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Short Research Paper

Mitochondrial genomes of the South American frogs *Eupsophus vertebralis* and *E. emiliopugini* (Neobatrachia: Alsodidae) and their phylogenetic relationships

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Abstract

We report the sequencing and compare the mitochondrial genomes of the South American ground frogs *Eupsophus vertebralis* and *E. emiliopugini* and reconstruct phylogenetic relationships among *Eupsophus* species. These genomes consist of 16,156 and 16,711 bp in length, respectively and contain 13 protein-coding genes, two ribosomal RNA genes, 22 transfer RNA genes (tRNA), and partial non-coding D-loop region. Both genomes share 94.5% identity with 879 variable sites. A phylogenetic analysis with other available mitogenomes recovered both species as the sister clade of *Alsodes gargola*. Sequences from *D-loop*, *CO1*, and *Cyt b*, amplified and sequenced with primers developed from the mitochondrial genomes, allowed us to reconstruct phylogenetic relationships among *Eupsophus* species. Since our report represents the first mitogenomes for the genus *Eupsophus*, we expect these data will be valuable for further studies on conservation genetics and on the evolution of Patagonian amphibians.

Key words: anurans, mitochondrial genome, *Eupsophus*, conservation

Introduction

The alsodid ground frogs genus *Eupsophus* is divided into the *roseus* (2n=30) and *vertebralis* (2n=28) groups, distributed throughout the temperate *Nothofagus* forests of South America [1, 2]. The *roseus* group is composed of *E. altor*, *E. roseus*, *E. calcaratus*, *E. contulmoensis*, *E. insularis*, *E. septentrionalis*, *E. migueli,* and *E. nahuelbutensis,* while the *vertebralis* group consists of *E. vertebralis* and *E. emiliopugini* [2]. The last two species are distributed mostly in Southern Chile, with marginal distribution in Argentina (Puelo Lake and Puerto Blest) [3]. *Eupsophus vertebralis* is included in Red List Category as Near Threatened [4], whereas *E. emiliopugini* as Least Concern [5]. The accelerated destruction and degradation of native *Nothofagus* forests, generally through fires and replacement with monocultures of exotic plant species, could render these species as vulnerable in the near future [6]. So far, no mitochondrial genomes for the genus *Eupsophus* have been described; here we provide mitogenomic data sets of the two species belonging to *vertebralis* group and reconstruct phylogenetic relationships using selected mitochondrial DNA segments from all *Eupsophus* species.

Adult specimens of *E. vertebralis* and *E. emiliopugini* were collected from Oncol (-39.69845, -73.32725) and Puyehue (-40,74448, -72,27309), respectively. These specimens were euthanized in accordance with Exempt Resolution No. 9244/2015 of Servicio Agrícola y Ganadero, Ministerio de Agricultura, Gobierno de Chile and included in herpetological collection from Instituto de Ciencias Marinas y Limnológicas, Univerisidad Austral de Chile (vouchers ICMLH495 and ICMLH499 for *E. vertebralis* and *E. emiliopugini*, respectively). Mitochondria were isolated from liver using the Thermo Scientific Mitochondria Isolation Kit (Cat. No.

89801) and their DNA extracted with the DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69504). The extracted DNA was used to construct an Ilumina paired-end (PE) genomic library and this library was sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA). Quality control of PE reads was carried out using Trim Galore! v0.4.5 [7] and BBMap v37.90 [8]. A subset of 1.7M trimmed reads were used for reconstructing the mitochondrial genomes with MITObim v1.7 [9]. We used *12S rRNA*, *tRNA-Val* and *16S rRNA* sequences from *Eupsophus altor* (JX204194) and the mitochondrial genome of *Telmatobius bolivianus* (JF703234) as reference sequences. A total of 38,652 (for *E. vertebralis*) and 9,192 (for *E. emiliopugini*) individual mitochondrial reads were obtained with a mean coverage of 468- and 102-fold, respectively. Mitochondrial genomes were annotated in Geneious vR9 (Biomatters Ltd., Auckland, New Zealand) by aligning them to the mitogenome of *T. bolivianus* (JF703234) and *Alsodes gargola* (JX564852).

Mitogenome sizes of 16,156 and 16,711 bp for *E. vertebralis* and *E. emiliopugini* were obtained, respectively (GenBank Accession Numbers: MH070027 and MH070028). Both sequences comprise 37 genes, including 13 protein coding genes (PCGs), 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes, and a partial sequence from the Control Region (*D-loop*) (Fig. 1, Table 1). Mitogenomic base composition was A (27.9%), T (32.4%), C (19.1%), G (20.6%), and GC (39.7%) for *E. vertebralis* and A (29.4%), T (32.4%), C (19.1%), G (19.1%), and GC (38.2%) for *E. emiliopugini*. Both mitogenomes exhibited the usual mitochondrial gene order for neobatrachian frogs [10]. This order is characterized by having the Control Region located between *Cyt b* and a block of tRNAs (*tRNAPro*, *tRNAGln*, *tRNAAla*, *tRNAAsn*), as well as other block of tRNAs (*tRNAGlu*, *ND6*, *ND5*, *tRNASer*, and *tRNAHis*) located downstream from *Cyt b* (Fig. 1).

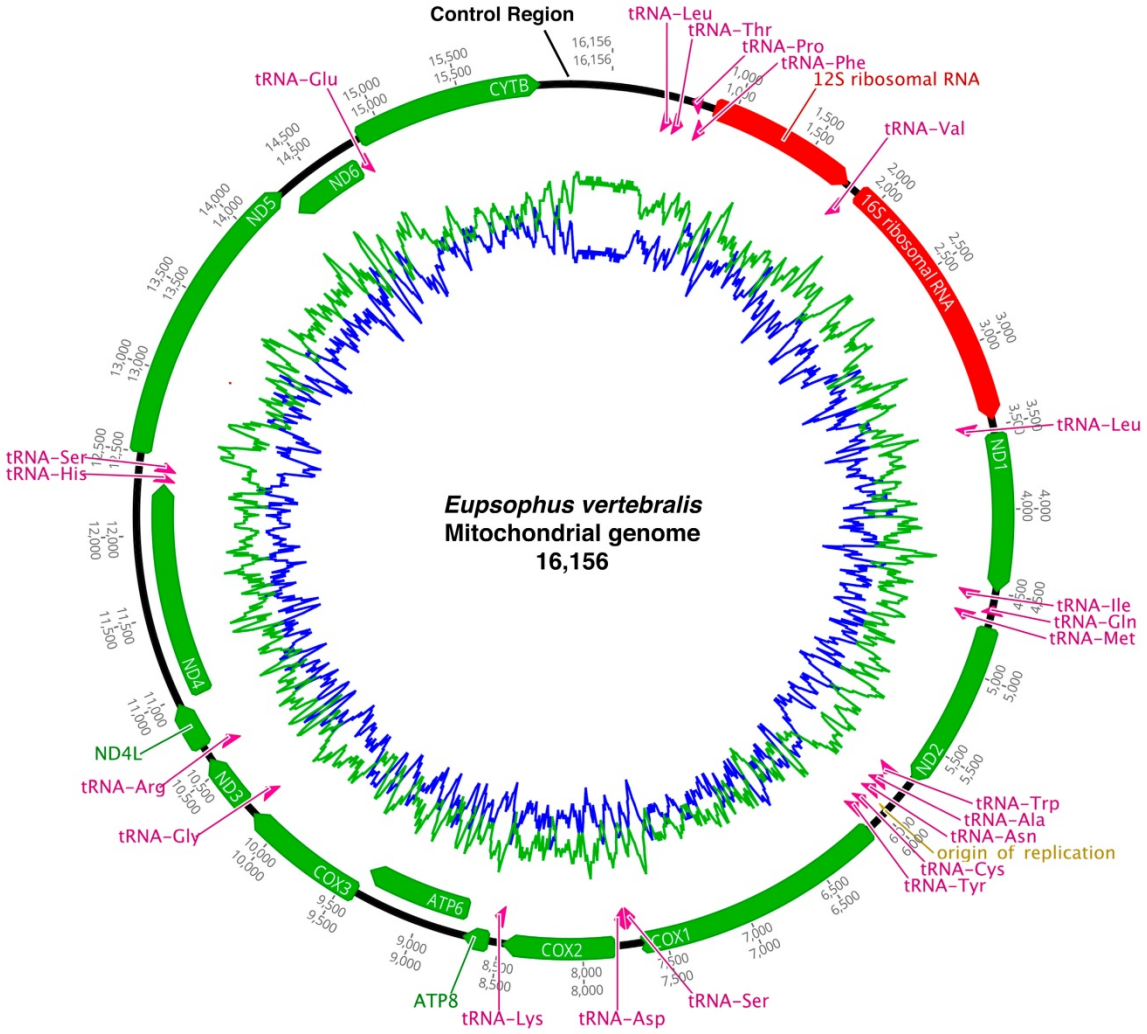

Figure 1. Mitochondrial genome structure of *Eupsophus vertebralis*. The same structure, consisting of 16,711 bp, was obtained for *E. emiliopugini*. Circular genome is shown for didactic purposes, although a segment of the Control Region remains unknown. Genes are indicated as arrows clockwise (heavy strand encoded) and arrows counter-clockwise (light strand encoded). The protein coding regions are labeled in *green*, tRNA genes are labeled in *pink*, and rRNA genes are labeled in *red* colors. GC (*blue*) and AT (*green*) content are shown.

Table 1. Organization of the mitochondrial genomes of *Eupsophus vertebralis* and *E. emiliopugini*. Strand, start codon, and gaps+/overlaps are identical for both species. Variable sites corresponding to the number of bases pairs do not coincide between both species.

Gene	*E. vertebralis*			*E. emiliopugini*			Strand	Start codon	Stop codon	Gaps+/overlaps (nt)	Variable sites
	From	To	Size	From	To	Size					
Control region	1 16004	598 16156		1 16,575	1,171 16,711		L			0	712
tRNALeu1	599	670	72	1,172	1,243	72	H			0	1
tRNAThr	671	743	73	1,244	1,316	73	H			0	0
tRNAPro	743	811	69	1,316	1,384	69	L			0	0
tRNAPhe	811	879	69	1,384	1,451	68	H			0	3
12S	880	1,809	930	1,452	2,380	929	H			0	3
tRNAVal	1,810	1,878	69	2,381	2,449	69	H			0	0
16S	1,879	3,473	1,595	2,450	4,044	1,595	H			0	10
tRNALeu2	3,474	3,546	73	4,045	4,117	73	H			0	0
ND1	3,547	4,507	961	4,118	5,078	961	H	?	T*	0	12
tRNAIle	4,508	4,578	71	5,079	5,149	71	H			0	3
tRNAGln	4,578	4,648	71	5,149	5,219	71	L			-1	0
tRNAMet	4,648	4,716	69	5,219	5,287	69	H			0	0
ND2	4,717	5,750	1,034	5,288	6,321	1,034	H	ATT	TA*	0	14
tRNATrp	5,751	5,820	70	6,322	6,391	70	H			0	1
tRNAAla	5,821	5,889	69	6,392	6,460	69	L			0	1
tRNAAsn	5,890	5,962	73	6,461	6,533	73	L			27	0
tRNACys	5,989	6,051	63	6,560	6,622	63	L			0	0
tRNATyr	6,052	6,121	70	6,623	6,692	70	L			2	0
CO1	6,123	7,667	1,545	6,694	8,238	1,545	H	GTG	AGG	0	13
tRNASer2	7,668	7,737	70	8,239	8,308	70	L			2	0
tRNAAsp	7,739	7,807	69	8,310	8,378	69	H			2	0
CO2	7,809	8,496	688	8,380	9,067	688	H	ATG	T*	0	11
tRNALys	8,497	8,568	72	9,068	9,139	72	H			0	0
ATP8	8,569	8,733	165	9,140	9,304	165	H	ATG	TAA	-9	1
ATP6	8,724	9,407	684	9,295	9,978	684	H	ATG	TAA	0	10
CO3	9,407	10,190	784	9,978	10,761	784	H	ATG	T*	0	13
tRNAGly	10,191	10,259	69	10,762	10,830	69	H			0	0
ND3	10,260	10,599	340	10,831	11,170	340	H	ATG	T*	0	2
tRNAArg	10,600	10,668	69	11,171	11,239	69	H			6	0
ND4L	10,674	10,973	300	11,245	11,544	300	H	ATG	TAA	-6	5
ND4	10,967	12,331	1,365	11,538	12,902	1,365	H	ATG	TAG	0	21
tRNAHis	12,332	12,399	68	12,903	12,970	68	H			0	1
tRNASer1	12,400	12,466	67	12,971	13,037	67	H			38	0
ND5	12,504	14,306	1,803	13,075	14,877	1,803	H	ATG	AGA	-16	28
ND6	14,290	14,784	495	14,861	15,355	495	L	ATG	AGA	0	0
tRNAGlu	14,785	14,852	68	15,356	15,423	68	L			3	1
Cyt b	14,855	16,003	1,149	15,426	16,574	1,149	H	ATG	TAG		13

? start codon not determined

* TAA stop codon is completed by the addition of 3' A residues to the mRNA

The phylogenetic position of *E. vertebralis* and *E. emiliopugini* was evaluated through Maximum Likelihood (ML) and Bayesian approaches using previously published mitogenome sequences of neobatrachians related to Alsodidae [11]. Mitogenomes of *Limnodynastes salmini* and *Crinia signifera* (Myobatrachidae) were used as outgroup [11]. Sequences of the two rRNAs, the concatenated tRNAs, and 13 protein-coding genes were aligned using Muscle [12] and Clustal W [13], implemented in Geneious. The best-fitting partitioning schemes and models of nucleotide substitution were selected using the Bayesian information criterion, implemented in PartitionFinder v1.1.0 [14]. ML analysis was performed using GARLI v2.0 with 1000 bootstrap replicates [15]. Both ML and Bayesian inference, performed in MrBayes v3.04b [16] and run for 1.0 x 10^7 generations sampled every 1000 steps, showed *E. vertebralis* and *E. emiliopugini* as a clade (Fig. 2A). This clade was related to *Alsodes gargola*, from the same family (Alsodidae) [2], as expected (Fig. 2A).

We used a combination of primers previously reported [17-19] and primers whose design was based on the mitochondrial genomes here reported (*Cyt b* F1 5′-AGCTACTGCAATCAACCCCC-3′, *Cyt b* F2 5′-AATGCACTACACTGCCGACA-3′ *Cyt b* R1 5′-GCTAAGACGCCTCCCAGTTT-3′), to amplify and sequence segments of *D-loop*, *CO1*, and *Cyt b* from the ten *Eupsophus* species (GenBank Accession Numbers: MH301116-MH301181). Phylogenetic analysis was carried out using Garli and MrBayes as described above. Sequences from *Batrachyla leptopus* and *A. gargola* were used as outgroup. The phylogenetic relationships among *Eupsophus* species were congruent with previous hypotheses [1, 3], showing two clades corresponding to *vertebralis* and *roseus* groups (Fig. 2B).

This is the first report of mitogenomes of species

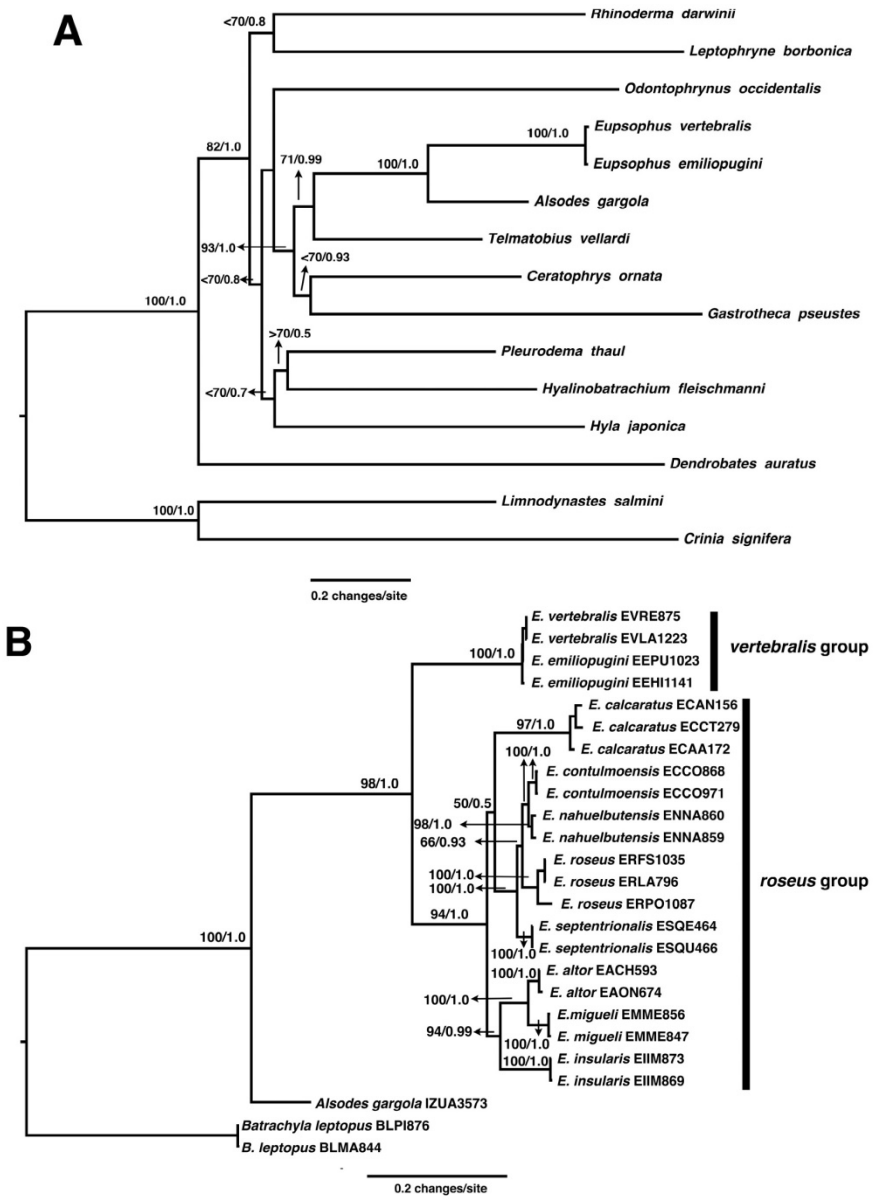

Figure 2. A. Phylogenetic relationships of neobatrachian species inferred from mitochondrial genomes, including the mitogenomes of *Eupsophus vertebralis* and *E. emiliopugini*. **B**. Phylogenetic relationships of *Eupsophus* species inferred from concatenated *D-loop*, *CO1*, and *Cyt b* markers. Both **A** and **B**, Maximum likelihood (ML) and Bayesian analyses depicted the same topology. ML bootstrap percentage /Bayesian posterior probabilities are shown at nodes.

in the genus *Eupsophus*. In this genus, seven of 10 species are included in the IUCN Red List, namely, *E. insularis* (Critically Endangered), *E. queulensis* (Vulnerable), *E. migueli*, *E. contulmoensis*, and *E. nahuelbutensis* (Endangered), and *E. vertebralis* and *E. roseus* (Near Threatened). Our data provides a baseline for further studies on these endemic, threatened and poorly known species.

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Competing Interests

The authors have declared that no competing interest exists.

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