being detected as research progresses (San Mauro et al., 2006; Mindell et al., 1998; Miya et al., 2001; Kumazawa and Endo, 2004; Kurabayashi et al., 2010).

As at December 6 2010 there were 97 species of amphibians for which the complete mitochondrial genome is available, and 47 species for which the mitochondrial genome is sequenced with little gaps (San Mauro et al., 2004a; Mueller et al., 2004; Kurabayashi et al., 2010; Zhang and Wake, 2009a; Zhang et al., 2005). Gene rearrangements have been frequently found in this group. Rearrangements of the gene cluster including $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$ in turn and being abbreviated as *WANCY* have been found in Gymnophiona and Caudata (San Mauro et al., 2006; Mueller et al., 2004). The *TLPF* gene cluster including $tRNA^{Leu}$ (CUN), $tRNA^{Thr}$, $tRNA^{Pro}$ and $tRNA^{Phe}$ in turn is rearranged in neobatrachian families (Sumida et al., 2001; Cao et al., 2006; Arnason et al., 2004). Rearrangement of the

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ND5 gene has been found in *Euphlyctis hexadactylus* (Alam et al., 2010), *Hoplobatrachus tigerinus* (Alam et al., 2010) and *Fejervarya* (Liu et al., 2005) of Dicroglossidae, the Mantellidae (Kurabayashi et al., 2006) and Rhacophoridae (Sano et al., 2005). Two $tRNA^{Met}$ genes have been reported in the Dicroglossidae (Liu et al., 2005; Ren et al., 2009; Zhang et al., 2009; Zhou et al., 2009) and *Mantella* of the Mantellidae (Kurabayashi et al., 2006). In addition, five species, *Euphlyctis hexadactylus* (Alam et al., 2010), *Hoplobatrachus tigerinus* (Alam et al., 2010), *Rhacophorus schlegelii* (Sano et al., 2005), *Mantella madagascariensis* (Kurabayashi et al., 2006) and *Aneides hardii* (Mueller et al., 2004), possess duplicated CR, and *Polypedates megacephalus* has lost the *ATP8* and *ND5* genes (Zhang et al., 2005).

Because of its high abundance in cells, fast mutation rate, maternal inheritance and limited recombination (Curole and Kocher, 1999; Zardoya and Meyer, 2000; Saccone et al., 1999), mtDNA has been widely used in studies of the phylogenetics and phylogeography of various taxa. Variations such as gene rearrangements, genetic code changes, simplification of *tRNA* and *rRNA* structures, peculiar mechanisms of replication and transcription, and base compositional bias, have occurred in a lineage-specific manner and are expected to lead to new findings (Pereira, 2000; Gissi et al., 2006; Kurabayashi et al., 2010). It's worthy to research phylogenetic meaning of these variations.

The genus *Nanorana* includes *N. pleskei*, *N. parkeri* and *N. ventrapunctatus* and is native to the Qinghai-Tibet Plateau at altitudes of 3,000 to 4,500 m (Fei et al., 2005, 2009; Chen et al., 2005). *Nanorana pleskei* is the type species of this genus and distributed in the eastern Qinghai-Tibet Plateau (Fei et al., 2009). Complete sequencing of the mtDNA in this species may provide insight into the genetic foundation for its acclimatization to the high altitude environment and the phylogenetic status and intraspecific relationships within this group (Roelants et al. 2004; Jiang and Zhou 2001; 2005; Jiang et al. 2005; Che et al. 2009).

We sequenced the complete mitochondrial genome of *Nanorana pleskei* and summarized the structural variations that have been found in the 145 amphibian mitochondrial genomes reported thus far. We reconstructed the phylogenetic relationships of amphibians using the concatenated sequences of 11 protein-coding genes from 145 amphibian mitochondrial genomes, on the basis of which the evolutionary characteristics of the mitochondrial genomes in amphibians were evaluated.

1 Materials and Methods

1.1 Sampling and experiment

A sample from *Nanorana pleskei* (Voucher Number CIB20080515-1) was obtained from Shiqu, Sichuan, China (N32°30.116', E98°23.932'; 4,280 m above sea level). The mitochondrial genome was extracted from fresh liver and muscle according to the protocol of Xia et al (2002) and Zhang et al. (2000). The isolated mtDNA genome was used for PCR amplification.

We amplified overlapping fragments that covered the entire mitochondrial genome of *Nanorana pleskei* using PCR and cloning methods. Eleven overlapping fragments were amplified using 18 newly designed primers (Table 1), one pair of primers in the *12S rRNA* gene (Kocher et al., 1989) and one pair of primers in the *16S rRNA* gene (Simon et al., 1994). Of the designed primers, ten primers (Table 1) were designed based on the alignment of the complete mtDNA sequences of eight species, *Rana nigromaculata* (Sumida et al., 2001), *Fejervarya limnocharis* (Liu et al., 2005), *Limnonectes fujianensis* (NC_007440), *Rhacophorus schlegelii* (Sano et al., 2005), *Microhyla heymonsi* (AY458596), *Andrias davidianus* (Zhang et al., 2003a), *Buergeria buergeri* (San Mauro et al., 2004a), and *Xenopus laevis* (Roe et al. 1985). The other primers (Table 1) were designed according sequences obtained using the first ten primers.

PCR amplification reactions were run in 30 μl volumes containing 10–100 ng of template DNA, 3 μl 10× reaction buffer, 2 μl 25 mmol/L $MgCl_2$, 2 μl 2 mmol/L dNTPs, 1 μl 10 μmol/L each primer, 1 U *Taq* DNA polymerase, and sterile double distilled water to a final volume of 30 μl. Amplification was performed using a PTC-200 thermocycler with the following conditions: initial denaturation at 95°C for 4 min followed by 35 cycles of: denaturation at 95°C for 40 s, annealing at 50–55°C for 40 s for sequences <2,000 bp or for 60 s for sequences >2,000 bp, and extension at 68°C for 3 min for sequences <2,000 bp or for 7 min for sequences >2,000 bp , with a final extension at 68°C for 10 min. Negative controls were run for all amplifications. The annealing temperature varied from 50–55°C to optimize the quality of PCR products as necessary. Amplification products were examined on 1.0% agarose gels, stained with ethidium bromide and photographed under ultraviolet light. PCR products were purified using Gel Extract Purification Kits (V-gene). Purified PCR products were directly sequenced on an automated DNA sequencer (Applied Biosystems, 3730). The sequences obtained from each sequencing reaction averaged 600 bp

Table 1 Primers used in amplifying and sequencing the complete mitochondrial genome of *Nanorana pleskei*

Primer Number	Location	Gene Location	Sequence (5'-3')	Primers corporation	Product length (bps)
W2	831bp	D-loop	GTCCAYAGATTCRSWTCCGTCAG	M7/W2	2072
W1	854bp		CTGACGGAWSYGAATCTRTGGAC	W1/W4	1218
W4	2520bp	$tRNA^{Leu\ (UUR)}$	AGCTTTTACTTGGRYTTGCACCAAG	W3/W8	4526
M1		12S rRNA	ACAGTCCACGAAACTTAAGAGC	W5/W10	2183
W3	6691bp	ND2	CACCACACCCACGAGCCATTGAAGC	W7/W12 (including M5/M6)	3593
W6	6709bp		TTGTGGCGGCTTCAATGGCTCGTGG	M5/M6	1774
M3		ATP6	ATAGCCACCTCAGCCATACTAC	P7/W6	2209
W5	11267bp		CACCAAGCWCAYGCHTWYCACATAGT	P1/P8 (including M1/P8)	1617
W8	11293bp	COX3	ACTATGTGRWADGCRTGWGCTTGGTG	M1/P8	317
M2			AGGATTATTCCGTATCGCAGGC	M3/M2	362
W7	13421bp		TGACTHCCHAAAGCHC AYGTHGAAGC	M7/M4	496
W10	13437bp		TATWGADCCDGCRATDGGKGCTTC		
M5		ND4	TATACCAGGGCTCCGATGAAG		
M7			TTTTGAAGCCTCTCTGGTC		
M4			AATTAAGGCGGCAACAACCACC		
M6		ND6	AATTGGGTTACTGGGTGTAGC		
W12	17101bp	Cyt b	ATKACDGTDGCNCCYCARAANGATAT		
M9			GGCGTAGTTCTGCTGCTTCTAGTMATAGC		

In sequence of primers, Y = C or T, R = A or G, W = A or T, K = G or T, S = C or G, M = A or C, H = A or T or C, D = A or T or G, N = A or G or C or T. The primers coding with 'W' were designed according to the alignment complete mitochondrial genome sequences of eight frogs (see text), and the primers coding with 'M' were designed according to the first determined sequences in this work. The upstream primers were with odd numbers, and the downstream primers with even numbers.

in length, and each sequence overlapped the next contig by 50–100 bp. The segment of 1,280 bp including part of D-loop, $tRNA^{Leu}$ (CUN) and $tRNA^{Thr}$ genes was cloned and sequenced.

1.2 Sequence analyses

Nucleotide sequences were analyzed with Lasergene version 5.0.1 (Nystuen, 2001). The protein-coding, *tRNA* and two *rRNA* genes were sequenced by comparison with homologous sequences of the above eight species. The *tRNA* genes were also identified by their cloverleaf secondary structure and anti-codon sequences, which were inferred using tRNAscan-SE 1.21 (Schattner et al., 2005). The complete mitochondrial genome sequence of *Nanorana pleskei* reported was deposited in GenBank under the accession number (HQ324232).

1.3 Amphibian mitochondrial genome types

We downloaded 97 complete mitochondrial genomes and 47 partial mitochondrial genomes of amphibians that had been deposited in GenBank before December 6, 2010 (Appendix). We compared and analyzed the mtDNA of these species, together with that of *Nanorana pleskei,* with respect to length and gene organization, based on which, mitochondrial genome types were defined.

1.4 Phylogenetic analyses

We investigated the phylogenetic relationships of these amphibians based on the concatenated sequences of 11 mitochondrial protein-coding genes from the 145 amphibian mitochondrial genomes sequenced thus far. Considering the lack of ATP8 and ND5 genes in *Polypedates megacephalus* mitochondrial genome (Zhang et al., 2005), we excluded these two genes in the phylogenetic analyses. Two fishes (*Latimeria chalumnae* and *Protopterus dolloi*) were selected as outgroups based on Zhang and Wake (2009a), and their complete mitochondrial genomes were also downloaded for phylogenetic analyses.

To avoid artificial bias in refining alignments, we

used Gblocks (Castresana, 2000) to extract regions of defined sequence conservation from these 11 protein-coding gene alignments. The parameter settings used in Gblock are: minimum number of sequences for a conserved position 22; minimum number of sequences for a flanking position 35; maximum number of contiguous nonconserved positions 8; minimum length of a block 5; gaps allowed. Finally, a DNA dataset combining all 11 Gblock-refined alignments was generated.

Maximum parsimony (MP) and maximum likelihood (ML) methods were used to reconstruct the phylogenetic relationships. The MP analyses, implemented in PAUP 4.0b10 (Sworfford, 2002), involved heuristic searches of 1000 random-addition replicates using tree bisection-reconnection (TBR) branch swapping with characters being unordered and equally weighted and gaps treated as missing data. The GTR+I+G model was selected as the best-fit model for the datasets by likelihood ratio tests implemented in jModeltest 0.1.1 (Posada, 2008) under the corrected Akaike information criterion (AICc; Hurvich and Tsai, 1989). Using the parameter values provided by jModeltest, ML analyses were implemented in PhyML version 3.0 (Guindon and Gascuel, 2003) with the default tree search settings. To assess the branch supports (bs) in the MP and ML trees, we used non-parametric bootstrapping with heuristic searches of 1,000 replicates in both the MP and ML analyses (Felsenstein, 1985; Felsenstein and Kishino, 1993; Hedges, 1992). Tree topologies with bootstrap values 70% or greater were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993), and those between 50% and 70% as weakly supported.

2 Results

2.1 *Nanorana pleskei* mitochondrial genome

2.1.1 Genome organization

The complete mitochondrial genome sequence of *Nanorana pleskei* is 17,660 bp in length, containing 13 protein-coding, two *rRNA*, and 23 *tRNA* genes, as well as non-coding regions that include the CR, OL and nucleotides separating different genes. The gene arrangement of *N. pleskei* is shown in Figure 1. There are 29 genes encoded on the heavy strand and nine genes on the light strand. Gene locations are given in Table 2. The base composition of the light strand is A: 28%, C: 26%, G: 15% and T: 30%. In comparison to the mtDNA sequences typical of vertebrates, a tandem duplication of the $tRNA^{Met}$ gene and a rearrangement of the $tRNA^{Thr}$ (*T*), $tRNA^{Pro}$ (*P*), and $tRNA^{Leu\ (CUN)}$ (*L2*) genes were found (Fig. 1).

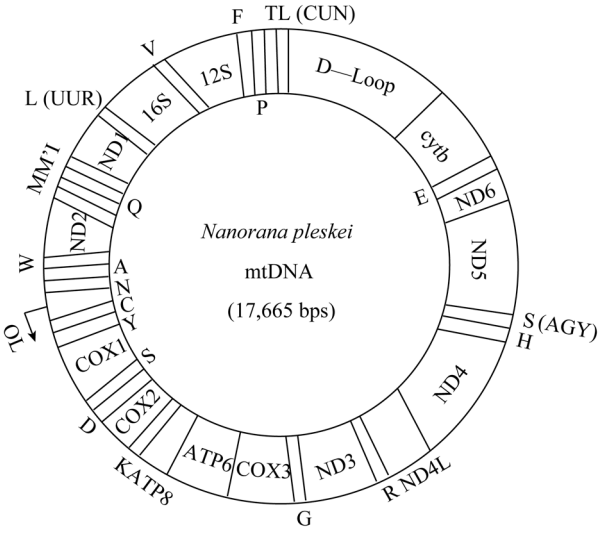

Fig. 1 Gene organization of the mitochondrial genome of *Nanorana pleskei*

tRNA genes are denoted by the single letter amino acid code and those encoded by the heavy and light strands are shown outside and inside the circle respectively. All protein-coding genes are heavy strand-encoded with the exception of *ND6* gene. M and M', a tandem duplication of $tRNA^{Met}$ genes; OL, the replication origins of light strand.

2.1.2 Non-coding regions

The OL comprising 30 nucleotides (TACTTCTCCC GGTTATGGAAAAAACGGGAG) in total is located between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes (Table 2), and it could be folded into a hairpin and loop structure, as 14 bases at both ends form a palindrome, and the remaining 13 bases form a loop containing nine consecutive cytosines. Moreover, three bases at the 3' end of the motif are also shared by the $tRNA^{Cys}$ gene. The CR of this new mitochondrial genome is located between the *Cyt b* and $tRNA^{Leu\ (CUN)}$ genes and contains a 124-bp unit threefold repeat at the 5' end that spans 372 bp, and all of these repeat units have the same nucleotide sequences, which are composed of: ATTTAATGTTTTTTATACATGCTA TGTATAATCAACATTAACAGTTATTCCCCATGCAT ATCTTTTCCGACCTACTATCTTAATTTTACTATAG TCTTAATTATCTAATATAGTATTTATGCAT. In addition, there is a non-coding sequence of 44 bases between the *tRNA-Ser* (AGY) and *ND5* genes and a non-coding sequence of nine bases between the $tRNA^{Met}$ gene and its duplicate gene.

2.1.3 Ribosomal RNA and protein-coding genes

The length of the *12S* and *16S rRNA* genes in *Nanorana pleskei* are 934 and 1,591 bps respectively; these *rRNA* genes are situated between the $tRNA^{Phe}$ and $tRNA^{Leu\ (UUR)}$ genes and separated by the $tRNA^{Val}$ gene.

Table 2 Gene organization and gene location of the complete mitochondrial genome of *Nanorana pleskei*

Gene	Start position	Stop positon	Size		Codon		Spacer (+)/
			bp	aa	Start	Stop	Overlap (-)
tRNA$^{Leu\ (CUN)}$	1	81	81				
tRNA^{Thr}	73	140	68				9
tRNA^{Pro} (L)	141	209	69				
tRNA^{Phe}	209	278	70				-1
12S rRNA	279	1212	934				
tRNA^{Val}	1213	1282	70				
16S rRNA	1283	2873	1591				
tRNA$^{Leu\ (UUR)}$	2874	2946	73				
ND1	2947	3904	958	319	GTG	T	
tRNA^{Ile}	3905	3975	71				
tRNA^{Gln} (L)	3975	4045	71				-1
New-tRNA^{Met}	4046	4114	69				
tRNA^{Met}	4124	4192	69				9
ND2	4193	5227	1035	344	ATT	TAG	
tRNA^{Trp}	5226	5295	70				-2
tRNA^{Ala} (L)	5296	5365	70				
tRNA^{Asn} (L)	5368	5440	73				2
Origin-L	5441	5470	30				
tRNA^{Cys} (L)	5471	5536	66				
tRNA^{Tyr} (L)	5537	5603	67				
COX1	5608	7158	1551	516	ATA	AGG	4
tRNA$^{Ser\ (UCN)}$ (L)	7150	7220	71				-9
tRNA^{Asp}	7221	7289	69				
COX2	7292	7976	685	228	ATG	T	2
tRNA^{Lys}	7977	8046	70				
ATP8	8049	8210	162	53	ATG	TAA	2
ATP6	8204	8885	682	227	ATG	T	-7
COX3	8886	9669	784	261	ATG	T	
tRNA^{Gly}	9670	9738	69				
ND3	9739	10098	360	120	GTG	-	
tRNA^{Arg}	10097	10165	69				-2
ND4L	10167	10451	285	94	ATG	TAA	1
ND4	10445	11807	1363	454	ATG	T	-7
tRNA^{His}	11808	11876	69				
tRNA$^{Ser\ (AGY)}$	11877	11944	68				
ND5	11989	13812	1824	608	ATG	T	44
ND6 (L)	13798	14295	498	165	ATG	AGA	1
tRNA^{Glu} (L)	14296	14364	69				
Cyt b	14372	15517	1146	381	ATG	TAG	7
D-Loop	15518	17660	2143				

"(L)" indicate a gene encoded on the light strand. "aa" is amino acid.

All 13 protein-coding open reading frames (ORFs) found in the mitochondrial genome of *Nanorana pleskei* possess the same organization as are found in most vertebrates. Two reading frame overlaps of protein-coding genes were observed, one of which was between *ATP8* and *ATP6*, sharing seven nucleotides; the other was between *ND4L* and *ND4*, also with seven nucleotides shared. Additionally, there was one overlap between protein-coding and *tRNA* genes in *COX1* and $tRNA^{Ser\,(UCN)}$ (L) with nine nucleotides shared. Most mitochondrial protein-coding genes begin with an ATG start codon, but *ND1* and *ND3* begin with GTG, *ND2* with ATT, and *COX1* with ATA. The genes *COX2*, *COX3*, *ATP6*, *ND1*, *ND4*, and *ND5* terminate at an incomplete stop codon of T; the gene *ND3* has no stop codon; the stop codon of *ATP8* and *ND4L* is TAA; *ND2* and *Cytb* terminate with TAG; *COX1* with AGG; and *ND6* with AGA (Table 2).

2.1.4 Transfer RNA genes

In the mitochondrial genome of *Nanorana pleskei*, there are 23 *tRNA*s genes, which range from 66 to 81 bp in size, including a tandem duplication of the $tRNA^{Met}$ genes. All *tRNA* genes can fold into the canonical cloverleaf secondary structure with the same anti-codon usage as reported in other vertebrates.

A tandem duplication of $tRNA^{Met}$ genes with a similarity of 85.8% was observed, both of which are 69 bp (Fig. 2). These two $tRNA^{Met}$ genes are the same in their DHU loop and the anti-codon region. Their amino acid acceptor stems are almost identical, except for one differentiated nucleotide. In the TψC stem a substitution in the duplicated $tRNA^{Met}$ gene allows for the formation of an additional base pairing compared to $tRNA^{Met}$ (Fig. 2). In addition, two differences (C-T and A-G) were found in the anti-codon loop between two genes. These two genes bear uniform anti-codons and have a similar cloverleaf-shaped conformation.

2.2 Characteristics of Amphibia mitochondrial genomes

2.2.1 Mitochondrial genome type

Twenty-four mitochondrial genome types were identified based on gene organizations for 145 amphibian mitochondrial genomes (Fig. 3; Appendix). The type 1 is the typical type of the amphibian mitochondrial genomes, being present in 108 species; the type 11 is the second, characterizing 11 species belonging to the Neobatrachia (Figs. 4, 5); the types 5, 16, 17, and 20 are each present in two species; and the other types are each present in only one species (Appendix). The *Nanorana pleskei* mitochondrial genome belongs to the type 20, as does *Quasipaa spinosa* (Appendix).

Excluding the type 1, there is no type commonly possessed by any two orders of Amphibia (Table 3). There are 15 types from 37 species in the Anura having the highest diversity on mitochondrial genome types among the three orders so far, though the number of genera and species of this order, for which the mitochondrial genomes have been sequenced, is less than that in the Caudata (Table 3). Within the Anura, an unexpected high diversity on mitochondrial genome types was found in the Ranoidea, in which the types 12 to 24 were found in 17 species (Figs. 4, 5). There are nine types from 95 species in the Caudata, seven of which are found in 22 species of the Plethodontidae, but only type 1 was found in 37 species of Salamandridae, and type 10 was in 20 species of the Hynobiidae (Figs. 4, 5).

2.2.2 Gene rearrangement

Among amphibian mitochondrial genomes, some gene clusters and their location and arrangement are conserved (E.g *F-12S-V-16S-L1*, *ND1-I*, *Q-M* and *COX1~S2*) (Fig. 3). Compared with type 1, gene positions changed frequently in other types of the mitochondrial genomes. The frequency of translocation, deletion, and duplication of *tRNA* genes is far higher than that of other kinds of genes (Table 4). The organizational changes of the protein-coding genes are mainly translocations; there is a tandem duplication of the *ND5* gene in *Hoplobatrachus tigerinus* and a pseudo *ND5* gene in *Euphlyctis hexadactylus* (Fig. 3; Appendix); and the protein-coding *ATP8* and *ND5* genes were found to be deleted only in *Polypedates megacephalus* (Fig. 3; Appendix). There are eleven species presenting translocations of the protein-coding genes, while there are 35 species presenting *tRNA* gene translocations, especially of $tRNA^{Leu\,(CUN)}$, $tRNA^{Thr}$, and $tRNA^{Pro}$. The $tRNA^{Met}$ gene has the highest frequency of duplications, all of which are found in Ranoidea species (Fig. 3; Appendix; Figs. 4, 5).

2.2.3 Mitochondrial genome size

Of the 98 amphibian species having complete mitochondrial genomes, *Gegeneophis ramaswamii* (Gymnophiona) has the smallest mitochondrial genome size of 15,897 bp, and *Ensatina eschscholtzii* (Caudata) has the largest mitochondrial genome size of 22,816 bp. The size of the mitochondrial genomes of all species in the Gymnophiona are less than 18,000 bp, and most of them are 15,000–16,000 bp. The size of the genomes of all species in the Caudata and Anura are more than 16,000 bp, and the mitochondrial genome sizes of the species in the Caudata exhibit the largest range of variation, though most of them are within 16,000–17,000 bp, while most of the Anura fall within 17,000–18,000 bp.

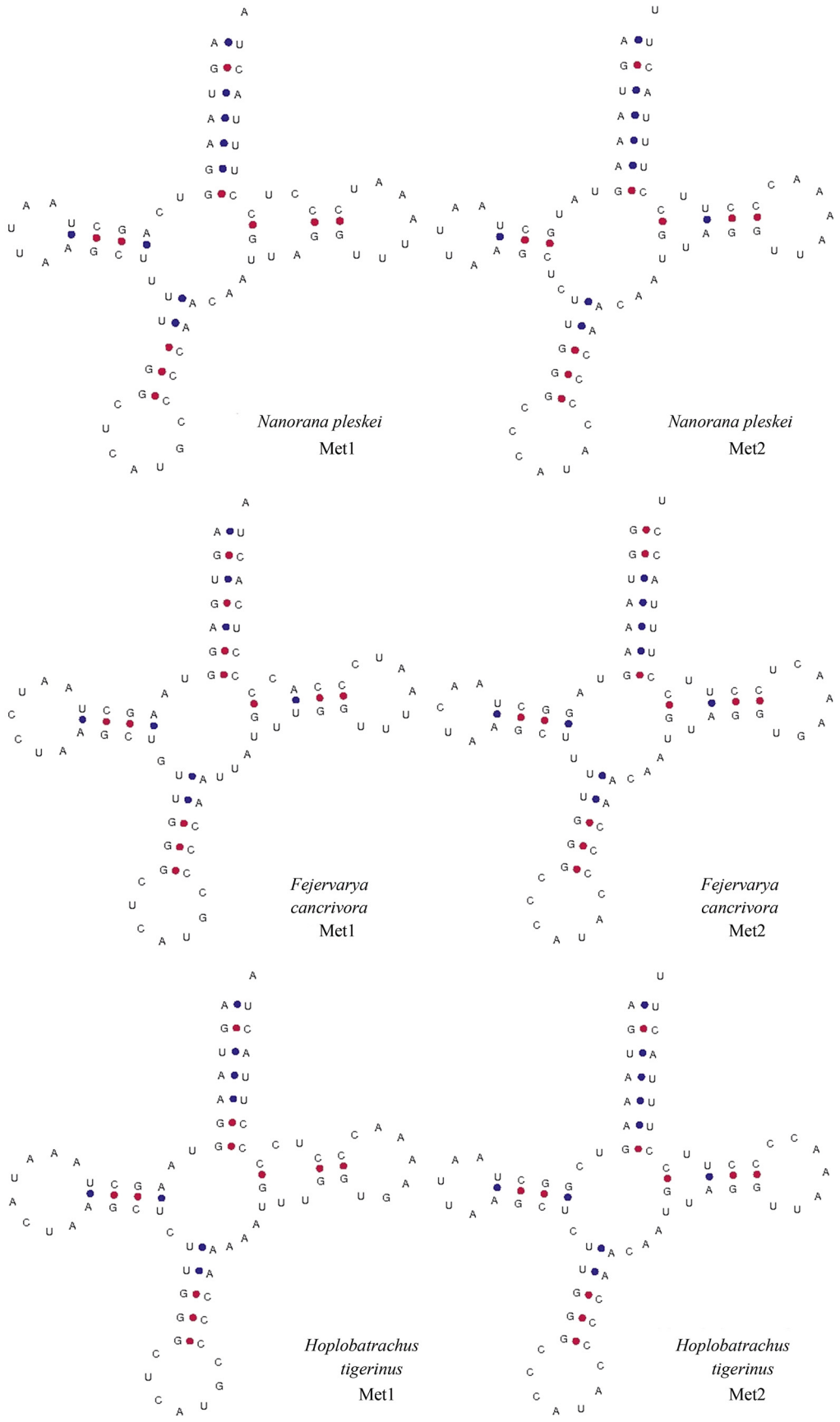

Fig. 2 Putative secondary structures of the two copies of *tRNA^{Met}* genes in nine amphibian species

Met1, the upstream copy of *tRNA^{Met}* gene; Met2, the downstream copy of *tRNA^{Met}* gene.

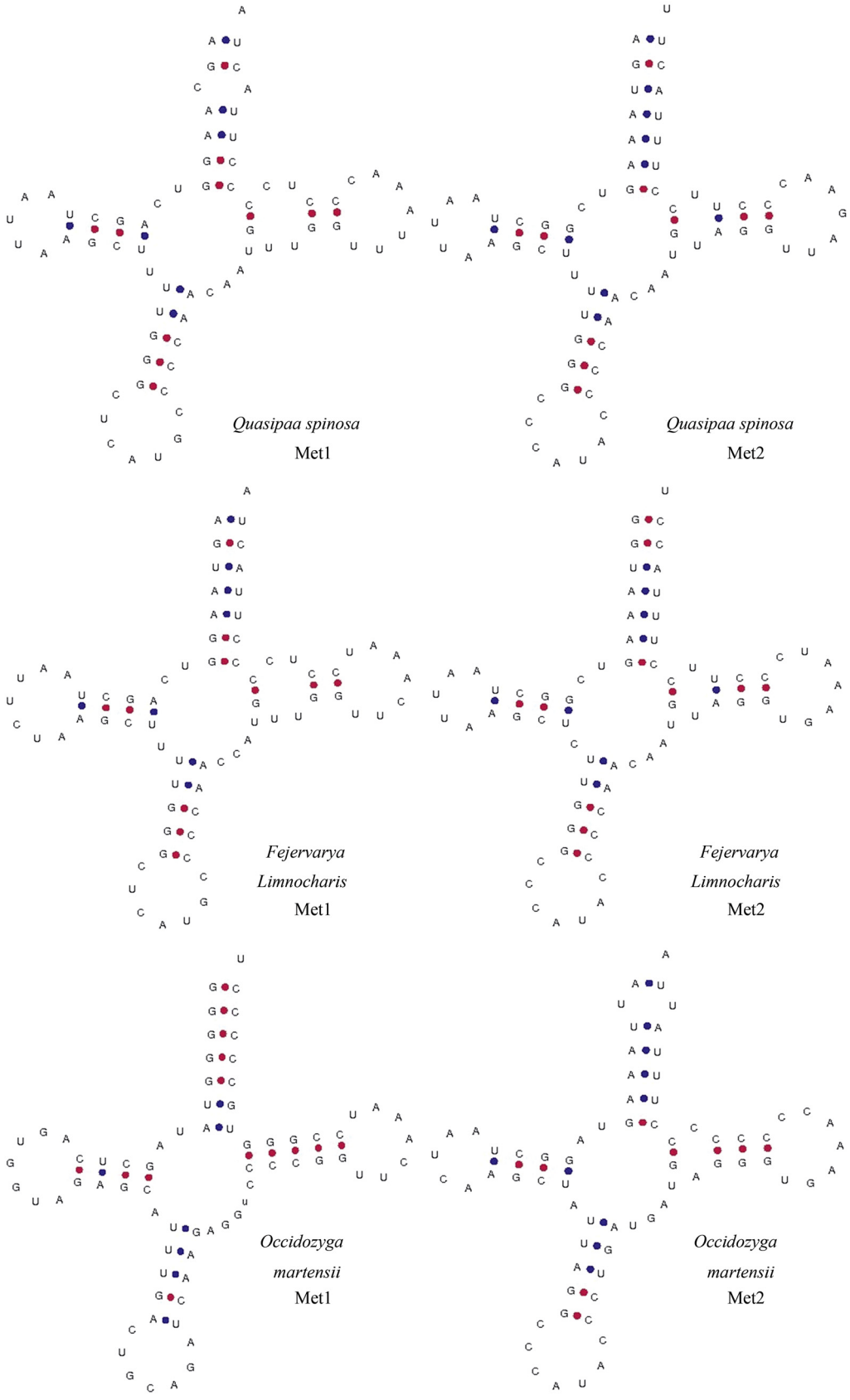

Fig. 2 Putative secondary structures of the two copies of *tRNA*^{Met} genes in nine amphibian species (Continued)

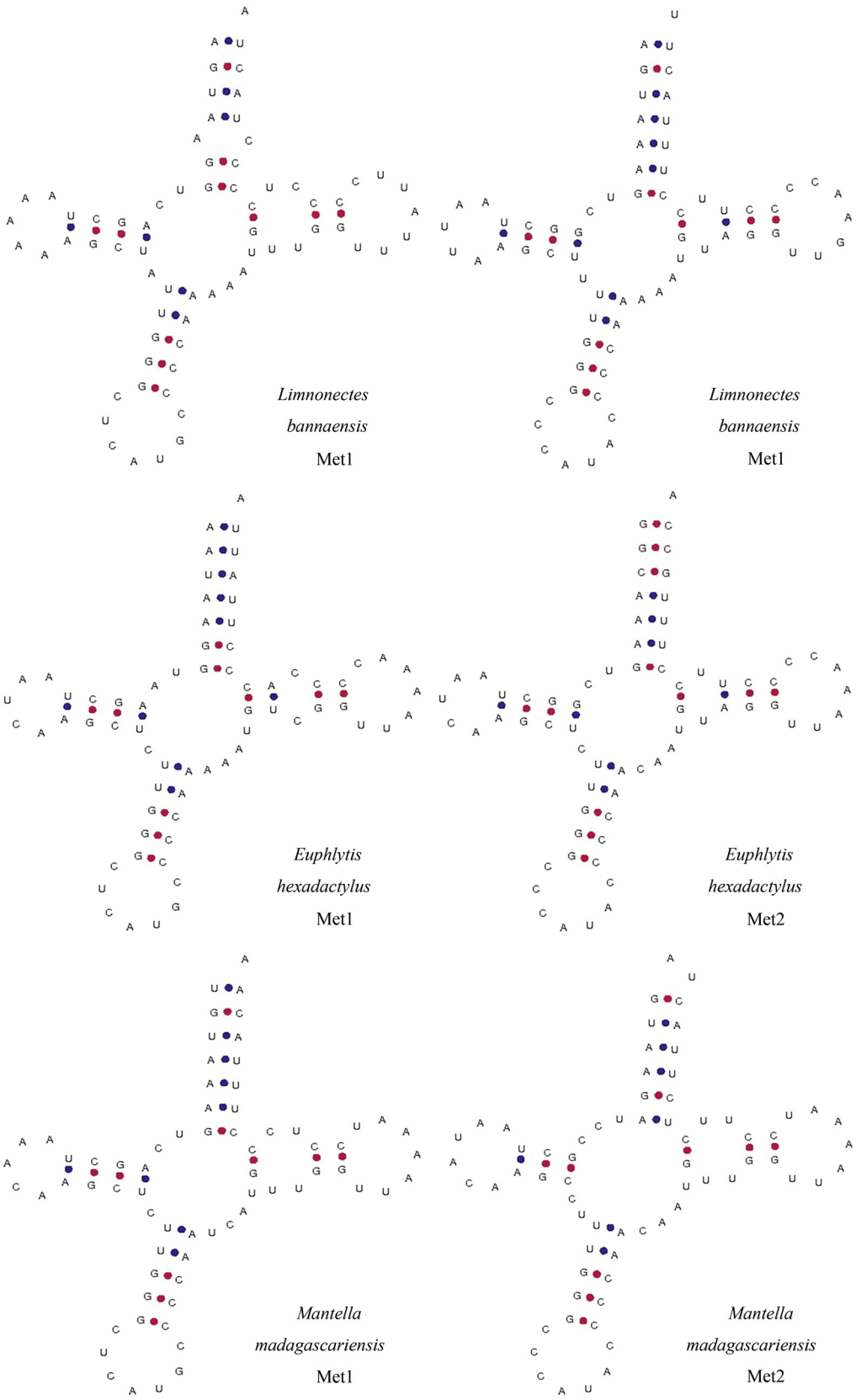

Fig. 2 Putative secondary structures of the two copies of *tRNA^{Met}* genes in nine amphibian species (Continued)

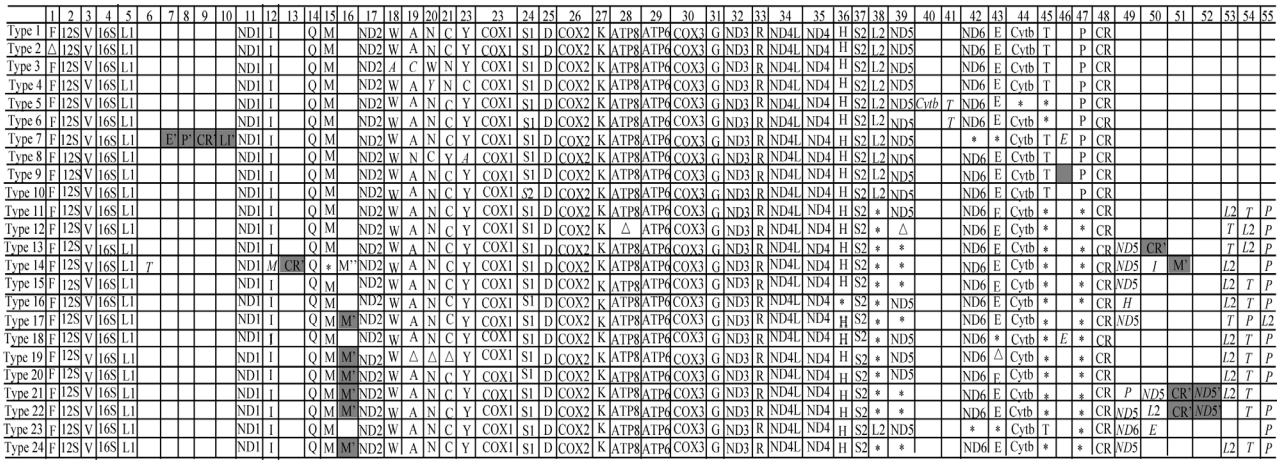

Fig. 3 Gene organization of 24 mitochondrial genome types defined from mitochondrial genomes of 145 amphibian species
Compared to the typical type 1, "*" means that the gene in this location translocate to the other site, italics means one gene translocate from other site, the drop shadow means a gene duplication, a triangle means absence of a gene, and M" means a pseudogene of *tRNA^{Met}*. L1: L (UUR), L2: L (CUN), S1: S (UCN), S2: S (AGY).

Table 3 The mitochondrial genome types contained in the three orders of Amphbia, respectively

Order	Mitochondrial genome type	Number of families, genera, and species
Gymnophiona	1, 2, 3	4, 12, 13
Caudata	1, 4, 5, 6, 7, 8, 9, 10	8, 51, 95
Anura	1, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	12, 24, 37

Type number refers to Fig. 3.

Table 4 The frequency of gene changes of the other 23 mitochondrial genome types on comparison with the type 1 referring to Fig. 3

Gene change	*ATP8*	*ND5*	*ND6*	*CYTB*	*D-loop*	*t-Thr*	*t-Leu (CUN)*	*t-Pro*	*t-Met*	*t-Asn*
Translocation		8	2	2		28	27	27	1	
Duplication		2			5	1		1	9	
Absence	1	1								1
Gene change	*t-Tyr*	*t-Glu*	*t-Cys*	*t-Leu (UUR)*	*t-Ala*	*t-Ile*	*t-His*	*t-Phe*	*t-ser1*	*t-ser2*
Translocation.	1	3	1		2	1	2		1	1
Duplication		1		1						
Absence		1	1		1			1		

2.3 Phylogenetic relationships of amphibians

The mtDNA dataset of the 147 species combining 11 protein-coding gene sequences contains 8042 nucleotides, including 5,780 variable sites and 5,159 parsimony-informative sites. The MP analyses yielded only one maximally parsimonious tree (Fig. 4), with a length of 136,836 (CI = 0.0900, RI = 0.4609). The ML analysis under the GTR+G+I model produced a topology (Fig. 5) with ln *L* = -504,903.1153 [gamma shape parameter with four discrete rate categories (G) = 0.92; proportion of invariable sites (I) = 0.33].

The MP tree was mainly identical in topology to the ML tree, but with some differences in unstable lineages (Figs. 4, 5), most of the differences presenting in the Gymnophiona. The monophyly of the Amphibia was strongly supported in all trees (MP/ML: bs = 92/100), which included three major clades corresponding to the three orders Gymnophiona, Caudata and Anura, each of them was well supported as monophyly in all trees (bs > 80). The relationship (Gymnophiona (Caudata, Anura)) was supported in all trees (bs = 61/77).

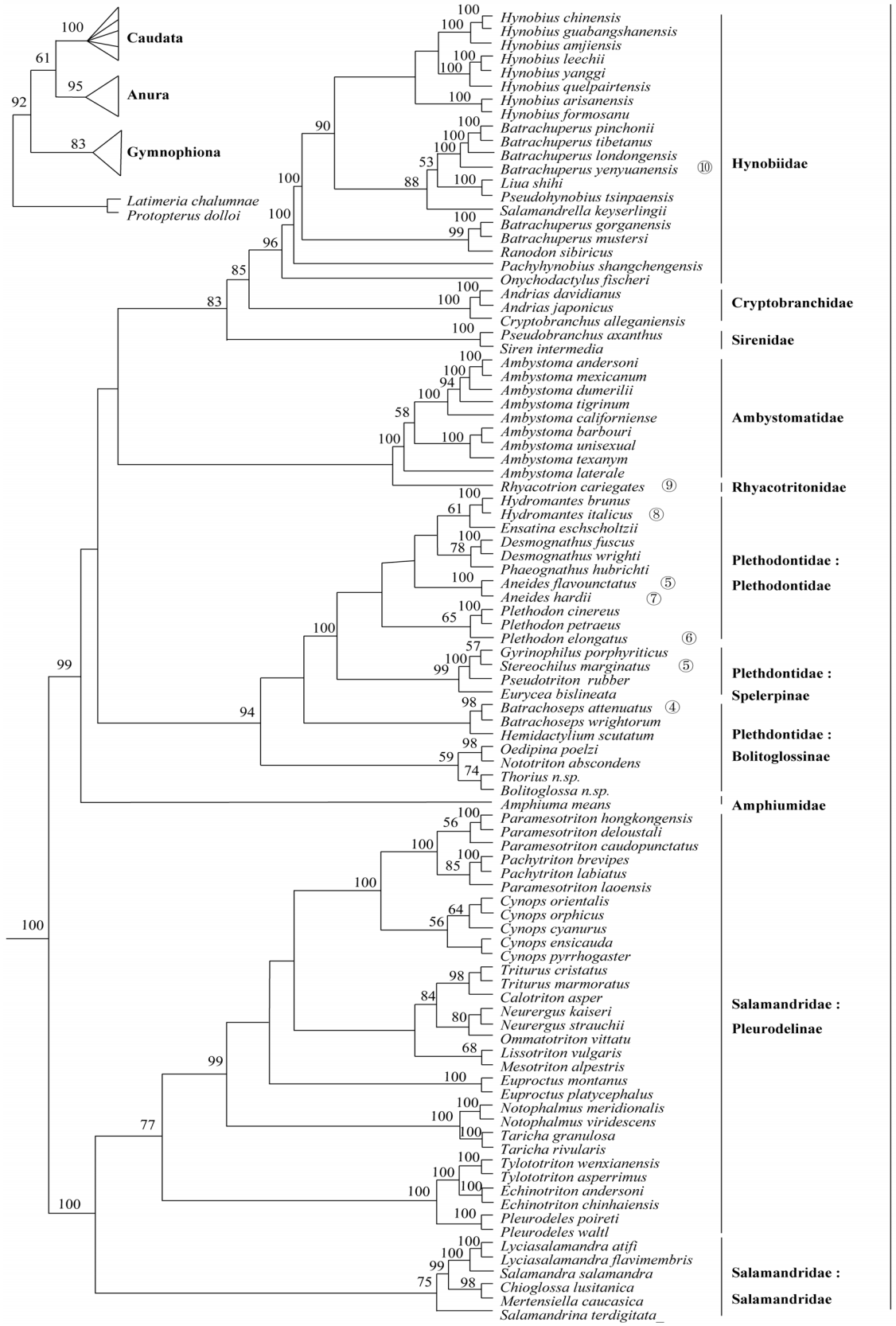

Fig. 4 Maximum parsimony (MP) tree (tree length = 136,836, CI = 0.0900, RI = 0.4609) based on combining 11 protein-coding gene sequences of 145 amphibian species and two fishes

Numbers on branches represent bootstrap supports (1000 replicates). The numbers in circles behind some species means the mitochondrial genome types in Fig. 3; the mitochondrial genomes of the other species without circles belong to type 1.

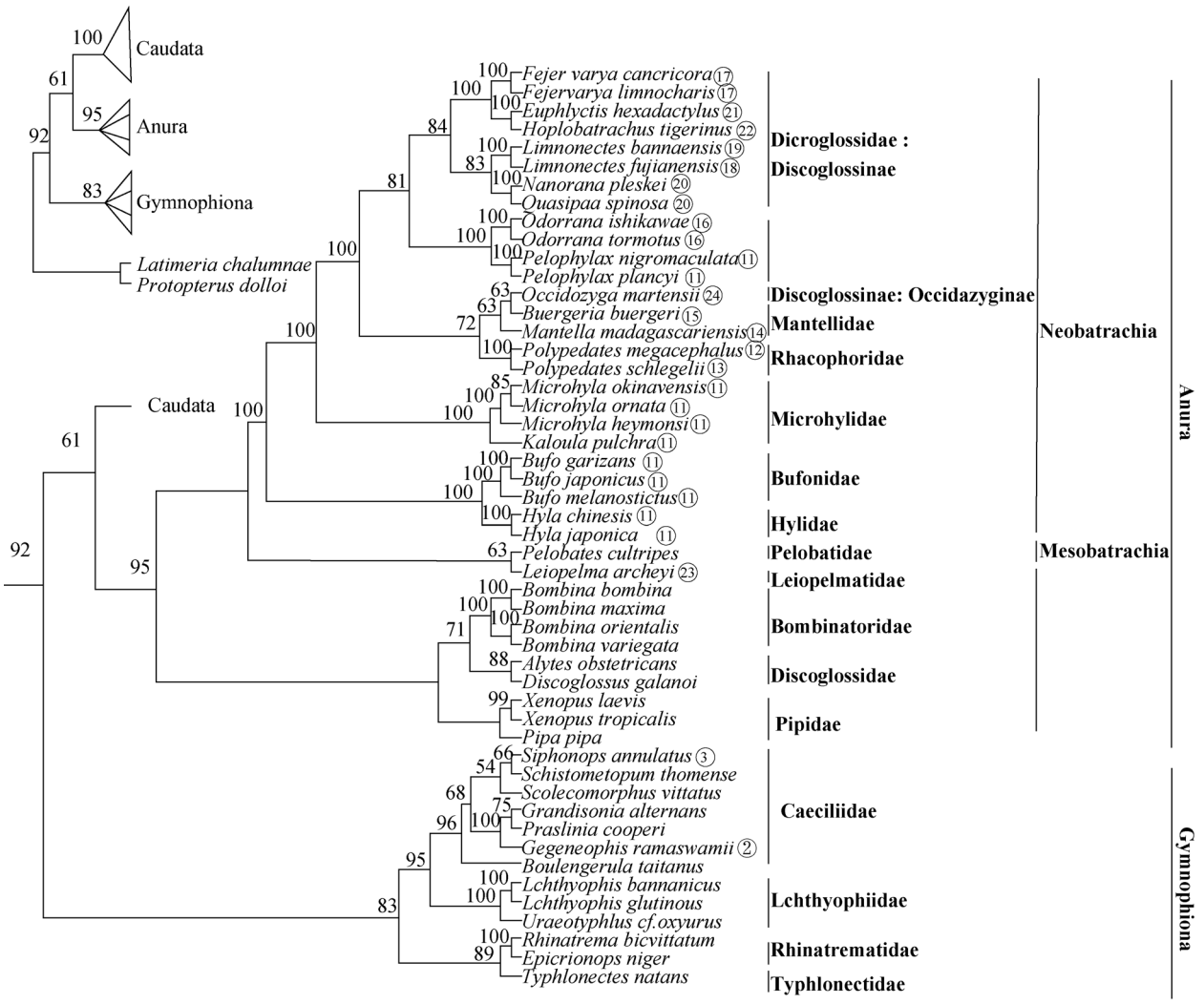

Fig. 4 Maximum parsimony (MP) tree (tree length = 136,836, CI = 0.0900, RI = 0.4609) based on combining 11 protein-coding gene sequences of 145 amphibian species and two fishes (Continued)

In the Gymnophiona, the relationships among families were different between the MP and ML trees (Figs. 4, 5): MP tree (Fig. 4) showed that the Rhinatrematidae was sister to Typhionectidae (bs = 89) and Ichthyophlidae sister to Caeciliidae (bs = 95), but ML tree showed that the Rhinatrematidae firstly diverged from the others, and the second was Ichthyophlidae, Typhionectidae was sister group of Caeciliidae (bs = 97).

In the Anura, the topologies were mostly identical between the MP and ML trees, except the location of some lineages, such as the Leiopelmatidae and Occidozyginae (Figs. 4, 5). In Figure 5, each family was supported as a monophyly (all bs > 98). The monophyly of the Neobatrachia (bs = 100) and Mesobatrachia get strong support, while the Archaeobatrachia is paraphyletic, better than polyphyly in MP tree. The Archaeobatrachia is firstly diverged, and then is the Mesobatrachia. In the Neobatrachia, the monophyly consisting of the families Hylidae and Bufonidae (all bs = 100) was firstly differentiated from the others, followed by the Microhylidae. The third monophyly of the Neobatrachia (all bs = 100) contained families Rhacophoridae, Mantellidae, Ranidae and Discoglossidae, but their relationships were uncertain because of the unstable location of the genus *Occidozyga* of Occidozyginae. The position of *Occidozyga martensii*, representing the subfamily Occidozyginae, is not firmly clustered with the other subfamily Dicroglossinae of the same family. In the Dicroglossidae excluding the *Occidozyga*, the relationships among six genera here were well resolved as ((*Fejervarya* (*Euphlyctis*, *Hoplobatrachus*)), (*Limnonectes* (*Quasipaa*, *Nanorana*))) (all bs > 90).

In the Caudata, the monophyly of each of the eight families here was well supported in all trees (all bs > 80), and their relationships was well suggested as

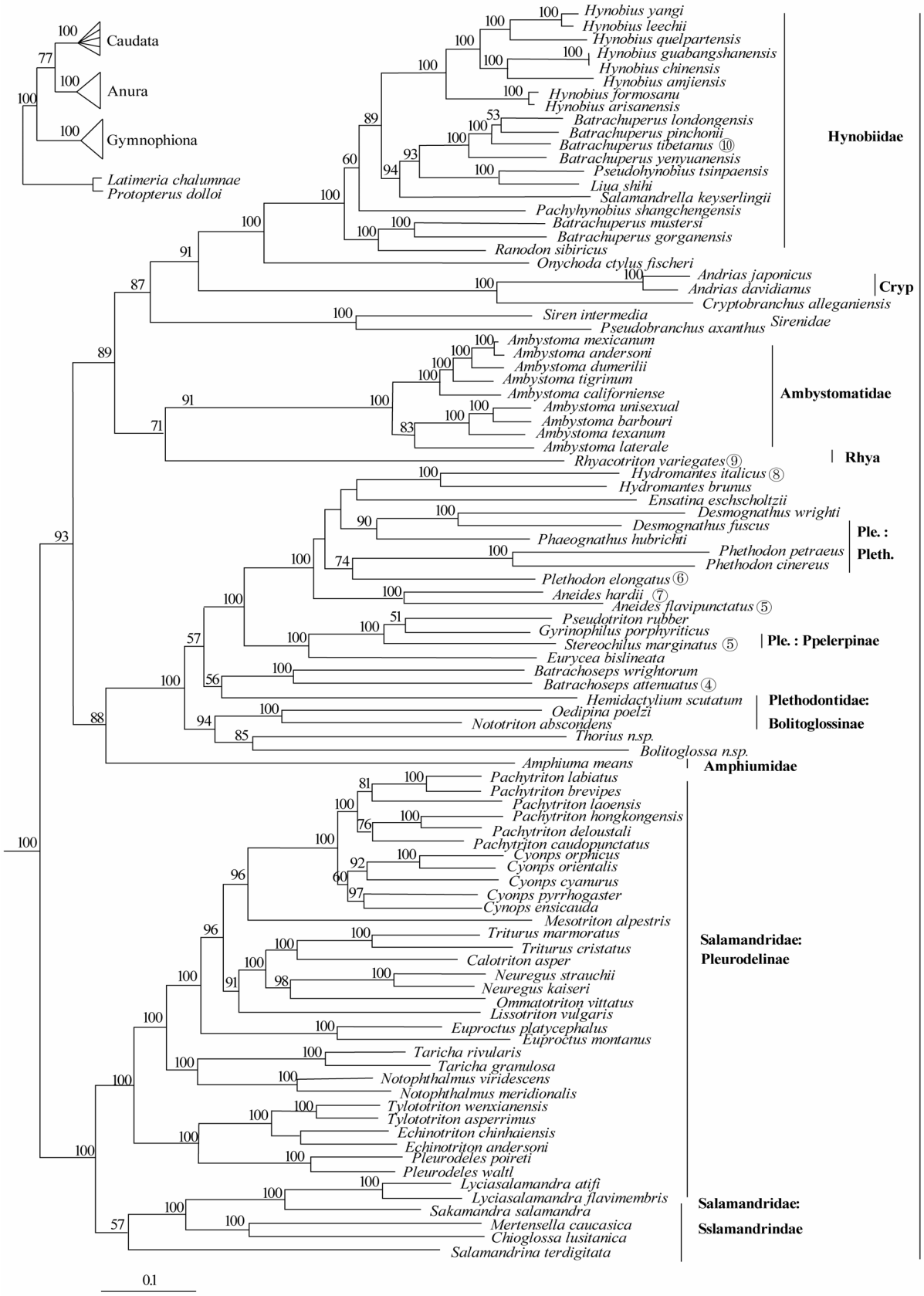

Fig. 5 Maximum likelihood (ML) tree (−ln*L* = 504,903.1153) based on combining 11 protein-coding gene sequences of 145 amphibian species and two fishes

Numbers on branches represent bootstrap supports (1000 replicates). The numbers in circles behind some species means the mitochondrial genome types in Fig. 3; the mitochondrial genomes of the other species without circles belong to type 1.

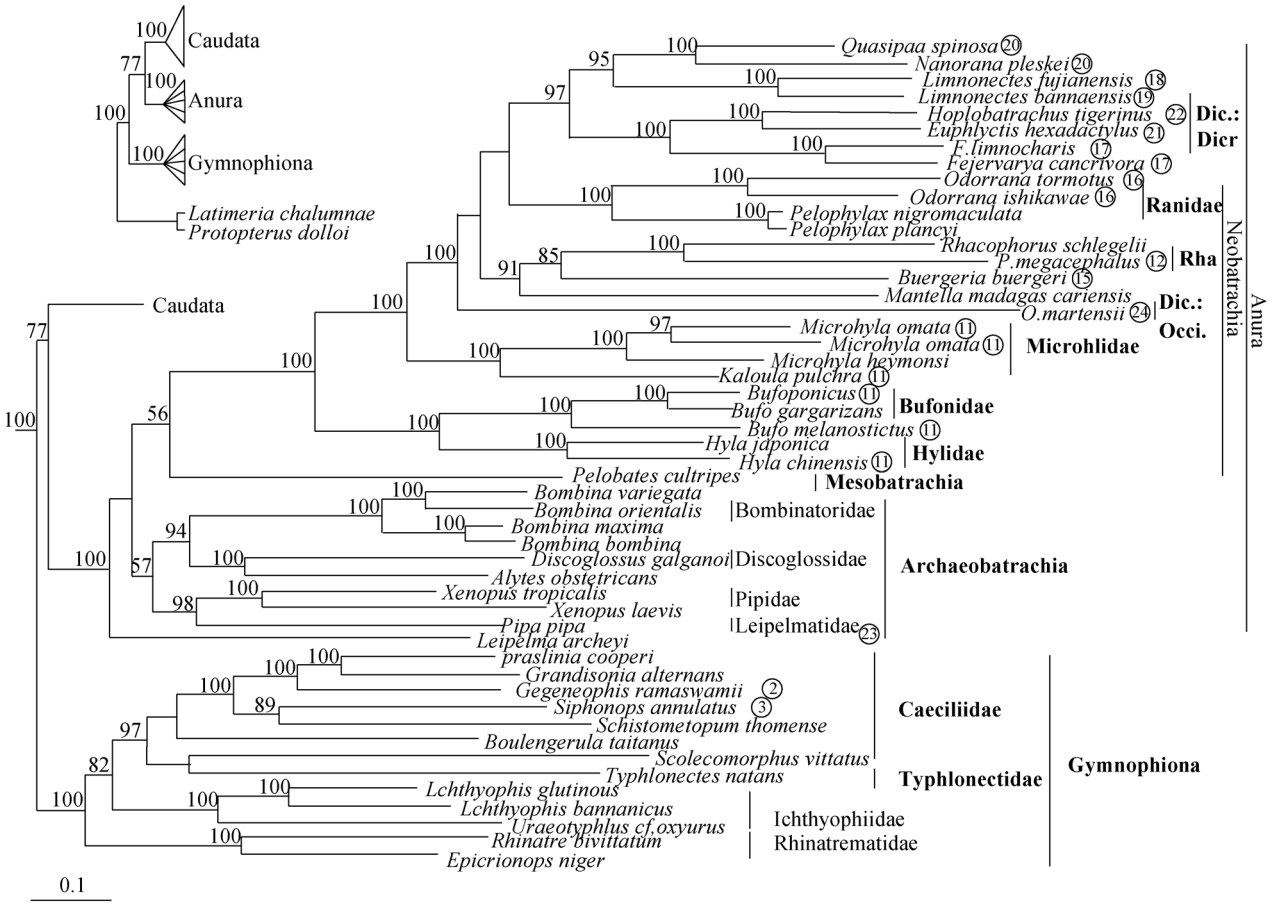

Fig. 5 Maximum likelihood (ML) tree (−ln*L* = 504,903.1153) based on combining 11 protein-coding gene sequences of 145 amphibian species and two fishes (Continued)

(Salamandridae ((Amphiumidae, Plethodontidae), ((Rhyacotritonidae, Ambystomatidae), (Sirenidae (Cryptobranchidae, Hynobiidae))))) in the ML tree (all bs > 70) (Fig. 5), while only the monophyly (Sirenidae (Cryptobranchidae, Hynobiidae)) was strongly supported by the MP tree (bs = 83; Fig. 4). So we adopt the ML tree to interpret the relationships among families of Caudata as in Gymnophiona and Anura (Fig. 5).

3 Discussion

3.1 Tandem duplication of the *tRNA*^{Met} genes

Macey et al. (1998) first reported the tandem duplication of the mitochondrial *tRNA*^{Thr} and *tRNA*^{Pro} genes in the reptile *Bipes biporus*. Recently, *tRNA*^{Thr}, *tRNA*^{Met}, *tRNA*^{Leu}, *tRNA*^{Glu} and *tRNA*^{Pro} genes were also found to be copied in some amphibians (Fig. 3; Appendix). Within the Amphibia, the duplication of the *tRNA*^{Met} gene was found in *Nanorana pleskei* in this study, and a similar discovery was made in *Fejervaya limnocharis* (Liu et al., 2005), *F. cancrivora* (Ren et al., 2009), *Limnonectes bannaensis* (Zhang et al., 2009), *Quasipaa spinosa* (Zhou et al., 2009), *Euphlyctis hexadactylus* (Alam et al., 2010), *Hoplobatrachus tigerinus* (Alam et al., 2010), *Occidozyga martensii* (NC_014685) and *Mantella* (Kurabayashi et al., 2006). Though the secondary structures of the *tRNA*^{Met} gene and its duplication are similar in these species (Fig. 2), there are two modes of evolution for this duplication, one of which is tandem duplication, as found in the Dicroglossidae, and the other is non-tandem duplication, as found in *Mantella* of Mantellidae (Fig. 3; Appendix). To date, the tandem duplication of the *tRNA*^{Met} genes has been found only in the Dicroglossidae in the Amphibia, supporting the suggestion that the tandem duplication of the *tRNA*^{Met} genes might be a synapomorphy of the Dicroglossidae.

The sequence similarity between the *tRNA*^{Met} gene and its duplicate is higher in *Nanorana pleskei* than in other amphibian species (Table 5) and may exhibit much higher function than in the other species. S-adenosylmethionine (SAM) is an important metabolite, and as a methyl group donor, it participates in many reactions. It is utilized in three kinds of key metabolic pathways, including transmethylation, polyamine synthesis and transsulfuration (Mathur et al., 1992; Shelly,

Table 5 The sequence similarity between the *tRNA^{Met}* gene and its tandem duplicated one in different species

species	*Nanorana pleski*	*Quasipaa spinosa*	*Limnonectes bannaensis*	*Fejervarya limnocharis*	*Fejervarya cancrivora*
The similarity	85.80%	84.06%	78.26%	73.61%	68.06%
species	*Mantella madagascariensis*	*Euphlyctis hexadactylus*	*Hoplobatrachus tigerinus*	*Occidozyga martensii*	
The similarity	78.57%	70.27%	74.68%	60%	

The sequences of the *tRNA^{Met}* genes of other species except *Nanorana pleski* were retrieved from other studies: *Quasipaa spinosa* (Zhou et al., 2009), *Limnonectes bannaensis* (Zhang et al., 2009), *Fejervarya limnocharis* (Liu et al., 2005), *F. cancrivora* (Ren et al., 2009), *Mantella madagascariensis* (Kurabayashi et al., 2006), *Euphlyctis hexadactylus* (Alam et al., 2010), *Hoplobatrachus tigerinus* (Alam et al., 2010), *Occidozyga martensii* (NC_014685).

2000; Yu et al., 2003). Its functions of conferring resistance to low temperature, strong drought and high salinity have been confirmed in many studies, in particular, in plants such as *Glycine soja* (Fan el at., 2008), *Populus deltoids* (Wu and Liang, 2005), and *Oryza sativa* (Frank el at., 1994). Therefore, duplication of the *tRNA^{Met}* genes may play a role in regulating the anabolic stability of SAM, which could take on an important function in *N. pleskei*, probably allowing this species to inhabit the harsh plateau environment characterized by low temperature, drought and high salinity.

3.2 Phylogenetic relationships

Our results support the monophyly of the living amphibians (Figs. 4, 5; Amphibia of Cannatella and Hillis, 1993) (Trueb and Cloutier, 1991; Zardoya and Meyer, 2000; Zhang et al., 2003b; Frost et al., 2006; Roelants et al., 2007). The monophyly of the three orders, Gymnophiona, Caudata, and Anura, were also well supported (Figs. 4, 5). Feller and Hedges (1998) proposed the relationships among the three orders as ((Gymnophiona, Caudata) Anura). However, the (Gymnophiona (Caudata, Anura)) relationships were supported in our ML and MP trees (Figs. 4, 5), as has been proposed by the majority of morphology and molecular studies (Trueb and Cloutier, 1991; Frost et al., 2006; Roelants et al., 2007; Zardoya and Meyer, 2001; Schoch and Milner, 2004; San Mauro, 2010). Thus, (Gymnophiona (Caudata, Anura)) might represent the true relationship.

Gymnophiona Our analyses showed that the *Uraeotyphlus* is a sister group to the *Ichthyophis*, in agreement with the conventional view supporting that the *Uraeotyphlus* was a member of the Ichthyophiidae (Frost et al., 2006; Gower et al., 2002; Zhang and Wake, 2009b). Previous studies (Nussbaum, 1977, 1979; Duellman and Trueb, 1986; San Mauro et al., 2004b; San Mauro et al., 2005; Frost et al., 2006; Roelants et al., 2007; Zhang and Wake, 2009b) showed that the Rhinatrematidae represented the monophyletic sister taxon of the remaining caecilians, including the Ichthyophiidae, Typhlonectidae, and Caeciliidae. The *Scolecomorphus* was clustered together with the Caeciliidae in all analyses (Figs. 4, 5), an observation consistent with previous analyses based on DNA sequences (Wilkinson et al., 2003; Frost et al., 2006; Roelants et al., 2007; Zhang and Wake, 2009b; San Mauro et al., 2009) and morphological data (Wake, 1993; Wilkinson, 1997). The position of the Typhlonectidae is uncertain in the two trees (Figs. 4, 5); it was clustered with the Rhinatrematidae at the base of the Gymnophiona in the MP tree (Fig. 4), while it was imbedded in the Caeciliidae in the ML tree (Fig. 5) in consistent with the results of Zhang and Wake (2009b). But the new work of Wilkinson et al. (2011) supported it as a family. Therefore, it is suitable to keep Typhlonectidae as the familial rank, for the time being, though the elucidation of the relationships among the families above requires additional evidence.

Caudata Our analyses supported the monophyly of each family (Salamandridae, Plethodontidae, Rhyacotritonidae, Ambystomatidae, Cryptobranchidae, Hynobiidae, Sirenidae and Amphiumidae; Figs. 4, 5), consistent with previous studies (Frost et al., 2006; Roelants et al., 2007; Gao and Shubin, 2001; Larson and Dimmick, 1993; Wiens et al., 2005; Roelants and Bossuyt, 2005; San Mauro et al., 2005; Zhang and Wake, 2009a; Mueller and Boore, 2005). However, on the relationships among these families, our MP and ML trees showed different topologies from previous work that suggested that the relationships among the eight families were (Sirenidae, (Hynobiidae, Cryptobrancjidae), ((Ambystomatidae, Salamandridae), (Rhyacotritonidae, Amphiumidae, Plethodontidae))); our analyses indicated that the relationships were mainly (Salamandridae (Amphiumidae, Plethodontidae, ((Rhyacotritonidae, Ambystomatidae), (Sirenidae (Hynobiidae, Cryptobranchidae))))) (Figs. 4, 5). The phylogenetic relationships among families of Caudata requires further research.

Anura The phylogenetic topology of the 37 species

investigated here was similar between the MP and ML trees (Figs. 4, 5), and similar to those in some previous studies (Frost et al., 2006; Roelants et al., 2007; Roelants and Bossuyt, 2005; Zhou et al., 2009; Kurabayashi et al., 2010; Irisarri et al., 2010), supporting the three suborders classification (Archaeobatrachia, Mesobatrachia and Neobatrachia). This also has morphological support (Ford and Cannatella, 1993). Therefore, we adopted the suborder Mesobatrachia, which was represented by the family Pelobatidae here, though Ren et al. (2009) and Zhang et al. (2009) showed that the Archaeobatrachia including Pelobatidae was a monophyly, and did not adopt the suborder Mesobatrachia.

Among the Archaeobatrachia, the Leiopelmatidae was indicated as the most basic clade of the Anura in our ML tree (Fig. 5) (Ford and Cannatella, 1993; Frost et al., 2006; Roelants et al., 2007; Roelants and Bossuyt, 2005).

The Neobatrachia (Figs. 4, 5) has been supported by our work and previous studies (Frost et al., 2006; Roelants et al., 2007; Roelants and Bossuyt, 2005). Within the Neobatrachia, the basal position of the group (Hylidae + Bufonidae) was consistently supported by our analyses and previous studies listed above. The Mantellidae were indicated close to the Rhacophoridae in different studies, and together formed as a sister group of the Ranidae (Frost et al., 2006; Roelants et al., 2004; Ren et al., 2009); while (Mantellidae + Rhacophoridae) is a sister group to (Ranidae + Dicroglassidae) in our results (Figs. 4, 5) and some published work based on analyses of complete mtDNA (Zhang et al., 2009; Zhou et al., 2009).

In the Dicroglossidae, Frost et al. (2006) supported the relationships ((*Fejervarya*, *Nanorana*), *Limnonects*), but our results strongly supported (*Fejervarya* (*Nanorana*, *Limnonects*)), an observation consistent with previous analyses based on *12S rRNA* and *16S rRNA* gene sequences (Jiang and Zhou, 2005). Noticeably, previous studies (Frost et al., 2006; Roelants et al., 2007; Roelants and Bossuyt, 2005) indicated that the *Occidozyga* was clustered into the Dicroglassidae, but in our ML tree, the species *Occidozyga martensii* represented an independent lineage from the Dicroglassidae (Fig. 5), and even in the MP tree, unexpectedly, it was clustered into the Mantellidae (Fig. 4). More *Occidozyga* species are needed to recognize the position of this genus in the life tree.

3.3 Evolution of amphibian mitochondrial genomes

Mitochondrial genome arrangements may reflect phylogenetic relationships (Boore and Brown, 1998; Macey et al., 2000; Kurabayashi et al., 2006). Many gene rearrangements found in amphibian mitochondrial genomes occur in a lineage-specific manner (Kurabayashi and Ueshima, 2000; Mabuchi et al., 2004; Kurabayashi et al., 2008). In amphibians, the type 1 of mitochondrial gene arrangement is identically presented in most taxa of the Amphibia, as well as in many other vertebrates (Wolstenholme, 1992; Boore, 1999), suggesting that this gene arrangement type is the common ancestral type of the Amphibia. Similarly, type 11 is the ancestral type of the Neobatrachia (Figs. 4, 5; Appendix). There are two types of mitochondrial genome (the types 2 and 3) endemic to the Gymnophionaboth are present in the family Caeciliidae, which is a top lineage in the phylogenetic tree (Figs. 4, 5). According to the principle of cladistics, these two derived types lack parsimony information. Similarly, among the seven types endemic to the Caudata and the 14 types endemic to the Anura, the types 5, 11, 16, 17, and 20 are parsimony informative. The fact that there is no synapomorphy type of the mitochondrial genome shared by any two of the three orders indicates that the type of the mitochondrial genome cannot yet supply sufficient phylogenetic information to determine the relationships of the three orders, though they can support their monophyly.

Based on the gene arrangements of the mitochondrial genome types (Fig. 3) and their distribution on the phylogenetic trees (Figs. 4, 5), the arrangement of the *LTPF tRNA* gene cluster located upstream of the *12S rRNA* gene (Fig. 3) is proposed to be a derived arrangement from the typical of vertebrates and to be a synapomorphic character of the neobatrachia, which experienced a single origin from the Archeobatrachia type (Figs. 4, 5). This has also been suggested by other studies (Ren et al., 2009; Zhang et al., 2009; Zhou et al., 2009). In addition, the arrangement of the *LTPF* gene cluster is different in some taxa, such as in the Mantellidae and the *Fejervarya* of the Dicroglassidae. These two rearrangements of the *LTPF* are proposed to have evolved from the neobatrachian type and to have experienced two steps of evolution from the typical type for vertebrates.

In contrast, the arrangement of *WANCY*, another *tRNA* gene cluster, is conserved in most mitochondrial genome types of amphibians (Fig. 3; Appendix). The three rearrangements of this cluster, *ACWANY*, *WAYNC*, and *WNCYA*, are found in *Siphonops annulatus* (Gymnophiona: Caeciliidae), *Batrachoseps attenuatus* (Cau-

data: Plethodontidae), and *Hydromantes brunus* (Caudata: Plethodontidae), respectively, but these three species are distinctly clustered in distant lineages (Figs. 4, 5). Thus, it is proposed that the rearrangement of *WANCY* in these lineages should be of independent origins.

The duplication of the CR regions presents in *Euphlyctis hexadactylus* and *Hoplobatrachus tigerinus* supporting the common origin of these two genera (Figs. 3, 4, 5; Appendix). However, this character also presents in *Aneides hardii*, *Rhacophorus schlegelii* and *Mantella madagascariensis* falling into distant lineages (Fig. 3; Appendix; Figs. 4, 5). Thus, this character should be of independent origin for these distant lineages.

The *ND5* gene was relocated to the 3' end of a control region in some mitochondrial genomes, including that of the *Rhacophorus schlegelii* (type 13), *Mantella madagascariensis* (type 14), *Buergeria buergeri* (type 15), *Fejervarya limnocharis* and *F. cancrivora* (type 17), *Euphlyctis hexadactylus* (type 21), *Hoplobatrachus tigerinus* (type 22), and *Occidozyga martensii* (type 24). *Hoplobatrachus tigerinus* possessed a tandem duplication of *ND5* gene and *Euphlyctis hexadactylus* possessed a pseudo *ND5* gene (Alam et al., 2010; Appendix). Conversely, an unusual loss of the *ND5* gene was reported for the mitochondrial genome of *P. megacephalus*. Based on our phylogenetic results and previous studies (Kurabayashi et al., 2006, 2010; Ren et al., 2009; Zhou et al., 2009), the distinct relationship between (mantellids + rhacophorids) and the dicroglossinids indicates that rearrangement of the *ND5* gene in these two lineages seems to have experienced an independent origin.

Based on the mitochondrial genome gene arrangements (Fig. 3) and their distribution on the phylogenetic trees (Figs. 4, 5), the duplication of the $tRNA^{Met}$ genes presented in seven species of Dicroglossidae (Fig. 3; Appendix) should be a synapomorphy of the Dicroglossidae, an observation consistent with other studies (Ren et al., 2009; Zhang et al., 2009; Zhou et al., 2009; Alam et al., 2010). The similarity of the structure of each pair of $tRNA^{Met}$ genes in each species (Fig. 2) indicates that they should have similar functions. The mantellids also possess a duplication of the $tRNA^{Met}$ gene (Kurabayashi et al., 2006), but the position of the duplicated $tRNA^{Met}$ gene, located after the *ND5* gene, is different from that in the Dicroglossidae (Fig. 3; Appendix). Therefore, the duplication of the $tRNA^{Met}$ genes in the Dicroglossidae and mantellids originated independently.

There were two hypotheses to explain the evolution of similarity between duplicated *tRNA* genes in the family Dicroglossidae. The first explanation suggests that in the first the family obtained the duplicated $tRNA^{Met}$ genes with low similarity, then the duplicated genes differentiated by mutation in different lineages, and then one derived lineage, *Nanorana pleskei*, got its high similarity by selection. The second explanation suggests that first the family obtained the duplicated genes, then the duplicated genes differentiated by mutation in all the species, and then one derived lineage, *N. pleskei*, "recovered" its similarity by selection.

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References

Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR et al., 1981. Sequence and organization of the human mitochondrial genome. Nature 290: 457–465.

Alam MS, Kurabayashi A, Hayashi Y, Sano N, Khan MR et al., 2010. Complete mitochondrial genomes and novel gene rearrangements in two dicroglossid frogs, *Hoplobatrachus tigerinus* and *Euphlyctis hexadactylus*, from Bangladesh. Genes & Genetic Systems 85 (3): 219–232.

Arnason U, Gullberg A, Janke A, Joss J, Elmerot C, 2004. Mitogenomic analyses of deep gnathostome divergences: A fish is a fish. Gene 333: 61–70.

Boore JL, 1999. Animal mitochondrial genomes. Nucleic Acids Research 27: 1767–1780.

Boore JL, Brown WM, 1998. Big trees from little genomes: Mitochondrial gene order as a phylogenetic tool. Current Opinion in Genetics Development 8: 668–674.

Cannatella DC, Hillis DM, 1993. Amphibian relationships: Phylogenetic analysis of morphology and molecules. Herpetological Monographs 7: 1–7.

Cao SY, Wu XB, Yan P, Hu YL, Su X et al., 2006. Complete nucleotide sequences and gene organization of mitochondrial genome of *Bufo gargarizans*. Mitochondrion 6 (4): 186–193.

Castresana J, 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540–552.

Che J, Hu JS, Zhou WW, Murphy RW, Papenfuss TJ et al., 2009. Phylogeny of the Asian spiny frog tribe Paini (Family Dicroglossidae) sensu Dubois. Molecular Phylogenetics and Evolution 50 (1): 59–73.

Chen GY, Liu JY, Jiang JP, Xie F, Zheng ZH, 2005. Systematics and distribution of the Genus *Nanorana*. Herpetologica Sinica 10: 47–51.

Curole AP, Kocher TD, 1999. Mitogenomics: Digging deeper with complete mitochondrial genomes. Trends in Ecology & Evolution 14: 394–398.

Desjardins P, Morais R, 1990. Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. Journal of Molecular Biology 212: 599–634.

Duellman WE, Trueb L, 1986. Biology of Amphibians. New York: McGraw-Hill.

Fan JP, Bai X, Li Y, Ji W, Wang X et al., 2008. Cloning and Function Analysis of Gene SAMS from *Glycine soja*. Acta Agronomica Sinica 34 (9): 1581–1587.

Fei L, Ye CY, Huang YZ, Jiang JP, Xie F, 2005. An Illustrated Key to Chinese Amphibians. Sichuan Chengdu: Sichuan Publishing House of Science and Technology.

Fei L, Hu SQ, Ye CY, Huang YZ, 2009. Fauna Sinica: Amphibia. Vol 3. Anura. Beijing: Science Press.

Fei L, Ye CY, Jiang JP, 2010. Phylogenetic systematic of Ranidae. Herpetologica Sinica 12: 1–43.

Feller AE, Hedges SB, 1998. Molecular evidence for the early history of living amphibians. Molecular Phylogenetics and Evolution 9: 509–516.

Felsenstein J, 1985. Confidence-limits on phylogenies-an approach using the bootstrap. Evolution 39: 783–791.

Felsenstein J, Kishino H, 1993. Is there something wrong with the bootstrap on phylogeny? A reply to Hillis and Bull. Systematic Zoology 42: 193–200.

Ford LS, Cannatella DC, 1993. The major clades of frogs. Herpetological Monographs 7: 94–117.

Frank VB, Rudy D, Jan G, 1994. Characterization of an S-adenosylme-thionine synthetase gene in rice. Plant Physiology 105: 1463–1464.

Frost DR, Grant T, Faivovich J, Bain RH, Haas A et al., 2006. The amphibian tree of life. Bulletin of the American Museum of Natural History 297: 1–371.

Gao K, Shubin NH, 2001. Late Jurassic salamanders from northern China. Nature 410: 574–577.

Gissi C, San Mauro D, Pesole G, Zardoya R, 2006. Mitochondrial phylogeny of Anura (Amphibia): A case study of congruent phylogenetic reconstruction using amino acid and nucleotide characters. Gene 366: 228–237.

Gower DJ, Kupfer A, Oommen OV, Himstedt W, Nussbaum RA et al., 2002. A molecular phylogeny of ichthyophiid caecilians (Amphibia: Gymnophiona: Ichthyophiidae): Out of India or out of South East Asia? Proceedings of the Royal Society of London - Series B: Biological Sciences 269: 1563–1569.

Guindon S, Gascuel O, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704.

Hedges SB, 1992. The number of replications needed for accurate estimation of the bootstrap *P* value in phylogenetic studies. Molecular Biology and Evolution 9: 366–369.

Huelsenbeck JP, Hillis DM, 1993. Success of phylogenetic methods in the four-taxon case. Systematic Biology 42: 247–264.

Hurvich CM, Tsai CL, 1989. Regression and time series model selection in small samples. Biometrika 76: 297–307.

Inoue JG, Miya M, Tsukamoto K, Nishida M, 2003. Evolution of the deep-sea gulper eel mitochondrial genomes: Large-scale gene rearrangements originated within the eels. Molecular Biology and Evolution 20: 1917–1924.

Irisarri I, San Mauro D, Green DM, Zardoya R, 2010. The complete mitochondrial genome of the relict frog *Leiopelma archeyi*: Insights into the root of the frog Tree of Life. Mitochondrial DNA 21: 173–182.

Jiang JP, Dubois A, Ohler A, Tillier A, Chen XH et al., 2005. Phylogenetic relationships of the tribe Paini (Ranidae, Anura, Amphibia) based on partial sequences of mitochondrial 12S and 16S rRNA genes. Zoological Science 22: 353–362.

Jiang JP, Zhou KY, 2001. Evolutionary relationships among Chinese Ranid frogs inferred from mitochondrial DNA sequences of 12S rRNA genes. Acta Zoologica Sinica 47 (1): 38–44.

Jiang JP, Zhou KY, 2005. Phylogenetic relationships among Chinese ranids inferred from sequence data set of 12S and 16S rDNA. Herpetological Journal 15: 1–8.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S et al., 1989. Dynamics of mitochondrial DNA evolution in mammals: Amplification and sequence with conserved primers. Proceedings of the National Academy of Sciences of the United States of America 86: 6169–6200.

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

Kurabayashi A, Sumida M, Yonekawa H, Glaw F, Vences M et al., 2008. Phylogeny, recombination, and mechanisms of stepwise mitochondrial genome reorganization in mantellid frogs from Madagascar. Molecular Biology and Evolution 25: 874–891.

Kurabayashi A, Ueshima R, 2000. Complete sequence of the mitochondrial DNA of the primitive opisthobranch gastropod *Pupa strigosa*: Systematic implication of the genome organization. Molecular Biology and Evolution 17: 266–277.

Kurabayashi A, Usuki C, Mikami N, Fujii T, Yonekawa H et al., 2006. Complete nucleotide sequence of the mitochondrial genome of a Malagasy poison frog *Mantella madagascariensis*: Evolutionary implications on mitochondrial genomes of higher anuran groups. Molecular Phylogenetics and Evolution 39 (1): 223–236.

Kurabayashi A, Yoshikawa N, Sato N, Hayashi Y, Oumi S et al., 2010. Complete mitochondrial DNA sequence of the endangered frog *Odorrana ishikawae* (family Ranidae) and unexpected diversity of mt gene arrangements in ranids. Molecular Phylogenetics and Evolution 56: 543–553.

Larson A, Dimmick W, 1993. Phylogenetic relationships of the salamander families: an analysis of congruence among morphological and molecular characters. Herpetological Monographs 7: 77–93.

Liu ZQ, Wang YQ, Su B, 2005. The mitochondrial genome organization of the rice frog *Fejervarya limnocharis* (Amphibia: Anura): A new gene order in the vertebrate mtDNA. Gene 346: 145–151.

Mabuchi K, Miya M, Satoh TP, Westneat MW, Nishida M, 2004. Gene rearrangements and evolution of tRNA pseudogenes in the mitochondrial genome of the parrotfish (Teleostei: Perciformes: Scaridae). Journal of Molecular Evolution 59: 287–297.

Macey JR, Schulte II JR, Larson A, Papenfuss TJ, 1998. Tandem duplication via light-strand synthesis may provide a precursor for mitochondrial genome rearrangement. Molecular Biology

and Evolution 15: 71–75.

Macey JR, Schulte JA, Larson A, 2000. Evolution and phylogenetic information content of mitochondrial genomic structural features illustrated with acrodont lizards. Systematic Biology 49: 257–277.

Mathur M, Satpathy M, Sachar RC, 1992. Phytohormonal regulation of S-adenosyl- methionine synthetase by gibberellic acid in wheat aleurones. Biochimica et Biophysica Acta 1137 (3): 338–348.

Mindell DP, Sorenson MD, DimcheV DE, 1998. Multiple independent origins of mitochondrial gene order in birds. Proceedings of the National Academy of Sciences of the United States of America 95: 10693–10697.

Miya M, Kawaguchi A, Nishida M, 2001. Mitogenomic exploration of higher teleostean phylogenies: A case study for moderate-scale evolutionary genomics with 38 newly determined complete mitochondrial DNA sequences. Molecular Biology and Evolution 18: 1993–2009.

Mueller RL, Boore JL, 2005. Molecular mechanisms of extensive mitochondrial gene rearrangement in plethodontid salamanders. Molecular Biology and Evolution 22: 2104–2112.

Mueller RL, Macey JR, Jaekel M, Wake DB, Boore JL, 2004. Morphological homoplasy, life history evolution, and historical biogeography of plethodontid salamanders inferred from complete mitochondrial genomes. Proceedings of the National Academy of Sciences of the United States of America 101 (38): 13820–13825.

Nussbaum RA, 1977. Rhinatrematidae: A new family of caecilians (Amphibia: Gymnophiona). Occasional Papers of the Museum of Zoology, University of Michigan 682: 1–30.

Nussbaum RA, 1979. The taxonomic status of the caecilian genus *Uraeotyphlus* Peters. Occasional Papers of the Museum of Zoology, University of Michigan 687: 1–20.

Nystuen A, 2001. Lasergene 5.0.1. Biotech Software and Internet Report 2 (6): 239–244.

Pereira SL, 2000. Mitochondrial genome organization and vertebrate phylogenetics. Genetics and Molecular Biology 23 (4): 745–752.

Posada D, 2008. jModelTest: Phylogenetic Model Averaging. Molecular Biology and Evolution 25: 1253–1256.

Ren Z, Zhu B, Ma EB,Wen J, Tu TY et al., 2009. Complete nucleotide sequence and gene arrangement of the mitochondrial genome of the crab-eating frog *Fejervarya cancrivora* and evolutionary implications. Gene 441: 148–155.

Roe BA, Ma DP, Wilson RK, Wong JF, 1985. The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. Journal of Biological Chemistry 260 (17): 9759–9774.

Roelants K, Bossuyt F, 2005. Archaeobatrachian paraphyly and Pangaean diversification of crown-group frogs. Systematic Biology 54: 111–126.

Roelants K, Gower DJ, Wilkinson M, Loader SP, Biju SD et al., 2007. Global patterns of diversification in the history of modern amphibians. Proceedings of the National Academy of Sciences of the United States of America 104: 887–892.

Roelants K, Jiang JP, Bossuyt F, 2004. Endemic ranid (Amphibia: Anura) genera in southern mountain ranges of the Indian subcontinent represent ancient frog lineages: Evidence from molecular data. Molecular Phylogenetics and Evolution 31: 730–740.

Saccone C, Giorgi CD, Gissi C, Pesole G, Reyes A, 1999. Evolutionary genomics in Metazoa: The mitochondrial DNA as a model system. Gene 238: 195–209.

San Mauro D, 2010. A multilocus timescale for the origin of extant amphibians. Molecular Phylogenetics and Evolution 56: 554–561.

San Mauro D, Gower DJ, Massingham T, Wilkinson M, Zardoya R et al., 2009. Experimental design in caecilian systematics: Phylogenetic information of mitochondrial genomes and nuclear rag1. Systematic Biology 58: 425–438.

San Mauro D, Garcia-Paris M, Zardoya R, 2004a. Phylogenetic relationships of discoglossid frogs (Amphibia: Anura: Discoglossidae) based on complete mitochondrial genomes and nuclear genes. Gene 343 (2): 357–366.

San Mauro D, Gower DJ, Oommen OV, Wilkinson M, Zardoya R, 2004b. Phylogeny of caecilian amphibians (Gymnophiona) based on complete mitochondrial genomes and nuclear RAG1. Molecular Phylogenetics and Evolution 33 (2): 413–427.

San Mauro D, Gower DJ, Zardoya R, Wilkinson M, 2006. A hotspot of gene order rearrangement by tandem duplication and random loss in the vertebrate mitochondrial genome. Molecular Biology and Evolution 23: 227–234.

San Mauro D, Vences M, Alcobendas M, Zardoya R, Meyer A, 2005. Initial diversification of living amphibians predated the breakup of Pangaea. American Naturalist 165: 590–599.

Sano N, Kurabayashi A, Fujii T, Yonekawa H, Sumida M, 2005. Complete nucleotide sequence of the mitochondrial genome of the Schlegel's tree frog *Rhacophorus schlegelii* (family Rhacophoridae): Duplicated control regions and gene rearrangements. Genes & Genetic Systems 80: 213–214.

Schattner P, Brooks AN, Lowe TM, 2005. The tRNAscan-SE, snoscan and snoGPS web servers for the detection of *tRNA*s and snoRNAs. Nucleic Acids Research 33: 686–689.

Schoch RR, Milner AR, 2004. Structure and implications of theories on the origins of lissamphibians. In: Arratia G, Wilson MV, Cloutier R ed. Recent Advances in the Origin and Early Radiation of Vertebrates: 345–377. Mu¨nchen: Pfeil.

Shelly C, 2000. S-adenosylmethionine. The International Journal of Biochemistry & Cell Biology 32: 391–395.

Simon C, Frait F, Beckenbach A, Crespi B, Liu H et al., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87: 651–701.

Sumida M, Kanamori Y, Kaneda H, Kato Y, Nishioka M et al., 2001. Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. Genes & Genetic Systems 76 (5): 311–325.

Swofford DL, 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), version 4.0 beta. Sunderland, MA: Sinauer Associates.

Trueb L, Cloutier R, 1991. A phylogenetic investigation of the inter- and intrarelationships of the Lissamphibia (Amphibia: Temnospondyli). In: Schultze HP, Trueb L ed. Origins of the Higher Groups of Tetrapods: Controversy and Consensus: 223-313. Ithaca, NY: Cornell University Press.

Tzeng CS, Hui CF, Shen SC, Huang PC, 1992. The complete nucleotide sequence of the *Crossostoma lacustre* mitochondrial genome: Conservation and variations among vertebrates.

Nucleic Acids Research 20: 4853–4858.

Wake MH, 1993. Non-traditional characters in the assessment of caecilian phylogenetic relationships. Herpetological Monographs 7: 42–55.

Wiens JJ, Bonett RM, Chippindale PT, 2005. Ontogeny discombobulates phylogeny: Paedomorphosis and higher-level salamander relationships. Systematic Biology 54: 91–110.

Wilkinson M, 1997. Characters, congruence and quality: A study of neuroanatomical and traditional data in caecilian phylogeny. Biological Reviews of the Cambridge Philosophical Society 72: 423–470.

Wilkinson M, San Mauro D, Sherratt E, Gower DJ, 2011. A nine-family classification of caecilians (Amphibia: Gymnophiona). Zootaxa 2874: 41–64.

Wilkinson M, Loader SP, Gower DJ, Sheps JA, Cohen BL, 2003. Phylogenetic relationships of African caecilians (Amphibia: Gymnophiona): Insights from mitochondrial rRNA gene sequences. African Journal of Herpetology 52: 83–92.

Wolstenholme DR, 1992. Animal mitochondrial DNA: Structure and evolution. International Review of Cytology-A Survey of Cell Biology 141: 173–216.

Wu Y, Liang YR, 2005. Research progress in effect of S-adenosy-L-methionine on physiological metabolism in tea plant. Journal of Tea Science 31 (2): 8587.

Xia YL, Liu YQ, Lu C, 2002. Principles and methods for the extraction of mitochondrium DNA of animals. Newsletter of Sericultural Science 22 (3): 24–29.

Yu ZL, Yang S, Cai J, Yuan ZY, 2003. Development in S-Adenosyl-L-methionine. The Chinese Pharmaceutical Journal 34 (1): 49–52.

Zardoya R, Meyer A, 2000. Mitochondrial evidence on the phylogenetic position of caecilians. Genetics 155 (2): 765-775.

Zardoya R, Meyer A, 2001. On the origin of and phylogenetic relationships among living amphibians. Proceedings of the National Academy of Sciences of the United States of America 98: 7380–7383.

Zhang JF, Nie LW, Wang Y, Hu LL, 2009. The complete mitochondrial genome of the large-headed frog *Limnonectes bannaensis* (Amphibia: Anura), and a novel gene organization in the vertebrate mtDNA. Gene 442: 119–127.

Zhang P, Chen YQ, Liu YF, Zhou H, Qu LH, 2003a. The complete mitochondrial genome of the Chinese giant salamander *Andrias davidianus* (Amphibia: Caudata). Gene 311: 93–98.

Zhang P, Chen, YQ, Zhou H, Wang XL, Qu LH, 2003b. The complete mitochondrial genome of a relic salamander *Ranodon sibiricus* (Amphibia: Caudata) and implications for amphibian phylogeny. Molecular Phylogenetics and Evolution 28: 620–626.

Zhang P, Wake DB, 2009a. Higher-level salamander relationships and divergence dates inferred from complete mitochondrial genomes. Molecular Phylogenetics and Evolution 53: 492–508.

Zhang P, Wake MH, 2009b. A mitogenomic perspective on the phylogeny and biogeography of living caecilians (Amphibia: Gymnophiona). Molecular Phylogenetics and Evolution 53: 479–491.

Zhang P, Zhou H, Liang D, Liu YF, Chen YQ et al., 2005. Mitogenomic perspectives on the origin and phylogeny of living amphibians. Systematic Biology 54: 391–400.

Zhang WG, Zhao YQ, Lai SY, Li JQ , Zhao JY, 2000. Study on the separation and purification of mitochaondrial DNA of inner mongolia cashmere goat and analyses of RFLP on IT. Journal of Inner Mongolia Agricultural University 21 (1): 23–28.

Zhou Y, Zhang JR, Zheng RQ, Yu BG, Yang G, 2009. Complete nucleotide sequence and gene organization of the mitochondrial genome of *Paa spinosa* (Anura: Ranoidae). Gene 447: 86–96.

Appendix The mitochondrial genome types of the 145 species of the Amphibia

Type	Number of genes	Number of Species	Species composition
1	38	108	*Ranodon sibiricus* (Zhang et al., 2003b); *Paramesotriton hongkongensis*, *Ichthyophis bannanicus*, *Bombina bombina* (Zhang et al., 2005); *Typhlonectes natans* (Zardoya & Meyer, 2000); *Rhinatrema bivittatum*, *Ichthyophis glutinosus*, *Uraeotyphlus cf. oxyurus*, *Scolecomorphus vittatus* (San Mauro et al., 2004b); *Andrias davidianus* (Zhang et al., 2003a); *Ambystoma mexicanum* (Arnason et al., 2004); *Lyciasalamandra atifi* (Zardoya and Meyer, 2001); *Ambystoma andersoni*, *Ambystoma dumerilii*, *Ambystoma tigrinum, Ambystoma californiense* (Samuels et al., 2005); *Andrias japonicus* (AB208679); *Ambystoma laterale*, *Bolitoglossa n. sp.*, *Desmognathus fuscus*, *Desmognathus wrighti*, *Eurycea bislineata*, *Gyrinophilus porphyriticus*, *Hemidactylium scutatum*, *Phaeognathus hubrichti*, *Plethodon cinereus*, *Plethodon petraeus*, *Batrachoseps wrightorum, Pseudotriton ruber*, *Ensatina eschscholtzii*, *Thorius n. sp.*, *Oedipina poelzi*, *Hydromantes italicus*, *Nototriton abscondens* (Mueller et al., 2004); *Discoglossus galgano*, *Bombina orientalis, Aytes obstetricans pertinax* (San Mauro et al., 2004a); *Xenopus laevis* (Roe et al., 1985); *Hynobius arisanensis* (NC_009335); *Bombina variegate* (Szymura et al., 2000); *Hynobius formosanus*, *Batrachuperus mustersi*, *Onychodactylus fischeri*, *Hynobius chinensis*, *Batrachuperus gorganensis*, *Batrachuperus tibetanus*, *Batrachuperus pinchonii*, *Salamandrella keyserlingii*, *Liua tsinpaensis* (*=Pseudohynobius tsinpaensis)*, *Pachyhynobius shangchengensis*, *Hynobius leechii*, *Liua shihi*, *Batrachuperus londongensis*, *Hynobius amjiensis* (Zhang et al., 2006); *Pelobates cultripes* (Gissi et al., 2006); *Bombina maxima* (AY974191); *Hynobius quelpartensis* (NC_010224); *Xenopus tropicalis* (NC_006839); *Hynobius yangi* (NC_013825); *Hynobius guabangshanensis* (NC_013762); *Ambystoma barbouri*, *Ambystoma texanum, Ambystoma unisexual* (Bi and Bogart, 2010)*; Amphiuma means, Cryptobranchus alleganiensis*, *Siren intermedia*, *Pseudobranchus axanthus* (Zhang and Wake, 2009a)*, Calotriton asper, Chioglossa lusitanica, Cynops cyanurus, Cynops ensicauda, Cynops orientalis, Cynops orphicus, Cynops pyrrhogaster, Echinotriton andersoni, Echinotriton chinhaiensis, Euproctus montanus, Euproctus platycephalus, Lyciasalamandra flavimembris, Lissotriton vulgaris, Mertensiella caucasica, Mesotriton alpestris, Neurergus kaiseri, Neurergus s. strauchii, Notophthalmus meridionalis, Notophthalmus viridescens, Ommatotriton vittatus, Pachytriton brevipes, Pachytriton labiatus, Paramesotriton caudopunctatus, Paramesotriton deloustali, Paramesotriton laoensis, Pleurodeles poireti, Pleurodeles waltl, Salamandra salamandra, Salamandrina terdigitata, Taricha granulosa, Taricha rivularis, Triturus cristatus, Triturus marmoratus, Tylototriton asperrimus, Tylototriton wenxianensis* (Zhang et al., 2008); *Schistometopum thomense, Praslinia cooperi, Pipa pipa, Grandisonia alternans, Epicrionops niger, Boulengerula taitanus* (Zhang and Wake, 2009b)
2	37	1	*Gegeneophis ramaswamii* (San Mauro et al., 2006)
3	38	1	*Siphonops annulatus* (San Mauro et al., 2006)
4	38	1	*Batrachoseps attenuatus* (Mueller et al., 2004)
5	38	2	*Aneides flavipunctatus* (NC_006327)*, Stereochilus marginatus* (Mueller et al., 2004)
6	38	1	*Plethodon elongatus* (Mueller et al., 2004)
7	42	1	*Aneides hardii* (Mueller et al., 2004)
8	38	1	*Hydromantes brunus* (Mueller et al., 2004)
9	39	1	*Rhyacotriton variegatus* (NC_007633)
10	38	1	*Batrachuperus yenyuanensis* (Zhang et al., 2006)
11	38	11	*Hyla chinensis*, *Kaloula pulchra*, *Microhyla heymonsi* (Zhang et al., 2005); *Pelophylax nigromaculatus* (= *Rana nigromaculata*; Sumida et al., 2001); *Pelophylax plancyi* (= *Rana plancyi*; NC_ 009264); *Bufo gargarizans* (Cao et al., 2006); *Bufo melanostictus* (Zhang et al., 2005); *Bufo japonicus*, *Hyla japonica*, *Microhyla okinavensis* (Igawa et al., 2008); *Microhyla ornata* (NC_009422)
12	36	1	*Polypedates megacephalus* (Zhang et al., 2005)
13	39	1	*Rhacophorus schlegelii* (Sano et al., 2005)
14	41	1	*Mantella madagascariensis* (Kurabayashi et al., 2006)
15	38	1	*Buergeria buergeri* (San et al., 2004)
16	38	2	*Odorrana tormotus* (= *Amolops tormotus*, Su et al., 2007) ; *Orodrrana ishiikawae* (Kurabayashi et al., 2010)
17	39	2	*Fejervarya limnocharis* (Liu et al, 2005); *Fejervarya cancrivora* (Ren et al., 2009)
18	38	1	*Limnonectes fujianensis* (NC_007440)
19	35	1	*Limnonectes bannaensis* (Zhang et al., 2009)
20	39	2	*Quasipaa spinosa (= Paa spinosa*; Zhou et al., 2009); *Nanorana pleskei* (this study)
21	40	1	*Euphlyctis hexadactylus* (Alam et al., 2010)
22	40	1	*Hoplobatrachus tigerinus*(Alam et al., 2010)
23	38	1	*Leiopelma archeyi* (Irisarri et al., 2010)
24	39	1	*Occidozyga martensii* (GU177877)

The name of species have been revised according to Frost (2010), their original name in literature present in parentheses.