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The mitochondrial genome of *Brachycephalus brunneus* (Anura: Brachycephalidae), with comments on the phylogenetic position of Brachycephalidae

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ABSTRACT

The mitochondrial genome of *Brachycephalus brunneus* was determined by next-generation sequencing of mitochondrial DNA. Without its control region, it has a total length of 15,485 bp, consisting of 37 genes: 13 protein-coding genes, 2 rRNA genes, and 22 tRNA genes. Except for eight tRNAs and the *nd6* gene, all other mitochondrial genes are encoded on the heavy strand. ATG and ATC act mainly as the initial codon in 10 protein-coding genes, whereas *nd2* and *cox1* use ATT and *nad3* uses ATA. Gene order is generally consistent with that observed in closely-related families. The cloverleaf structures for trnS1 and *trnC* lacked the DHU-stem and DHU-loop, respectively. Phylogenetic analyses of mitogenomes of closely-related families indicate that Brachycephalidae is more closely-related to Craugastoridae than to Eleutherodactylidae. This is the first sequenced mitochondrial genome for the entire Brachycephalidae and can provide the basis for the development of mitochondrial markers for other members of the family, including many species that are critically endangered.

1. Introduction

Brachycephalidae is an intriguing family of Neotropical anurans. The first brachycephalid species was described by Spix in 1824 within *Bufo ephippium*, but soon afterwards was established as a monotypic genus by Fitzinger (1826). *Brachycephalus* was believed to be most closely related to *Atelopus* based on similarities in its pectoral girdle similarities (Griffiths, 1959). Later, McDiarmid (1971) placed *Brachycephalus* in its own family based mostly on the lack of a Bidder's organ, which could indicate phylogenetic proximity to Bufonidae. However, following extensive phylogenetic analyses and taxonomic reassignments (e.g. Frost et al., 2006; Hedges et al., 2008), the modern composition of Brachycephalidae now includes

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Brachycephalus and *Ischnocnema* (Hedges et al., 2008). *Brachycephalus* is composed of 30 currently recognized species (Frost, 2016), and includes miniaturized, highly endemic frog species distributed mostly in montane forests of the Atlantic Forest of south and southeastern Brazil (Pie et al., 2013; Bornschein et al., 2016), whereas *Ischnocnema* is currently composed of 33 species and has a broader geographical distribution throughout central and southern Brazil, northern Argentina, and possibly into adjacent Paraguay (Frost, 2016).

Recent studies have placed Brachycephalidae as closely related to two hyloid families, namely Craugastoridae and Eleutherodactylidae, but the relationships among these three families is still uncertain. For instance, although some studies place Brachycephalidae as more closely related to Craugastoridae (Darst and Cannatella, 2004; Frost et al., 2006), another study suggested that Craugastoridae is more closely related to Eleutherodactylidae than to Brachycephalidae (Pyron and Wiens, 2011). In this study, we determined the mitochondrial genome of *Brachycephalus brunneus* using next generation sequencing, the first brachycephalid mitogenome to date, and use it to assess the phylogenetic position of Brachycephalidae.

2. Materials and methods

2.1. Specimen

An adult of *Brachycephalus brunneus* was collected in the type locality of the species: Caratuva (25°14′33″S, 48°50′04″W), municipality of Campina Grande do Sul, state of Paraná, southern Brazil. Tissue samples were preserved in absolute ethanol and kept in −20 °C freezer until use. The corresponding specimen was deposited in the Museu de História Natural Capão da Imbuia (MHNCI), Curitiba, state of Paraná.

2.2. DNA extraction and library preparation

Total genomic DNA was extracted using PureLink™ Genomic DNA kit (Invitrogen™, USA), according to the manufacturer's instructions. The purified genomic DNA was quantified using the Qubit dsDNA HS assay Kit (Life Technologies, USA). mtDNA sequences of *B. brunneus* were obtained as off-target regions from another study (Pie et al., unpublished results) using target capture of ultraconserved elements (UCEs; see Faircloth et al., 2012).

2.3. Sequence and genome analysis

We assembled reads into the mtDNA sequence using Trinity (Grabherr et al., 2011), as implemented in PHYLUCE (Faircloth, 2015), which is part of the UCE-processing pipeline. As expected (Hung et al., 2013), the longest contig included the entire mtDNA genome except for the control region. Annotation was carried out using MITOS (Bernt et al., 2013). The sequin file generated from MITOS was submitted to NCBI (accession number KY355081).

2.4. Phylogenetic analysis

Mitogenomes of *Eleutherodactylus atkinsi* and *Craugastor augusti* were used as representatives of Eleutherodactylidae and Craugastoridae, respectively. These are the only mitogenomes of those families currently available on GenBank. As outgroups, we selected mitogenomes of four closely-related families, namely Centrolenidae, Leptodactylidae, Dendrobatidae, Bufonidae and Odontophrynidae (Table 1). Sequences for each gene were separately aligned using MUSCLE 3.8.31 (Edgar, 2004), although the relatively low number of indels would probably lead to nearly identical alignments using other programs. Analyses included all protein-coding, ribosomal and tRNA genes except $tRNA_{phe}$, $tRNA_{thr}$ and $tRNA_{pro}$, which were not included given that they are often not available from other frog genomes. Model selection and partitioning using PartitionFinder 1.1.0 (Lanfear et al., 2012) based on the Bayesian information criterion and the greedy search scheme. Phylogenetic analysis using Bayesian Inference (BI) was conducted using MrBayes 3.2 (Ronquist et al., 2012) with 2×10^6 MCMC generations with four chains and sampling every 200^{th} generation, with the first 25% of the chains being discarded as burnin. Maximum likelihood (ML) analyses using the same partitioning scheme were carried out using RAxML (Stamatakis et al., 2008, as implemented in http://embnet.vital-it.ch/raxml-bb/), with branch support obtained using 500 pseudoreplicates.

Table 1
Species used in phylogenetic analyses in the present study.

Species	Family	Accession Number
Anomaloglossus baeobatrachus	Dendrobatidae	NC_030054
Brachycephalus brunneus	Brachycephalidae	KY355081
Bufo tibetanus	Bufonidae	NC_020048
Craugastor augusti	Craugastoridae	JX564870
Eleutherodactylus atkinsi	Eleutherodactylidae	JX564864
Hyalinobatrachium fleischmanni	Centrolenidae	JX564869
Leptodactylus melanonotus	Leptodactylidae	JX564873
Odontophrynus occidentalis	Odontophrynidae	JX564880

3. Results

3.1. Mitogenome analysis and features

The total length of the *Brachycephalus brunneus* mitochondrial genome sequence is 15,485 bp. It consists of 13 protein-coding genes, 2 rRNA genes (*rrnS* and *rrnL*), 22 tRNA genes (the control region was not sequenced; Table 2). ATG and ATC act mainly as the initial codon in 10 protein-coding genes, whereas *nd2* and *cox1* use ATT and *nd3* uses ATA. With the exception of eight tRNAs and *nd6* gene, all other mitochondrial genes are encoded on the heavy strand (H strand). Spacing sequences ranged from one to 179 bp, with the latter being found between *atp6* and *cox3*. The rRNAs are located between *trnF* and *trnL2* genes and are separated by the *trnV* gene. The putative secondary structure for all tRNA genes of *B. brunneus* are shown in Fig. 1. As commonly observed in other metazoans, *trnS1* and *trnC* lacked the dihydrouracil (DHU) stem and loop, respectively (Fig. 1). Base composition across all genes was A = 30.0%, C = 25.5%, G 14.8%, and T 29.7%, but these values differed considerably between genes. tRNAs varied their GC content from 25.8% in *trnF* to over 51% in *trnK* and *trnY*. Protein-coding genes varied less in base composition, from 36% GC in *atp8* to 42.6 in *cox1*. Finally, a relatively higher GC content was found in ribosomal genes: 39.4 and 43.6 for *rrnL* and *rrnS*, respectively.

The total alignment for phylogenetic analysis was 14,927 bp. Model selection using PartitionFinder indicated an optimal partitioning scheme with six partitions, with tRNAs being assigned to three different partitions (one of which also including *rrnS* and *rrnL* genes), whereas protein-coding genes were assigned to three different partitions (Table 3). BI analysis using this partitioning scheme reached apparent stabilization of MCMC chains, with average standard deviation of split frequencies being lower than 0.0002 and ESS of estimated parameters almost invariably exceeding 300. The obtained phylogenetic relationships are indicated in Fig. 2. In particular, *B. brunneus* is more closely related to *Graugastor augusti* than to *Eleutherodactylus atkinsi*, with 100% bootstrap support and posterior probabilities, suggesting that Brachycephalidae is more closely-related to Craugastoridae than to Eleutherodactylidae.

Table 2
Characteristics of the mitochondrial genome of *Brachycephalus brunneus*.

Gene	Position		Length (bp)	Intergenic nucleotides	Strand	Nucleotides					
	From	To				A	C	G	T	G + C	A + T
cob	1	1110	1110		−	27.66	26.49	12.97	32.88	39.46	60.54
trnE	1125	1192	68	15	+	26.47	16.18	22.06	35.29	38.24	61.76
nd6	1193	1698	506	0	+	16.57	9.86	31.56	42.01	41.42	58.58
nd5	1701	3467	1767	3	−	30.45	28.07	11.09	30.39	39.16	60.84
trnS1	3516	3582	67	49	−	34.33	16.42	14.93	34.33	31.34	68.66
trnH	3583	3652	70	0	−	25.71	28.57	17.14	28.57	45.71	54.29
nd4	3658	5016	1359	6	−	30.32	27.74	11.77	30.17	39.51	60.49
nd4l	5013	5278	266	−3	−	28.46	28.84	11.61	31.09	40.45	59.55
trnR	5310	5378	69	32	−	33.33	27.54	15.94	23.19	43.48	56.52
nd3	5380	5727	348	2	−	29.31	25.57	14.94	30.17	40.52	59.48
trnG	5719	5785	67	−8	−	32.84	26.87	14.93	25.37	41.79	58.21
cox3	5787	6569	783	2	−	27.08	27.2	14.56	31.16	41.76	58.24
atp6	6748	7419	672	179	−	28.42	27.98	11.61	31.99	39.58	60.42
atp8	7416	7571	156	−3	−	36.54	30.77	5.13	27.56	35.9	64.1
trnK	7572	7641	70	0	−	31.43	30	21.43	17.14	51.43	48.57
cox2	7649	8320	672	8	−	31.99	25.3	14.43	28.27	39.73	60.27
trnD	8344	8412	69	24	−	36.23	21.74	14.49	27.54	36.23	63.77
trnS2	8414	8484	71	2	+	23.94	14.08	26.76	35.21	40.85	59.15
cox1	8486	10,016	1531	2	−	25.42	26.27	16.34	31.96	42.61	57.39
trnY	10,024	10,090	67	8	+	22.39	19.4	35.82	22.39	55.22	44.78
trnC	10,091	10,144	54	0	+	33.33	22.22	27.78	16.67	50	50
trnN	10,172	10,244	73	28	+	24.66	13.7	27.4	34.25	41.1	58.9
trnA	10,245	10,313	69	0	+	30.43	11.59	23.19	34.78	34.78	65.22
trnW	10,313	10,382	70	−1	−	36.23	26.09	14.49	23.19	40.58	59.42
nd2	10,396	11,415	1020	14	−	33.53	27.94	9.8	28.73	37.75	62.25
trnM	11,416	11,484	69	0	−	30.43	24.64	15.94	28.99	40.58	59.42
trnQ	11,484	11,554	71	−1	+	28.17	14.08	21.13	36.62	35.21	64.79
trnI	11,554	11,623	70	−1	−	27.14	24.29	25.71	22.86	50	50
nd1	11,631	12,548	918	8	−	28.1	28.54	12.2	31.15	40.74	59.26
trnL2	12,589	12,659	71	41	−	33.8	16.9	15.49	33.8	32.39	67.61
rrnL	12,655	14,185	1531	−4	−	35.73	22.66	16.79	22.82	39.45	60.55
trnV	14,186	14,255	70	0	−	38.57	22.86	12.86	25.71	35.71	64.29
rrnS	14,253	15,183	931	−2	−	33.4	25.56	18.05	22.99	43.61	56.39
trnF	15,184	15,249	66	0	−	45.45	13.64	12.12	28.79	25.76	74.24
trnP	15,249	15,317	69	−1	+	26.09	11.59	27.54	34.78	39.13	60.87
trnT	15,317	15,383	67	−1	−	35.82	19.4	20.9	23.88	40.3	59.7
trnL1	15,384	15,455	72	0	−	26.39	23.61	20.83	29.17	44.44	55.56

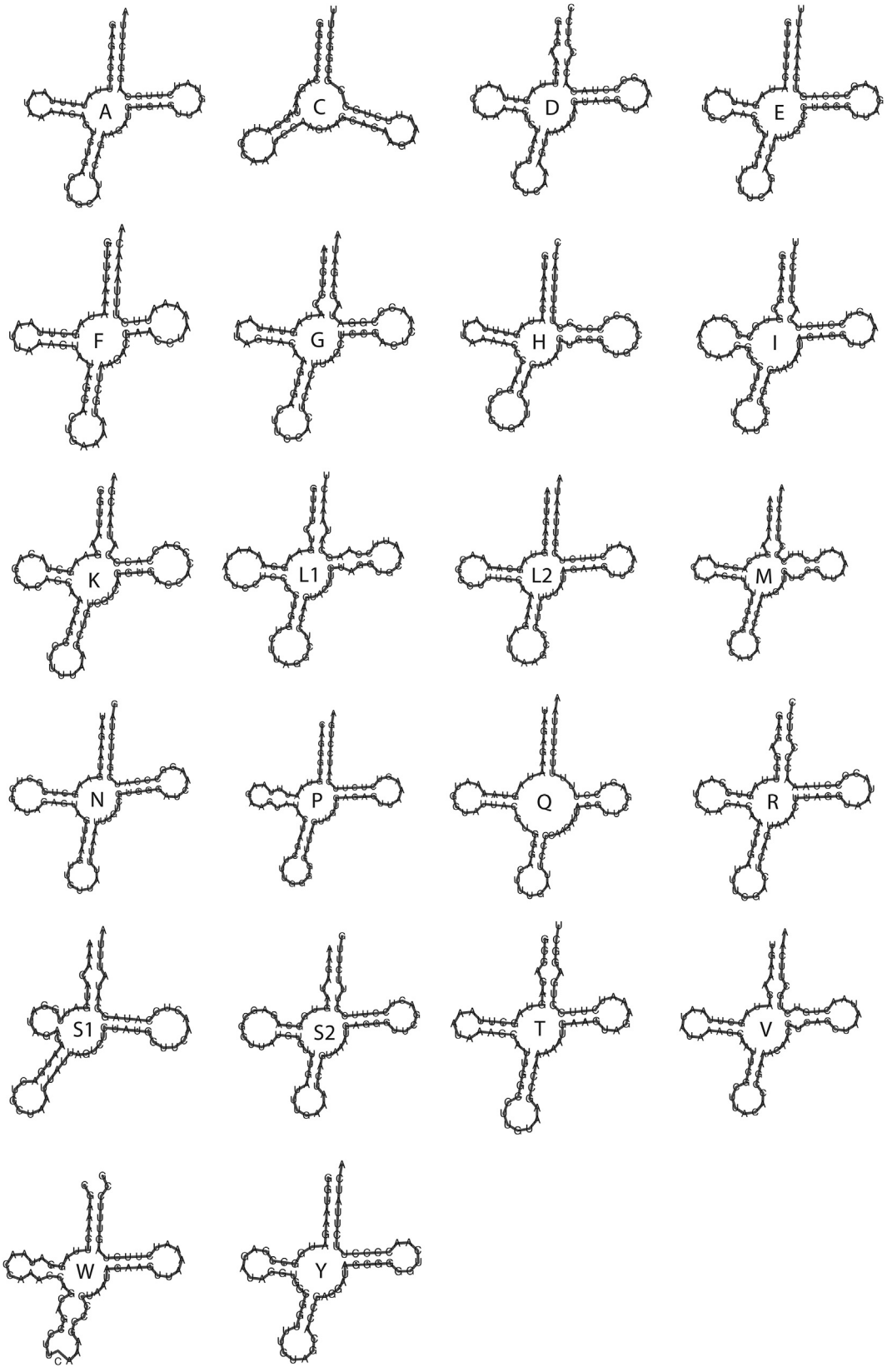

Fig. 1. Cloverleaf structure of the 22 inferred tRNAs in the mitogenome of *Brachycephalus brunneus*.

Table 3
Models of evolution for each partition of the studied alignment.

Partition	Best Model	Genes
1	GTR+Γ	*rrnS*, *rrnS*, *trnI*, *trnW*, *trnN*, *trnS2*, *trnK*
2	HKY+Γ	*trnV*, *trnL2*, *trnQ*, *trnA*, *trnD*, *trnG*, *trnR*, *trnH*, *trnS1*, *trnE*
3	GTR + I+Γ	*nd1*, *cox1*, *cox2*, *cox3*, *cob*
4	K80+Γ	*trnM*, *trnC*, *trnY*
5	GTR + I+Γ	*nd2*, *atp8*, *atp6*, *nd3*, *nd4L*, *nd4*, *nd5*
6	HKY+Γ	*nd6*

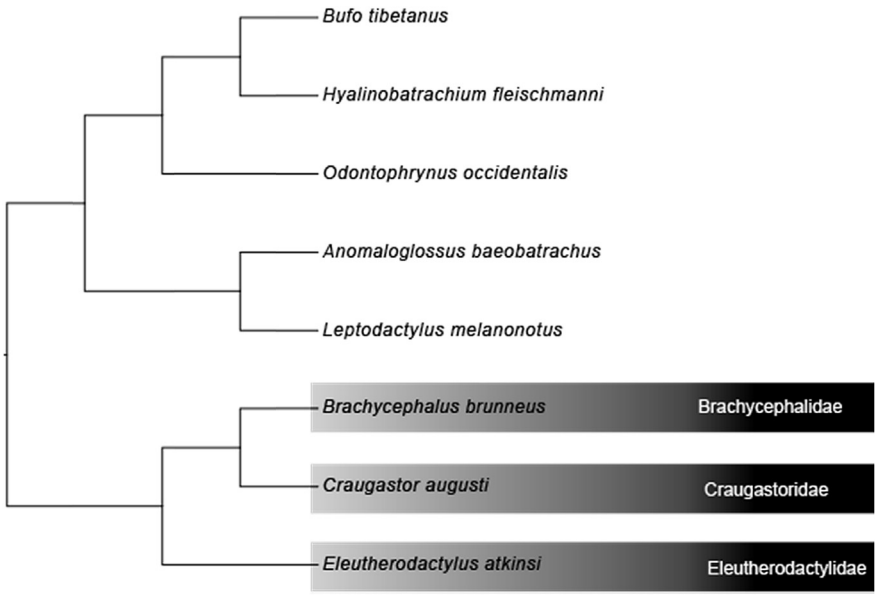

Fig. 2. Phylogenetic relationships between Brachycephalidae, Craugastoridae, and Eleutherodactylidae, as inferred based on Bayesian Inference analysis of mitogenomes of representatives of each family. All nodes received 100% posterior probabilities. Maximum likelihood analyses provided the same ingroup topology with 100% bootstrap support.

4. Discussion

The overall structure of the mitogenome of *Brachycephalus brunneus* shared similarities with closely-related neobatrachian lineages, including gene order (e.g. Cao et al., 2006; Wang et al., 2013; Zhang et al., 2013), codon usage (Cao et al., 2006), and low GC content (e.g. Cao et al., 2006; Lloyd et al., 2012). Interestingly, the loss of the DHU-stem and DHU-loop in *trnS1* and *trnC* is also shared with other anurans, such as the ranoid *Paa spinosa* (Zhou et al., 2009) and the microhylid *Microhyla butleri* (Yong et al., 2016).

The phylogenetic relationships inferred in the present study indicate that Brachycephalidae is more closely-related to Craugastoridae than to Eleutherodactylidae, contrary to some previous studies based on datasets with fewer genes (Pyron and Wiens, 2011). The agreement between our results and those of Darst and Cannatella (2004) and Frost et al. (2006) is not surprising, given that those studies themselves were based on mitochondrial DNA, particularly the *rrnS* and *rrnL* genes. Given the strong phylogenetic signal indicated in our analyses, it seems clear that the mitochondrial tree supports that Brachycephalidae and Craugastoridae are sister taxa. However, more extensive sampling, both of other species and of nuclear loci, are crucial to confirm this finding, particularly to confirm the monophyly of Eleutherodactylidae.

Finally, this study underscores the utility of using off-target sequences from sequence-capture methods, such as UCEs (Amaral et al., 2015). As the use of next-generation sequencing becomes more prevalent, the assembly of mitogenomes can become an inexpensive complementary marker that can be highly informative, not only in terms of sequence data, but also secondary structure and gene order (Zhang et al., 2013). In particular, gene reorganization has been detected many times across Neobatrachia (e.g. Xia et al., 2014) and their relatively low rates of homoplasy might provide valuable markers, particularly for higher-level relationships in Anura. Furthermore, the availability of the mitochondrial genome of *Brachycephalus brunneus* opens the possibility for the development of mitochondrial markers for other species of the family as well. Such markers can be used in intra and interspecific studies, including many microendemic species that are critically endangered.

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