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The complete mitochondrial genome of the relict frog *Leiopelma archeyi*: Insights into the root of the frog Tree of Life

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Abstract

Determining the root of the anuran Tree of Life is still a contentious and open question in frog systematics. Two genera with disjunct distributions have been traditionally considered the most basal among extant frogs: *Leiopelma*, which is endemic to New Zealand, and *Ascaphus*, which lives in North America. However, their specific phylogenetic position is rather elusive because each genus shows many autapomorphies, and together they retain many symplesiomorphic characters. Therefore, several alternative hypotheses have been proposed regarding the relative phylogenetic position of both *Leiopelma* and *Ascaphus*. In order to distinguish among these competing phylogenetic hypotheses, we sequenced the complete mitochondrial (mt) genome of *Leiopelma archeyi* and used it along with previously reported frog mt genomes (including that of *Ascaphus truei*) to infer a robust phylogeny of major anuran lineages. The reconstructed maximum likelihood and Bayesian inference phylogenies recovered identical topology, which supports the sister group relationship of *Ascaphus* and *Leiopelma*, and the placement of this clade at the base of the anuran tree. Interestingly, the mt genome of *L. archeyi* displays a novel gene arrangement in frog mt genomes affecting the relative position of cytochrome *b*, *trnT*, NADH dehydrogenase subunit 6, *trnE*, and *trnP* genes. The tandem duplication—random loss model of gene order change explains the origin of this novel frog mt genome arrangement, which is convergent with others reported in some fishes and salamanders. These results, together with comparative data for other available vertebrate mt genomes, provide evidence that the 5′ end of the control region is a hot spot for gene order rearrangement.

Keywords: *Leiopelmatidae, Anura, mitochondrial genome, gene rearrangement, molecular phylogeny, evolution*

Abbreviations: atp6 *and* atp8, *subunits 6 and 8 of the ATP synthase; bp, base pairs;* cob, *cytochrome* b*;* cox1–3, *subunits 1–3 of the cytochrome oxidase; mt, mitochondrial;* nad1–6, *NADH dehydrogenase subunits 1–6; NC, non-coding region;* O_L *and* O_H, *origins of light- and heavy-strand replication; PCR, polymerase chain reaction; rRNA, ribosomal ribonucleic acid;* rrnS *and* rrnL, *small and large subunits of the rRNA genes; tRNA, transfer ribonucleotide acid;* trnF, trnV, trnL, trnI, trnQ, trnM, trnW, trnA, trnN, trnC, trnY, trnS, trnD, trnK, trnG, trnR, trnH, trnE, trnT, *and* trnP *are transfer ribonucleotide acids for phenylalanine, valine, leucine, isoleucine, glutamine, methionine, tryptophan, alanine, asparagine, cysteine, tyrosine, serine, aspartic acid, lysine, glycine, arginine, histidine, glutamic acid, threonine, and proline, respectively*

Introduction

The genus *Leiopelma* Fitzinger 1861 comprises four extant and three extinct species and represents a relict and endemic group of frogs from New Zealand (Green et al. 1989). Along with the two species of the North American genus *Ascaphus*, they are considered the most basal extant Anura (Duellman and Trueb 1986; Green and Cannatella 1993; Hillis et al. 1993; Roelants and Bossuyt 2005; San Mauro et al. 2005;

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Frost et al. 2006), and therefore, they represent key lineages in understanding anuran evolution.

Both *Ascaphus* and *Leiopelma* share several morphological characters (Green et al. 1989) that were often used to hypothesize their sister group relationship. The most important one is the presence of amphicoelous vertebrae (i.e. with concavities on both anterior and posterior ends), which led to the clade being named the Amphicoela (Noble 1924, 1931; Ritland 1955; Green and Cannatella 1993). *Ascaphus* and *Leiopelma* also share the presence of nine presacral vertebrae, paired caudalipuboischiotibialis (tail-wagging) muscles, an epipubic cartilage, ribs not fused to the vertebrae, absence of vocal sacs, and the absence of a columella (Green and Cannatella 1993). However, most of these characters are now considered symplesiomorphies retained by both groups, and only the secondary loss of the columella may be a true synapomorphic character (Stephenson 1951). Ritland (1955) pointed out that the tail-wagging muscles are likely non-homologous to those of salamanders, and hence they may represent another synapomorphy for *Ascaphus* and *Leiopelma*. In addition, each genus exhibits its own unique apomorphies: *Ascaphus* has an intromittent organ for copulation in males (Duellman and Trueb 1986) and a highly modified torrent-dwelling tadpole, whereas *Leiopelma* has ventral inscriptional ribs (Noble 1931; Laurent 1986; Ford and Cannatella 1993) and lacks a feeding larval stage (Archey 1922; Altig and Johnston 1989; Bell and Wassersug 2003).

Even though *Ascaphus* and *Leiopelma* have always been considered basal living frogs, the possession of many symplesiomorphic characters along with their own autapomorphies have always hindered their specific phylogenetic relationships with respect to all other anuran lineages. Based on morphological evidence, the following groups have been proposed alternatively as the most basal anuran lineages and thus, sister group to all other anurans: (1) *Leiopelma* + *Ascaphus* + Discoglossoidea (including *Bombina*, *Barbourula*, *Discoglossus*, and *Alytes*) (Laurent 1979; Duellman and Trueb 1986), (2) *Ascaphus* (Ford and Cannatella 1993), (3) *Leiopelma* + *Ascaphus* (Lynch 1973), and (4) Pipoidea (Púgener et al. 2003). Ford and Cannatella (1993) proposed five putative synapomorphies to support *Ascaphus* as the sister group to the clade named Leiopelmatanura (*Leiopelma* + all other anurans). However, most studies supported a basal sister group relationship between *Leiopelma* and *Ascaphus* (Green et al. 1989; Báez and Basso 1996; Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006; Roelants et al. 2007; San Mauro 2010).

Moreover, this hotly debated question on the relative phylogenetic position of *Leiopelma* and *Ascaphus* is closely connected to that regarding the monophyly of "Archaeobatrachia" (containing the extant genera *Leiopelma*, *Ascaphus*, and the families Discoglossidae, Rhinophrynidae, Pipidae, Pelobatidae, and Pelodytidae, *sensu* Reig 1958; Duellman 1975). Early molecular analyses based on partial mitochondrial (mt) ribosomal ribonucleic acid (rRNA) gene sequences supported a monophyletic "Archaeobatrachia" as sister group of Neobatrachia (Hedges and Maxson 1993; Hay et al. 1995). However, recent morphological and molecular studies have supported the paraphyly of "Archaeobatrachia" with respect to Neobatrachia, even though the branching order varied among studies (Ford and Cannatella 1993; Haas 2003; Púgener et al. 2003; Hoegg et al. 2004; Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006; Gissi et al. 2006; Roelants et al. 2007). Furthermore, the questions on the paraphyly of "Archaeobatrachia" and whether or not *Leiopelma* and *Ascaphus* form a clade at the root of the anuran tree have been debated also in the context of biogeography. Feller and Hedges (1998) suggested the break up of the supercontinent Pangaea as the vicariant event that triggered the split between "archaeobatrachians" primarily distributed in Laurasia and neobatrachians originally in Gondwana. This would imply a post-Pangaean diversification of each group. In contrast, Roelants and Bossuyt (2005) and San Mauro et al. (2005) found strong support for the paraphyly of "Archaeobatrachia" with respect to the Neobatrachia, and the alternative hypothesis that initial anuran divergences, starting with the separation of the *Leiopelma* + *Ascaphus* clade from all other anurans, predated the continental fragmentation of Pangaea. Moreover, Roelants and Bossuyt (2005) suggested that the split between *Ascaphus* and *Leiopelma* was triggered by the early fragmentation of Pangaea in the Jurassic.

In order to distinguish among competing hypotheses regarding the root of the frog Tree of Life, we sequenced anew the complete mt genome of *Leiopelma archeyi*. The deduced amino acid sequences of the protein-coding genes of the *Leiopelma* mt genome were aligned with homologous sequences of previously reported anuran mt genomes and combined into a single sequence data set, which was used to infer phylogenetic relationships among major anuran lineages. This mitogenomic (Curole and Kocher 1999) approach follows many recent studies that have demonstrated the phylogenetic utility of complete mt genome sequence data for reconstructing statistically robust deep-level phylogenies, particularly in amphibians (e.g. Zardoya and Meyer 2001; Mueller et al. 2004; San Mauro et al. 2004a,b, 2009; Zhang et al. 2005; Zhang and Wake 2009).

Materials and methods

DNA extraction, polymerase chain reaction (PCR) amplification, cloning, and sequencing

The complete sequence of the mt genome of a single specimen of *L. archeyi* was determined. The specimen was collected in the Whareorino Forest, west of Te

Kuiti, New Zealand, by David M. Green (voucher DMG5136), and it is deposited in the Redpath Museum (Montréal, Canada) under catalog number RM2215. Total DNA was purified following standard phenol–chloroform extraction procedures (Sambrook et al. 1989). Several overlapping fragments covering the whole mt genome were amplified by polymerase chain reaction (PCR) using the primers and conditions reported in San Mauro et al. (2004b). Specific primers were designed to amplify the region between cytochrome *b* (*cob*) and the small subunit of the rRNA (*rrnS*) gene (LEI-P10F 5′-ACC CTA ATA GTA ATT GTC ACC-3′, LEI-P11F2 5′-TTC TGG GCC CTA GTA TCC AAC ACC TTA ATC C-3′, LEI-12SR2 5′-TGG CTG AGC CAG GTG TCT TGG GCT TAG-3′) because of the presence of a gene order rearrangement (see below). Other specific walking primers were designed to cover the total length of the control region (available from the authors upon request). PCR products were purified by ethanol precipitation and sequenced in an automated DNA sequencer (ABI PRISM 3700) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). If needed, PCR products were cloned into pGEM-T vectors (Promega, Madison, WI, USA) and sequenced using M13 universal primers. The obtained sequences averaged 700 base pairs (bp) in length and each sequence overlapped with the next contig by about 150 bp. Differences between overlapping regions were not observed. The complete mt genome sequence reported in this paper has been deposited in NCBI GenBank under accession number HM142901.

Molecular and phylogenetic analyses

The new mt genome sequence data were compared with previously sequenced frog and salamander mt genome sequences. Taxon sampling among available species in GenBank was designed to represent main lineages within extant Anura. One species was selected for each available non-neobatrachian family: *Alytes obstetricans* (NC_006688), *Ascaphus truei* (AJ871087), *Bombina orientalis* (NC_006689) *Discoglossus galganoi* (NC_006690), *Pelobates cultripes* (NC_008144), and *Silurana tropicalis* (NC_006839). Two species were chosen to represent each of the two most diverse lineages within Neobatrachia: *Duttaphrynus melanostictus* (NC_005794) and *Hyla chinensis* (NC_006403) for *Hyloides* and *Fejervarya limnocharis* (NC_005055) and *Pelophylax nigromaculata* (NC_002805) for *Ranoides* (*sensu* Frost et al. 2006). Four salamander species were used as outgroups: *Ambystoma mexicanum* (NC_005797), *Andrias davidianus* (NC_004926), *Lyciasalamandra atifi* (NC_002756), and *Ranodon sibiricus* (NC_004021).

Phylogenetic analyses were conducted using the 12 protein-coding genes encoded by the heavy strand of the mt genome. The light-strand-encoded NADH dehydrogenase subunit 6 (*nad6*) was not included in the analyses to eliminate possible bias due to the difference in the base composition between the two strands (Reyes et al. 1998). The deduced amino acid sequences of all 12 mt protein-coding genes were used in all phylogenetic analyses to avoid saturation at the nucleotide level (San Mauro et al. 2004a; Gissi et al. 2006). Separate amino acid alignments were produced for each of the 12 genes, and, in all cases, sequences were aligned manually against a previous database (San Mauro et al. 2004a; Gissi et al. 2006). The 12 amino acid alignments were combined into a single concatenated data set, and gaps and alignment ambiguities were excluded from it using Gblocks v0.91b (Castresana 2000) with default parameter settings.

The amino acid sequences deduced from the mt genomes of neobatrachians were highly divergent (producing long branches) compared to those of non-neobatrachians (Hoegg et al. 2004; San Mauro et al. 2004a). This unequal substitution rate among taxa may have severe effects on the reconstruction of the anuran phylogeny (long-branch attraction artifacts; Felsenstein 1978) based on complete mt genomes (San Mauro et al. 2004a; Gissi et al. 2006). Hence, in order to reduce biases in phylogenetic analyses, fast-evolving sites were excluded from the concatenated data set using the categories of the Γ distribution discrete approximation of rate heterogeneity as selective criterion. PAML v3.15 (Yang 1997) was employed to approximate the Γ distribution using eight discrete categories on the concatenated data set, and thus, each site of the alignment was assigned to one of the eight categories. All sites assigned to the two fastest evolving categories (with rates of evolution of 4.48 and 1.76 substitutions/site, respectively) were manually removed from the data set. The six slowest evolving categories (sites maintained in the final data set) have rates of evolution of less than 0.90 substitutions/site.

Phylogenetic relationships were estimated using maximum likelihood (Felsenstein 1981) and Bayesian inference (Huelsenbeck and Ronquist 2001). Maximum likelihood analysis was conducted with RAxML v7.0.4 (Stamatakis 2006) using the rapid hill-climbing algorithm (Stamatakis et al. 2007). For Bayesian inference, we used MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) running four simultaneous Markov chains for 10 million generations, sampling every 1000 generations, and discarding the first 1 million generations as burn-in (as judged by plots of maximum likelihood scores and low SD of split frequencies) to prevent sampling before reaching stationarity. Two independent Bayesian inference runs were performed to increase the chance of adequate mixing of the Markov chains and to increase the chance of detecting failure to converge. For both maximum likelihood and Bayesian inference, the best-fit model of amino acid substitution for the data set was identified using the Akaike information

criterion (Akaike 1973) as implemented in ProtTest v1.3 (Abascal et al. 2005) (the " + F" parameter was not included in the model comparison). The resulting best-fit model was mtREV (Adachi and Hasegawa 1996) + Γ (Yang 1994) + I (Reeves 1992). Support for internal branches was evaluated by non-parametric bootstrapping (Felsenstein 1985) with 1000 replicates (maximum likelihood) and by posterior probabilities (Bayesian inference). Eight alternative tree topologies (see results) were evaluated using the non-parametric approximately unbiased (AU) test (Shimodaira 2002) as implemented in Consel v0.1i (Shimodaira and Hasegawa 2001) with site-wise log-likelihoods calculated in PAML under a mtREV + Γ model and 1 million multiscale bootstrap replicates.

The MitoZoa database (http://mi.caspur.it/mitozoa; Lupi et al. 2010) was used to provide comparative information on the gene arrangement of the 1409 vertebrate mt genomes included as of February 2010 (release 2.0).

Results

Mitochondrial genome organization and structural features

The complete nucleotide sequence of the light strand of the mt genome of *L. archeyi* was determined. The total length of the newly sequenced genome was 16,593 bp long and, as for most metazoans (Boore 2000), it encoded for two rRNAs, 22 transfer ribonucleotide acids (tRNAs), and 13 protein-coding genes (Figure 1). All tRNAs could be folded into typical cloverleaf secondary structures with the known exception of *trnS* (*AGY*). Most protein-coding genes started with the codon ATG with the exception of subunit 1 of the cytochrome oxidase (*cox1*) (GTG), *nad1* (CTC), and *nad6* (TTG). Some genes had complete stop codons (TAA in subunits 6 and 8 of the ATP synthase, *nad4L*, and *nad5*; TAG in *cob*, *nad1*, and *nad2* ; AGG in *nad6*), whereas other genes (*cox1–3*, *nad3*, and *nad4*) ended with a single T, which presumably becomes functional by subsequent polyadenylation of the transcribed messenger RNAs (Ojala et al. 1981).

The gene order arrangement in the mt genome of *L. archeyi* departs from the vertebrate consensus mt gene arrangement (Boore 1999; Lupi et al. 2010) (Figure 1). The mt region upstream of the control region in *L. archeyi* is rearranged so that the *cob* and *trnT* genes are located immediately downstream of the *nad5* gene, and the *nad6*, *trnE*, and *trnP* genes are located between the control region and *trnF* (Figure 1). No changes in coding strand were observed for the rearranged genes. The control region is 858 bp long, and it contains three conserved sequence blocks (CSB-1, CSB-2, CSB-3; Walberg and Clayton 1981) that participate in the formation of a proper RNA primer in the process of replication of the mt DNA (Fernández-Silva et al. 2003), as well as three putative termination-associated sequences (TAS-1, TAS-2, TAS-3; Doda et al. 1981; MacKay et al. 1986). A 219 bp-long non-coding region was found between *trnP* and *trnF* (Figure 1). This region has two 46 bp-long non-tandem repeats separated by 27 nucleotides, 9 of which are also displayed by the two non-tandem repeats. Other intergenic spacers occurred between *nad6* and *trnE* (15 nucleotides) and between *trnE* and *trnP* (17 nucleotides). The putative origin of light-strand replication (O_L) was located within the WANCY tRNA cluster, between *trnN* and *trnC* genes, and had the potential to fold into a stem loop of secondary structure (Figure 1). The 5′-GCCGG-3′ motif, which in human mt DNA replication is involved the transition from RNA to DNA synthesis (Hixson et al. 1986), is entirely conserved in *L. archeyi*.

Phylogenetic analyses

Sites of ambiguous alignment as well as those with the fastest substitution rates were excluded rendering a final matrix of 2498 amino acid positions. Of these, 1703 were invariant and 441 were parsimony-informative. Both maximum likelihood ($-\ln L = 15{,}839.46$) and Bayesian inference ($-\ln L = 15{,}823.80$ for run1; $-\ln L = 15{,}823.93$ for run2) methods arrived at the same tree topology and only showed differences in branch lengths and levels of support (Figure 2).

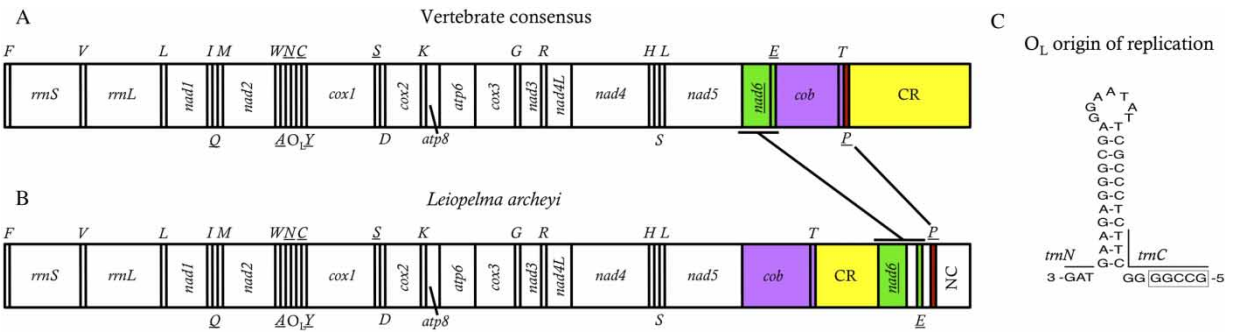

Figure 1. Gene organization of the consensus mt genome of vertebrates (A) and that of *L. archeyi* (B) Genes encoded by the light strand are underlined. Lines indicate translocated genes and rearranged genes are colored. (C) Proposed secondary structure for the O_L of *L. archeyi*. The 5′-GCCGG-3′ motif is indicated by a box. The lines show the overlapping regions with flanking transfer RNAs.

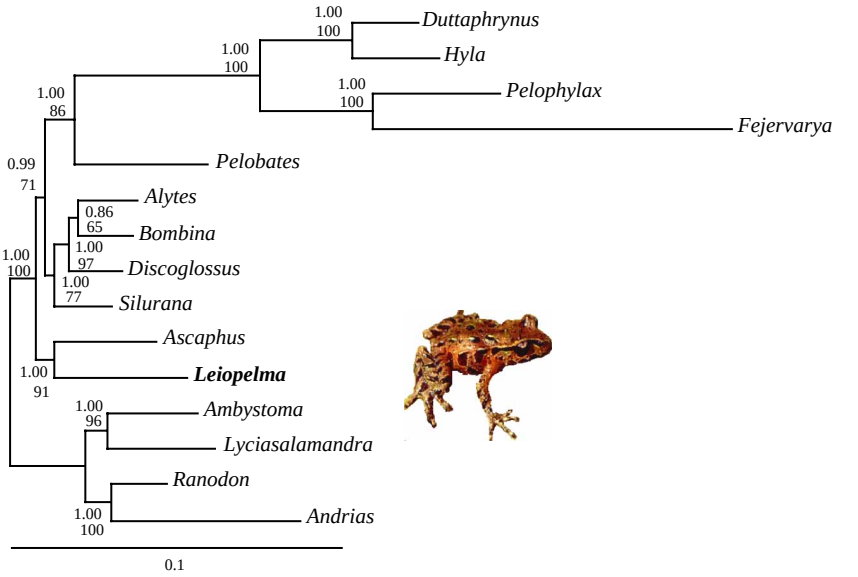

Figure 2. Anuran phylogeny (ML phylogram) inferred from a single concatenated data set of the deduced amino acid sequences of all mitochondrial protein-coding genes encoded by the heavy strand. The numbers above each node represent support for Bayesian inference (posterior probabilities; upper value) and maximum likelihood (RAxML bootstrap proportions; lower value). Scale bar represents substitutions/site. Picture by David M. Green showing a specimen of *L. archeyi* of the Whareorino Forest, west of Te Kuiti, New Zealand.

The reconstructed tree strongly supported the paraphyly of "Archaeobatrachia" with respect to Neobatrachia, the sister group relationship between *Leiopelma* and *Ascaphus*, and the basal position of this clade as sister to all other anuran lineages (Figure 2). Pipoidea (*Silurana*) was recovered as the sister group of Discoglossoidea with strong support. Internal relationships within Discoglossoidea were poorly resolved (*Discoglossus* was recovered as sister to *Alytes* + *Bombina*, but with low statistical support; Figure 2). The Pipoidea + Discoglossoidea clade was recovered as the sister group of a Pelobatoidea + Neobatrachia clade with strong support. Within Neobatrachia, two recognized clades (*Hyloides* and *Ranoides* [*sensu* Frost et al. 2006]) were recovered with high support (Figure 2).

According to the AU test for seven alternative rooting and branching phylogenetic hypotheses (Table I), the mt sequence data set rejected any topology that involved changes in the position of the root, i.e. hypotheses placing *Leiopelma* ($P = 0.023$), *Ascaphus* ($P = 0.037$), or *Silurana* ($P = 0.017$) alone at the base of the anuran tree. The "Mesobatrachia" hypothesis that implies a sister group relationship of *Silurana* and *Pelobates* was also significantly rejected ($P = 0.009$). Alternative hypotheses placing Pipoidea as the second major lineage branching off the anuran tree (San Mauro et al. 2005; Frost et al. 2006) or as the third major lineage branching off the anuran tree (Roelants and Bossuyt 2005; Roelants et al. 2007) could not be rejected (Table I). The recovered internal phylogenetic relationships of the discoglossids represented in our tree were not significantly different from those recovered by San Mauro et al. (2004a), i.e. a sister group relationship between *Alytes* and *Discoglossus* to the exclusion of *Bombina*; which is also supported by many other posterior studies (Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006; Gissi et al. 2006; Roelants et al. 2007).

Discussion

New mitochondrial gene order in anurans

The gene order found in the mt genome of *L. archeyi* departs from the consensus order of vertebrates

Table I. Log-likelihood values and *P* values of the AU tests for seven alternative rooting and branching scenarios. References are given below each alternative hypothesis.

Alternative hypotheses	$-\ln L$	AU test *P* value
Unconstrained tree	15,865.149	0.813
Leiopelma + all other anurans	15,875.565	*0.023*
Ascaphus + all other anurans	15,875.080	*0.037*
Ford and Cannatella (1993)		
Bombina + (*Discoglossus* + *Alytes*)	15,867.385	0.517
San Mauro et al. (2004a,b)		
Pipoidea + (Discoglossoidea + (Pelobatoidea + Neobatrachia))	15,874.319	0.076
San Mauro et al. (2005) and Frost et al. (2006)		
Discoglossoidea + (Pipoidea + (Pelobatoidea + Neobatrachia))	15,872.534	0.279
Roelants and Bossuyt (2005) and Roelants et al. (2007)		
"Mesobatrachia" (*Silurana* sister of *Pelobates*)	15,888.376	*0.009*
Ford and Cannatella (1993) and García-París et al. (2003)		
Silurana + all other anurans	15,884.661	*0.017*
Púgener et al. (2003)		

References are given below each hypothesis, and significant results ($p < 0.05$) are italicized.

(Boore 1999; Lupi et al. 2010) and it is clearly derived. Gene order rearrangements had only been reported in anurans among neobatrachians (Sano et al. 2005; Igawa et al. 2008; Kurabayashi et al. 2008), with the non-neobatrachian frogs conforming to the vertebrate consensus (Roe et al. 1985; San Mauro et al. 2004a; Gissi et al. 2006). The derived gene order found in *L. archeyi* can be explained as the result of a single ancestral tandem duplication of the mitogenomic region involving the genes *nad6*, *trnE*, *cob*, *trnT*, *trnP* and the control region ("CR 5′ region" hereafter), followed by arbitrary loss of redundant gene duplicates. Thus, it follows the tandem duplication—random loss model (TDRL) of gene order change (Moritz and Brown 1986, 1987; Moritz et al. 1987; Boore 2000), which is confidently regarded as the dominant mechanism of gene order rearrangement in vertebrate mt genomes (San Mauro et al. 2006).

The gene order found in *L. archeyi* is new in anurans, although convergent gene arrangements are found in two species of plethodontid salamanders (*Aneides flavipunctatus* and *Stereochilus marginatus*) and 14 species of eels (*Ophisurus macrorhynchos*, *Myrichthys maculosus*, *Coloconger cadenati*, *Derichthys serpentinus*, *Nessorhamphus ingolfianus*, *Cynoponticus ferox*, *Muraenesox bagio*, *Paraconger notialis*, *Ariosoma shiroanago*, *Conger myriaster*, *Nettastoma parviceps*, *Hoplunnis punctata*, *Facciolella oxyrhyncha*, and *Leptocephalus* sp.) among all available vertebrate mt genomes (Benson et al. 2007; Lupi et al. 2010). Previous studies have indicated that duplications are more likely to take place in close proximity to (or involving) replication origins due to mechanistic constraints (e.g. Moritz and Brown 1987; Mindell et al. 1998; Dowton and Austin 1999; Boore 2000). In fact, the WANCY region of the mt genome, which includes the replication origin of the light strand, has been shown to be a hot spot for gene order rearrangement (San Mauro et al. 2006). Alternative mechanisms such as secondary structures of tRNAs (Moritz and Brown 1987) or incorporation of the nascent chain of the heavy-strand replication (O_H) process (Zardoya et al. 1995) have been proposed as a source of duplications in the CR 5′ region. Moritz and Brown (1987) pointed out that most of the duplication boundaries found in lizards of the genus *Cnemidophorus* were at or near tRNA genes and suggested that tRNAs could act as promoters of gene duplications due to either secondary structure or sequence similarities between different tRNAs. During the replication of the heavy strand in the mt genome, most newly initiated chains are arrested by the TAS (Doda et al. 1981; MacKay et al. 1986) and their replication finishes downstream, shortly after the O_H (1350–1510 bp in *Xenopus laevis*; Roe et al. 1985). The newly synthesized chain remains associated with the template, thus, creating a triple-stranded structure known as the D-loop (Clayton 1982; MacKay et al. 1986; Bowmaker et al. 2003) that may be responsible for mt genome rearrangement by non-homologous recombination of the nascent chain (Zardoya et al. 1995). In fact, the phenomenon of replication fork arrest is a well-recognized prelude to genome rearrangement (Hyrien 2000; Rothstein et al. 2000; Bidnenko et al. 2002). Besides *L. archeyi*, more instances of gene order rearrangements occur in the CR 5′ region of vertebrates (online Table 1), and hence, this region may constitute another hot spot for gene order rearrangement. The present case of the CR 5′ region is fairly similar to that of the WANCY region in the sense that it also involves five rearranged genes (*cob*, *trnT*, *nad6*, *trnE*, *trnP*) in close proximity to one of the replication origins.

We identified the different mt gene orders involving the vertebrate CR 5′ region through searches in the MitoZoa (Lupi et al. 2010) and GenBank (Benson et al. 2007) databases. From the 1409 vertebrate mt genomes available in MitoZoa (Lupi et al. 2010), the vast majority (1100 entries) conformed to the vertebrate consensus. The remaining entries presented a distinct gene order, and 116 out of these involved the CR 5′ region. Among these 116 rearrangements in the control region, we found seven different types of derived gene orders. If we ignore those taxa in which some genes were deleted or duplicated, we can invoke a single tandem duplication followed by random loss of redundant genes as the mechanism that produced these seven types of rearrangements. The observed seven types of derived gene orders are the result of at least 12 independent rearrangement events that took place during the evolution of vertebrates (see online Table 1). The identical arrangement found in the two fish genera *Dallia* (first putative event of independent rearrangement) and *Rudarius* (second) seems to be a convergence due to the strong evidence of the distinct origin and monophyly of Esociformes (Nelson 2006) and Acanthomorpha, respectively (Stiassny 1986; Johnson and Patterson 1993; Wiley et al. 2000; Miya et al. 2003). *Ventrifossa garmani* (Satoh et al. 2006) represents a distinct derived gene order (third) from all other vertebrates. Birds show different convergences in gene order (Mindell et al. 1998), but a single independent event of rearrangement (fourth) seems to have taken place at the base of that clade. Nevertheless, this ancestral rearrangement of the mt genome of birds is convergent with that of the reptile *Rhineura floridana* (fifth) (Macey et al. 2004). All lampreys reported to date (sixth) share another divergent mt gene order. The frog *L. archeyi* (seventh) has a convergent gene order with 14 species of eels and two species of plethodontid salamanders. Eels of the suborder Congroidei include species with mt gene orders, both conforming to the consensus of vertebrates and with a similar gene order to *L. archeyi*, but for simplicity reasons, and because phylogenetic relationships within Congroidei still need to be resolved (Nelson 2006), we considered the latter as a single and independent event of mt genome rearrangement (eighth). As pointed out

by Mueller et al. (2004), the gene orders found in the plethodontid salamanders *S. marginatus* (9th) and *A. flavipunctatus* (10th) are the result of convergence. Other derived mt gene orders found correspond to three species of reptiles of the genus *Bipes* (11th) (Macey et al. 2004) and to the salamander *Plethodon elongatus* (12th) (Mueller et al. 2004). Following San Mauro et al. (2006), the conditional probability of at least one convergence given 12 independent rearrangements in the CR 5′ region is 0.95. Hence, the observed cases of convergence are not surprising, and strengthen the hypothesis that the CR 5′ region is a hot spot of gene order rearrangement.

Phylogenetic position of Leiopelma *and paraphyly of "Archaeobatrachia"*

Since, their description, both *Ascaphus* and *Leiopelma* have been considered to be the most basal of extant anurans (Duellman and Trueb 1986). In contrast to the paraphyletic relationship of *Ascaphus* and *Leiopelma* with respect to other anurans proposed by Ford and Cannatella (1993), our phylogenetic analyses support the sister group relationship between these two genera and place them at the base of the anuran tree (Figure 2). Several morphological (Lynch 1973; Green et al. 1989) and molecular (Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006; Roelants et al. 2007; San Mauro 2010) data support this hypothesis. The secondary loss of the columella (Stephenson 1951) and perhaps the tail-wagging muscles (Ritland 1955) appear to be the only morphological synapomorphies supporting the *Leiopelma* + *Ascaphus* clade (Amphicoela).

Our results strongly support the paraphyly of "Archaeobatrachia," which was proposed long ago by Noble (1931) and well corroborated by many studies since then (Reig 1958; Sokol 1975; Duellman and Trueb 1986; Hillis et al. 1993; Hoegg et al. 2004; Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006; Gissi et al. 2006; Roelants et al. 2007). Therefore, the monophyly of "Archaeobatrachia" supported by Hedges and Maxson (1993) and Hay et al. (1995) based on partial sequences of mt rRNA genes is likely spurious in the light of all recent molecular evidence. It seems likely that the limited data set employed by Hedges and Maxson (1993) and Hay et al. (1995) and the long branches exhibited by neobatrachians may have obscured the real phylogenetic signal (Roelants and Bossuyt 2005). The phylogenetic relationships within Discoglossoidea could not be fully resolved by our analyses, although a general agreement exists on the sister group relationship between *Discoglossus* and *Alytes* to the exclusion of *Bombina*. This finding was supported by several morphological (Púgener et al. 2003) and molecular (Biju and Bossuyt 2003; Hoegg et al. 2004; Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006) phylogenetic studies, and it was strongly supported previously by mt genome data (San Mauro et al. 2004a). The clade Pipoidea (represented by the genus *Silurana* in this study) is recovered as the sister group of Discoglossoidea in the reconstructed tree (Figure 2; Gissi et al. 2006). This phylogenetic hypothesis disagrees with two other reported hypotheses regarding the position of Pipoidea: (Pipoidea + (Discoglossoidea + (Pelobatoidea + Neobatrachia))) (San Mauro et al. 2005; Frost et al. 2006) and (Discoglossoidea + (Pipoidea + (Pelobatoidea + Neobatrachia))) (Roelants and Bossuyt 2005; Roelants et al. 2007). The AU test could not discriminate among the three hypotheses (Table I). Roelants and Bossuyt (2005) highlighted the complexity of the position of Pipoidea, and they could not choose between alternative branching orders for Discoglossoidea and Pipoidea. Additional sequence information of key anuran lineages is required to tackle this phylogenetic problem. Other hypotheses regarding the position of Pipoidea either as the sister group of Pelobatoidea to comprise the "Mesobatrachia" (Ford and Cannatella 1993; García-París et al. 2003) or as basal to all other anuran lineages (Púgener et al. 2003) are rejected by the AU test and are indeed discarded by most recent studies (Haas 2003; Roelants and Bossuyt 2005; San Mauro et al. 2005; Frost et al. 2006; Gissi et al. 2006; Roelants et al. 2007). In fact, any hypothesis assuming a basal split among anurans other than that comprising the *Leiopelma* + *Ascaphus* clade was rejected by the AU tests (Table I).

Salamanders are confidently thought to be the closest living relatives of anurans (Batrachia hypothesis; Milner 1988; Trueb and Cloutier 1991; Zardoya and Meyer 2001; San Mauro et al. 2005; Zhang et al. 2005; Frost et al. 2006; Carroll 2007; Hugall et al. 2007; Roelants et al. 2007; Ruta and Coates 2007; Zhang and Wake 2009; San Mauro 2010), and therefore, they are used in many studies, such as this one, to root anuran phylogenetic trees. However, the long branches exhibited by the mt genes of neobatrachians (Figure 2) may produce a long-branch attraction effect, rendering phylogenetic inference more difficult (Felsenstein 1978; Swofford 1996). The fast-evolving taxa (neobatrachians) may end up being grouped together and attracted to the outgroup taxa (salamanders), because the branch connecting the outgroup to the ingroup is the longest branch in the tree. The problems associated with the use of salamanders as outgroups of anuran phylogenies were already pointed out in previous molecular studies (García-París et al. 2003; San Mauro et al. 2004a; Gissi et al. 2006). Considering that the basal position of *Leiopelma* + *Ascaphus* as the sister group of all other anurans is here confidently confirmed, we would recommend the use of these two taxa instead of salamanders to root future anuran phylogenetic studies since this would help reducing the spurious effects of long-branch attraction.

Biogeographical implications of anuran phylogeny

The sister group relationship between *Leiopelma* and *Ascaphus* has sometimes been disputed due to their present disjunct geographical distribution (Green et al. 1989). *Leiopelma* is exclusively distributed in New Zealand, a relict of Gondwana, whereas *Ascaphus* occurs in the west coast of North America, which originated from Laurasia. Feller and Hedges (1998) proposed that the break up of the Pangaea supercontinent in the Mesozoic was the vicariant event that triggered the divergence of the "Archaeobatrachia" and Neobatrachia as the two major clades of frogs. However, neither the evident paraphyly of "Archaeobatrachia" (as recovered in this study) nor the sister group relationships of *Leiopelma* and *Ascaphus* and their current disjoint distributions are congruent with that hypothesis. Furthermore, recent molecular studies have pointed out a likely earlier origin of anurans, with initial splits predating the fragmentation of Pangaea (San Mauro et al. 2005; Roelants et al. 2007; San Mauro 2010). In contrast to the hypothesis of Laurasia as the center of diversification for "archaeobatrachians" and Gondwana for neobatrachians (Feller and Hedges 1998), a more or less widespread distribution of primitive anurans throughout Pangaea (Bossuyt and Roelants 2009) is consistent with the reported molecular dating, and it is reinforced by the fossil record of stem group anurans that has been recovered in both Laurasian- and Gondwanan-derived land masses (Estes and Reig 1973; Savage 1973; Duellman and Trueb 1986; Rage and Roček 1989; Shubin and Jenkins 1995; Evans and Borsuk-Bialynicka 1998). This latter hypothesis more easily explains current distribution patterns of some anurans, such as those of *Leiopelma* and *Ascaphus*, or the occurrence of pipid frogs only on southern landmasses (once part of Gondwana) and not in northern Laurasian-derived ones.

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