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International Journal of Biological Macromolecules 132 (2019) 461–469

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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The complete mitochondrial genome of *Dryophytes versicolor*: Phylogenetic relationship among Hylidae and mitochondrial protein-coding gene expression in response to freezing and anoxia

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ARTICLE INFO

Article history:
Received 24 January 2019
Received in revised form 28 March 2019
Accepted 28 March 2019
Available online 29 March 2019

Keywords:
Dryophytes
Mitogenome
Mitochondrial gene expression
Phylogeny

ABSTRACT

Dryophytes versicolor is one of the most extreme freeze-tolerant frogs from eastern North America. In this study, the mitochondrial genome of *D. versicolor* was sequenced to analyze the phylogenetic relationships among Hylidae and investigate mitochondrial gene expression in response to freezing and anoxia. The total length of the *D. versicolor* mitogenome is the longest known to date among the available family members of Hylidae. Both maximum likelihood (ML) and Bayesian inference (BI) analyses strongly supported *D. versicolor* as a sister clade to (*D. japonica* + *D. ussuriensis*) + (*D. suweonensis* + *D. immaculata* (KP212702)), and indicated that *Dryophytes* is monophyletic. Using the mitochondrial genome, gene expression analysis was performed using RT-qPCR in skeletal muscle samples, and determined that relative levels of *D. versicolor COX2* increased by 2.40 ± 0.23 fold in response to anoxia, but did not change with exposure to freezing. In addition, *ND3* transcript levels decreased in response to anoxia but remained constant during freezing. By contrast, *COX1* transcript levels decreased with exposure to freezing, but did not change under anoxic conditions. These results suggest that modulations of protein-coding mitochondrial genes of *D. versicolor* may play a role in the molecular response to freezing and anoxia tolerance.

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1. Introduction

Studies have sought to elucidate the phylogenetic relationships among *Hyla* in recent years. The *Dryophytes* (including 18 species) and *Hyla* (including 15 species) genera were thought to be sisters, as described by Duellman et al. [1] and Frost [2]. Studies by Faivovich et al. [3] suggested that three Eurasian species (*Hyla annectans*, *H. arborea*, *H. savignyi*), the Asian *H. japonica*, and eleven North American species of *Hyla* were clustered into one clade. Hua et al. [4] suggested that there were two clades of *Hyla* – one clade which included nine Eurasian species, and another clade which included thirteen North American species and three East Asian species (*H. immaculata*, *H. japonica*, *H. suweonensis*). *Dryophytes* was treated as a subgenus of *Hyla* by Fouquette and Dubois [5]. However, Duellman et al. [1] argued for two clades of Nearctic hylids, and suggested that *Dryophytes* and *Hyla* were two separate genera. Specifically, the genus *Hyla* was restricted to the Old World, and the genus *Dryophytes* was primarily from the New World and also included three additional species (*D. immaculatus*, *D. japonica*, *D. suweonensis*) in Asia [1]. Frost [2] suggested that *D. suweonensis* might be synonymous with *D. immaculata*, and *D. ussuriensis* might be a synonymous with *D. japonica*. As such, further studies that attempt to understand whether *Dryophytes* and *Hyla* are monophyletic are warranted.

Mitochondria are responsible for facilitating the citric acid cycle, and the formation of ATP through oxidative phosphorylation [6]. Compared to their nuclear genomes, anuran mitochondrial genomes are small, circular, and of approximately 16–27 kb in size [7,8]. The mitochondrial genome encodes 13 protein-coding genes, 22 tRNAs and 2 rRNA genes [9]. The mitochondrial genome has been increasingly used to reconstruct the phylogenetic relationships of Anura because of its simple genetic structure, maternal inheritance, and high evolutionary rate [7,10–13].

The gray treefrog, *Dryophytes versicolor* (formerly *Hyla versicolor*), is distributed throughout eastern North America [2]. It is a freeze-tolerant frog which can produce cryoprotectants (glucose and/or glycerol) in

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https://doi.org/10.1016/j.ijbiomac.2019.03.220
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response to low temperatures [14]. Although a rapidly growing literature dealing with the mitochondrial genomes of anurans was published to construct the phylogenetic relationship [7,10–13,15–41], few studies have included data on mitochondrial gene expression from mitochondria. Studies that focused on the protein or gene expression in freezing and/or anoxia tolerance did not focus on mitochondrial protein-coding genes, except for a few exceptions [42,43]. In the present study, the mitochondrial genome was not only used to study the phylogenetic relationships of *D. versicolor*, but also to investigate the relative expression of mitochondrial protein-coding genes in response to freezing or anoxia exposure, in order to better understand the molecular mechanisms of cold and anoxia tolerance in frogs.

2. Materials and methods

2.1. Animal treatments

Male gray treefrogs (*D. versicolor*) (mean mass of 7.9 ± 0.2 g) were captured from breeding ponds in the Limerick forest of Grenville Country, Ontario, Canada and transported to Carleton University. All animals were washed in a tetracycline bath and then housed in plastic boxes lined with damp sphagnum moss at 5 °C for one week before experimentation. Control animals were sampled from this condition. For freezing exposure, groups of frogs were placed in closed plastic trays lined with damp paper toweling and then transferred to an incubator set at −4 °C for 2 h to facilitate ice nucleation. Temperature was then raised to −3.5 °C for 1 h, and then to −2.5 °C. Frozen frogs were sampled after 24 h at −2.5 °C. For anoxia exposure, a group of acclimated frogs were transferred into sealed plastic jars (held on crushed ice) that had two ports in their lids: one to allow nitrogen gas to enter and the other to vent air/gas out. The jars were previously lined with damp paper toweling (wetted with water that was previously bubbled with nitrogen gas) and flushed with 100% nitrogen gas for ~15 min. Frogs were then added and jars were flushed again with nitrogen gas for ~15 min, then sealed and returned to the 5 °C incubator for 24 h. Control, frozen or anoxia exposed frogs were all euthanized by pithing, followed by rapid dissection and flash freezing of tissues in liquid nitrogen. Tissue samples were stored at −80 °C until use.

2.2. DNA extraction, PCR, and sequencing

Total DNA was extracted from the clipped toe of one specimen of *D. versicolor* using a DNeasy Tissue Kit (Qiagen, Germany). Overlapping fragments were amplified by normal PCR and long-and-accurate polymerase chain reaction (LA PCR) methods slightly modified from Ye et al. [44], Yu et al. [45] and Zhang et al. [7,30]. The PCR fragments were sequenced in both directions by the primer-walking method using an automated DNA sequencer (ABI 3730) (Sangon Biotech, Shanghai, China).

2.3. Sequence assembly and analysis

All sequences were checked by manual work and assembled using DNASTAR Package v.6.0 [46]. All tRNA genes, 13 protein-coding genes and 2 rRNA genes were identified by MITOS (http://mitos.bioinf.uni-leipzig.de/index.py). The locations of the 13 protein-coding genes and two rRNA genes were further determined by comparison with the closely related anurans downloaded from GenBank using Mega 7.0 [47]. All tRNA genes were further assessed by their cloverleaf secondary structure using tRNA-scan SE 1.21 [48] or determined by comparison with the homologous sequences of other anurans. CG View server v.1.0 [49] was used to draw the mitochondrial genome maps with GC content and GC skew. The skew in nucleotide composition was calculated by GC and AT skew, which were measured according to the formulae by Perna and Kocher [50], AT skew = (A − T) / (A + T); GC skew = (G − C) / (G + C). The complete mitochondrial genome of *D. versicolor* was deposited in the GenBank database with the accession number MH087467.

2.4. Molecular phylogenetic analyses

Phylogenetic analyses were performed with 14 available hylids including *D. versicolor* in this study. In total, it included the ingroup of 10 species from Hylidae [44,51–57] and the outgroup of *Nyctimystes kubori* [30], *Phyllomedusa tomopterna* [30], *Bokermannohyla alvarengai* [58] and *Osteocephalus taurinus* [30]. The amino acid sequences of 13 mt protein-coding genes were separately aligned in Mega 7.0 [47]. The alignments of the 13 mitochondrial protein-coding genes were concatenated and an alignment consisting of 3672 amino acid residues as the 13Paa dataset was recovered. An alignment of 11,016 nucleotides sites with 5331 variable and 4043 parsimonious informative sites was obtained using the amino acid alignment as the backbone. Saturation analysis was performed for subsets with the first, second, and third codon positions using DAMBE 4.2.13 [59]. The results showed that the third codon positions were slightly saturated. Thus, the third codon positions was included and a dataset deemed 13P consisting of 11,016 nucleotide sites was obtained.

The phylogeny was analyzed using the combined dataset 13P (nucleotides dataset) by the maximum likelihood (ML) and Bayesian inference (BI) methods. To improve the fits of the substitution model to the dataset of 13P, data partitioning schemes were compared according to the Bayesian Information Criterion (BIC) using the program PartitionFinder v1.0 [60]. The 13 coding-genes were set as 39 partitions in the dataset 13P. For the dataset 13P, 39 partitions were optimal: 13 protein-coding genes were partitioned by codons. The best substitution model of 39 partitions in thirteen different genes of dataset 13P was shown in Table 1. Since there was no appropriate model among the available substitution models in RaxML program, the GTR + I + G model was used for 13P with 39 partitions for ML analysis by the RaxML program with 1000 bootstrap replications [61]. The corresponding model was used according to PartitionFinder results in Bayesian analyses by MrBayes 3.1.2 [62]. During BI analysis, the following settings were applied: number of Markov chain Monte Carlo (MCMC) generations = ten million; sampling frequency = 1000; burn-in = 1000. The burn-in size was determined by checking convergences of −log likelihood (−ln L). Bayesian runs achieved sufficient convergence when the average standard deviation of split frequencies was below 0.01.

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from samples of frozen hind leg skeletal muscle of control, 24 h frozen, and 24 h anoxia treated *D. versicolor*. Extraction and cDNA synthesis were performed as previously described [63]. Briefly, frozen muscle samples were homogenized 1:20 w:v in TRI Reagent (Sigma Aldrich, Oakville, Canada) using a PowerGen 125 (Fisher Scientific, Ottawa, Canada) homogenizer. Subsequently, 200 μL

Table 1
The partition schemes and best-fitting models selected.

Nucleotide sequence alignments		
Subset	Subset partitions	Best model
Partition 1	ATP6_pos1, ATP8_pos1, ATP8_pos2, nd1_pos1, nd2_pos1, nd3_pos1, nd4L_pos1, nd4_pos1, nd5_pos1	GTR + I + G
Partition 2	ATP6_pos2, nd1_pos2, nd2_pos2, nd3_pos2, nd4L_pos2, nd4_pos2, nd5_pos2	TVM + G
Partition 3	ATP6_pos3, ATP8_pos3, cox1_pos3, cox2_pos3, cox3_pos3, cytb_pos3, nd1_pos3, nd2_pos3, nd3_pos3, nd4L_pos3, nd4_pos3, nd5_pos3	TrN + I + G
Partition 4	cox1_pos1, cox2_pos1, cox3_pos1, cytb_pos1	TrNef + I + G
Partition 5	cox1_pos2, cox2_pos2, cox3_pos2, cytb_pos2	HKY + I
Partition 6	nd6_pos1, nd6_pos2	HKY + G
Partition 7	nd6_pos3	TIM + I + G

Table 2
Primer sequences of the 13 mitochondrial protein-coding genes in this study.

Gene name	Forward primers	Reverse primers
ND1	*Hyla*-ND1-J1 TGACCTCTCGCCATAATATG	*Hyla*-ND1-N1 GACCTCCAGCGTATTCTAC
ND2	*Hyla*-ND2-J1 CACTGAATCCTAGCCTGAAT	*Hyla*-ND2-N1 TGCTGCTGCTTGAGTTAG
COX1	Hyla-COX1-J1 TTACAGTAGGCGGCTTAAC	Hyla-COX1-N1 CCTGCTATAATTGCGAATACC
Cox2	Hyla-COX2-J1 CTACGGACAATGTTCAGAGA	Hyla-COX2-N1 GAGGCAGAGGATCAGTCTA
ATP8	Hyla-ATP8-J1 CACAACTAGACCCTATACCAT	Hyla-ATP8-N1 TCAGGATTTGCTTAGACCTT
ATP6	Hyla-ATP6-J1 TCTCATCCAACTTATCTCCTC	Hyla-ATP6-N1 CAAGCATAGTCAGAAGAAGG
COX3	Hyla-COX3-J1 AGCAACAGCAGCATTCAT	Hyla-COX3-N1 AACAATATCCCGTCATCACT
ND3	Hyla-ND3-J1 CCATACGAATGCGGATTTG	Hyla-ND3-N1 TCAGGGTGTTGGGAGAAG
ND4L	Hyla-ND4L-J1 GACTTCTAGGACTCTCACTT	Hyla-ND4L-N1 TTAAGGCTCATAGACCAAGG
ND4	Hyla-ND4-J1 CCACGGCTTAGTATCATCAG	Hyla-ND4-N1 TCATCAGGCGGCAGATAA
ND5	Hyla-ND5-J1 CCTAACCACTTGCCTCTG	Hyla-ND5-N1 GCCGATTGCTACCACTAT
ND6	Hyla-ND6-J1 ACGATAACACCTACACTTCC	Hyla-ND6-N1 TGTATGGTGGTGCCTCTT
CYTB	Hyla-Cyt B-J1 CAATTCTTCGCTCCATTCC	Hyla-Cyt B-N1 GGTGGTTCGTTGGTTAGAT

of chloroform was added for every 1 mL TRI Reagent and samples were mixed vigorously before centrifugation at 13,000 ×*g* for 15 min at 4 °C. The aqueous layer was separated and combined with 500 μL of isopropanol for every 1 mL TRI Reagent used. Samples were vortexed and incubated at room temperature for 10 min before centrifugation at 13,000 ×*g* for 15 min at 4 °C. Supernatants were discarded and pellets were rinsed with 1 mL of 70% ethanol before resuspension in autoclaved water. The RNA concentration and quality was assessed spectrophotometrically by measurements at 260 and 280 nm using a Take3 apparatus (BioTek Instruments Inc., Winooski, USA).

For cDNA synthesis, 1 μg RNA was diluted in 10 μL autoclaved water, combined with 1 μL (200 ng/μL) oligo-dT, and incubated at 65 °C for 5 min. Samples were then chilled on ice for 1 min, and each was combined with 4 μL of 5× First Strand buffer, 2 μL 0.1 M DTT, 1 μL 10 mM dNTP (BioBasic, Markham, Canada), and 1 μL MMLV reverse transcriptase. Samples were then incubated at 42 °C for 50 min and then stored at −20 °C.

2.6. RT-qPCR primers design

Specific primers for reverse transcription quantitative polymerase chain reaction (RT-qPCR) were designed using the Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, USA) using the default parameters for a real-time qPCR assay. Amplicon lengths varied from 100 to 150 bp, melting temperatures were between 48 and 50 °C, and primer lengths were between 18 and 22 bp (Table 2).

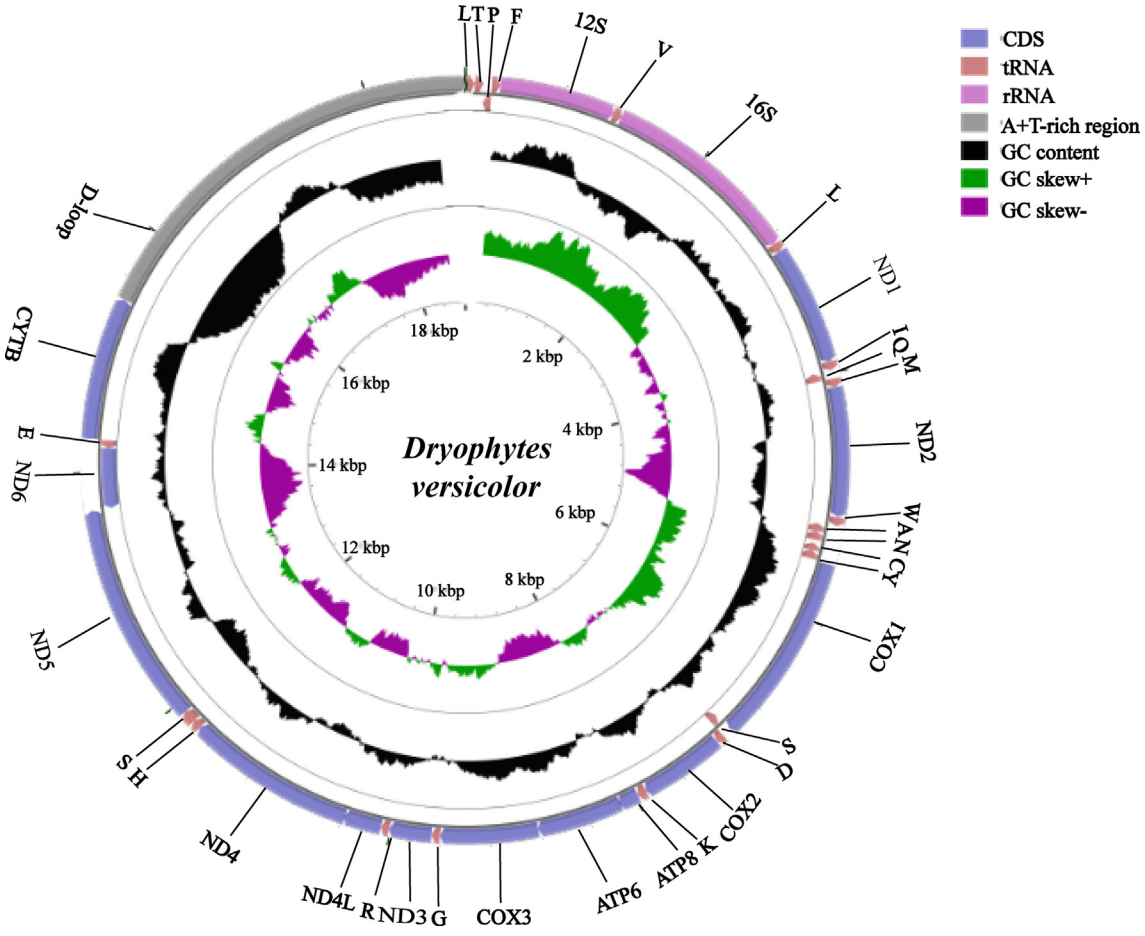

Fig. 1. Mitochondrial genome maps of *Dryophytes versicolor*. The first circle shows the gene map (PCGs, rRNAs, tRNAs and the AT-rich region) and the genes outside the map are coded on the majority strand (J-strand) whereas the genes inside the map are coded on the minority strand (Nstrand). The second circle shows the GC content and the third shows the GC skew. GC content and GC skew are plotted as the deviation from the average value of the entire sequence.

2.7. Relative mRNA quantification

Transcript levels of 13 protein-coding genes from mitochondrial DNA were relatively quantified using a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Reagents for RT-qPCR were prepared as previously described [64]. Serial dilutions of pooled cDNA from control, 24 h frozen, and 24 h anoxic were used to generate standard curves to test the primers for gene quantification. Conditions for RT-qPCR were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 57 °C for 20 s, and 72 °C for 20 s. Primer efficiencies determined from the standard curves were accounted for when calculating starting quantities of each target gene transcript. Relative mRNA quantification was done by calculating the ratio of starting quantity of target gene to starting quantity of reference gene in each sample. The mRNA levels of target genes were normalized against *β-actin* transcript levels. Only reactions that yielded a single peak during melt curve analysis were used for data quantification.

2.8. Data analysis in gene expression

Data were expressed as mean expression (mean ± SE) for each experimental condition, where $n = 4$ independent biological replicates represents samples generated from different animals. Data were plotted relative to the control mean that was set to 1. Statistical analysis of mRNA expression was determined using the Student's *t*-test, with $p < 0.05$ accepted as indicating a significant change, as compared to controls.

3. Results

3.1. General features of mitogenome of D. versicolor

Similar to most hylid mitogenomes, the mitochondrial genome of *D. versicolor* was a circular molecule of 18,800 bp in length (Table 3, Fig. 1). It comprised of 13 protein-coding genes (PCGs), two rRNAs (*rrnS* and *rrnL*), 22 tRNAs, and one control region (3385 bp) between *CYTB* and $tRNA^{Leu}$. The gene arrangement was identical to the ancestral mitogenome pattern of Hylidae. The overall base composition of the H strand for the mitogenome sequence of *D. versicolor* was as follows: A = 30.5%, G = 13.8%, T = 31.1%, and C = 24.6%. The A + T bases comprised 60.3% of the PCGs, 60.6% of the rRNAs and 67.4% of the putative control region, giving a total A + T content of 61. 6% in this mitogenome, which was higher than other species of *Dryophytes* and *Hyla* (57.8–60.9%) (Table S1). For the entire mitogenome of *D. versicolor*, the AT and GC skews for the H strand were −0.009 and −0.282, respectively, which was similar to other species of *Dryophytes* and *Hyla* (Table S1).

Twenty-eight genes were distributed on the heavy strand (H strand) excluding *ND6*, $tRNA^{Glu}$, $tRNA^{Pro}$, $tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$ and $tRNA^{Ser\ (UCN)}$ on the light strand (L strand). Overall, eight overlaps between adjacent genes were found in the *D. versicolor* mitochondrial genome with lengths ranging from 1 to 10 bp (Table 3). Seven base spacers between adjacent genes were also found with lengths ranging from 1 to 6 bp (Table 3).

The A + T contents of five *D. versicolor* protein-coding genes (*ATP8*, *ND1*, *ND4*, *ND5*, and *ND6*) were the highest among nine hylids species (Table S1). The A + T content of the *ATP8* gene was the highest among the 13 PCGs. In the mitochondrial genome of *D. versicolor*, the start codons were identified as follows: *ND2* with ATT, *COX1* with ATA, *COX2*, *COX3*, *ATP6*, *ATP8*, *ND4*, *ND4L*, *ND5*, *ND6* and *CYTB* with ATG, *ND3* with GTG, but *ND1* with TTG were found. Most genes had complete termination codons: AGA in *ND2*; AGG in *COX1* and *ND6*; TAA in *ATP6*, *ATP8*, *COX3*, *ND4* and *ND4L*; TAG in *CYTB*. However, incomplete termination codons (T) were found in *ND1*, *COX2*, *ND3* and *ND5*.

The Relative Synonymous Codon Usage (RSCU) of 13 protein-coding genes of the *D. versicolor* was shown in Fig. 2. Excluding stop codons, the mitochondrial genome of *D. versicolor* encoded 3760 amino acids, which is consistent among 8 species of *Hyla* and *Dryophytes* (3758–3764 amino acids). RSCU also suggested nucleotide composition bias in *D. versicolor*.

Table 3
The characteristic of mitochondrial genome of *Dryophytes versicolor*.

Gene/region	Start position	Stop position	Spacer (+) Overlap (−)	Length (bp)	Start codon	Stop codon	Strand
$tRNA^{Leu}$	1	72	+1	72			H
$tRNA^{Thr}$	74	141	0	68			H
$tRNA^{Pro}$	142	210	−1	69			L
$tRNA^{Phe}$	210	277	0	68			H
12S rRNA	278	1207	0	930			H
$tRNA^{Val}$	1208	1276	0	69			H
16S rRNA	1277	2874	0	1598			H
$tRNA^{Leu(UUR)}$	2875	2947	0	73			H
ND1	2948	3908	0	961	TTG	T	H
$tRNA^{Ile}$	3909	3979	−1	71			H
$tRNA^{Gln}$	3979	4049	−1	71			L
$tRNA^{Met(AUN)}$	4049	4117	0	69			H
ND2	4118	5152	+6	1035	ATT	AGA	H
$tRNA^{Trp}$	5159	5228	0	70			H
$tRNA^{Ala}$	5229	5297	0	69			L
$tRNA^{Asn}$	5298	5370	0	73			L
O_L	5371	5397	0	27			L
$tRNA^{Cys}$	5398	5461	0	64			L
$tRNA^{Tyr}$	5462	5531	+4	70			L
COI	5536	7077	+1	1542	ATA	AGG	H
$tRNA^{Ser(UCN)}$	7079	7149	+1	71			L
$tRNA^{Asp}$	7151	7219	+1	69			H
COII	7221	7908	0	688	ATG	T	H
$tRNA^{Lys}$	7909	7980	0	72			H
ATP8	7981	8145	−10	165	ATG	TAA	H
ATP6	8136	8819	−1	684	ATG	TAA	H
COIII	8819	9604	−1	785	ATG	TAA	H
$tRNA^{Gly}$	9604	9672	0	70			H
ND3	9673	10,012	0	339	GTG	T	H
$tRNA^{Arg}$	10,013	10,080	0	68			H
ND4L	10,081	10,383	−7	303	ATG	TAA	H
ND4	10,377	11,741	0	1365	ATG	TAA	H
$tRNA^{His}$	11,742	11,810	0	69			H
$tRNA^{Ser(AGY)}$	11,811	11,912	0	102			H
ND5	11,913	13,701	−3	1789	ATG	T	H
ND6	13,699	14,196	0	498	ATG	AGG	L
$tRNA^{Glu}$	14,197	14,264	+2	68			L
Cyt b	14,267	15,415	0	1149	ATG	TAG	H
D-loop	15,416	18,800		3385			H

3.2. The genetic distances and phylogenetic relationships of Dryophytes and Hyla

The genetic distance of each species of *Dryophytes* and *Hyla* with the *K2P* model is shown in Table S2. The interspecific genetic distance within *Dryophytes* varied from 0.9% to 13.7%, while the genetic distance of interspecies within *Hyla* ranged from 3.8% to 13.2%. The genetic distances between *H. tsinlingensis* (KU601448) and *H. tsinlingensis* (KP212702), *D. suweonensis* and *H. tsinlingensis* (KP212702), and *D. ussuriensis* and *D. japonica*, were 19.9%, 0.9%, and 2.7%, respectively.

The results from the two phylogenetic analyses (BI and ML) of 13 PCG genes yielded similar topologies except for the outgroup positions (*Bokermannohyla alvarengai*, *Phyllomedusa tomopterna*, *Nyctimystes kubori* and *Osteocephalus taurinus*) (Fig. 3). The phylogenetic analyses of nucleotide dataset with high values supported two clades of *Hyla* and *Dryophytes*. One clade showed that *D. versicolor* was a sister clade of (*D. suweonensis* + *H. tsinlingensis* (KP212702) = *D. immaculata*) + (*D. ussuriensis* + *D. japonica*)). The other clade showed that *H. chinensis* was a sister clade of ((*H. annectans* + *H. tsinlingensis* (KU601448)) + *H. sanchiangensis*).

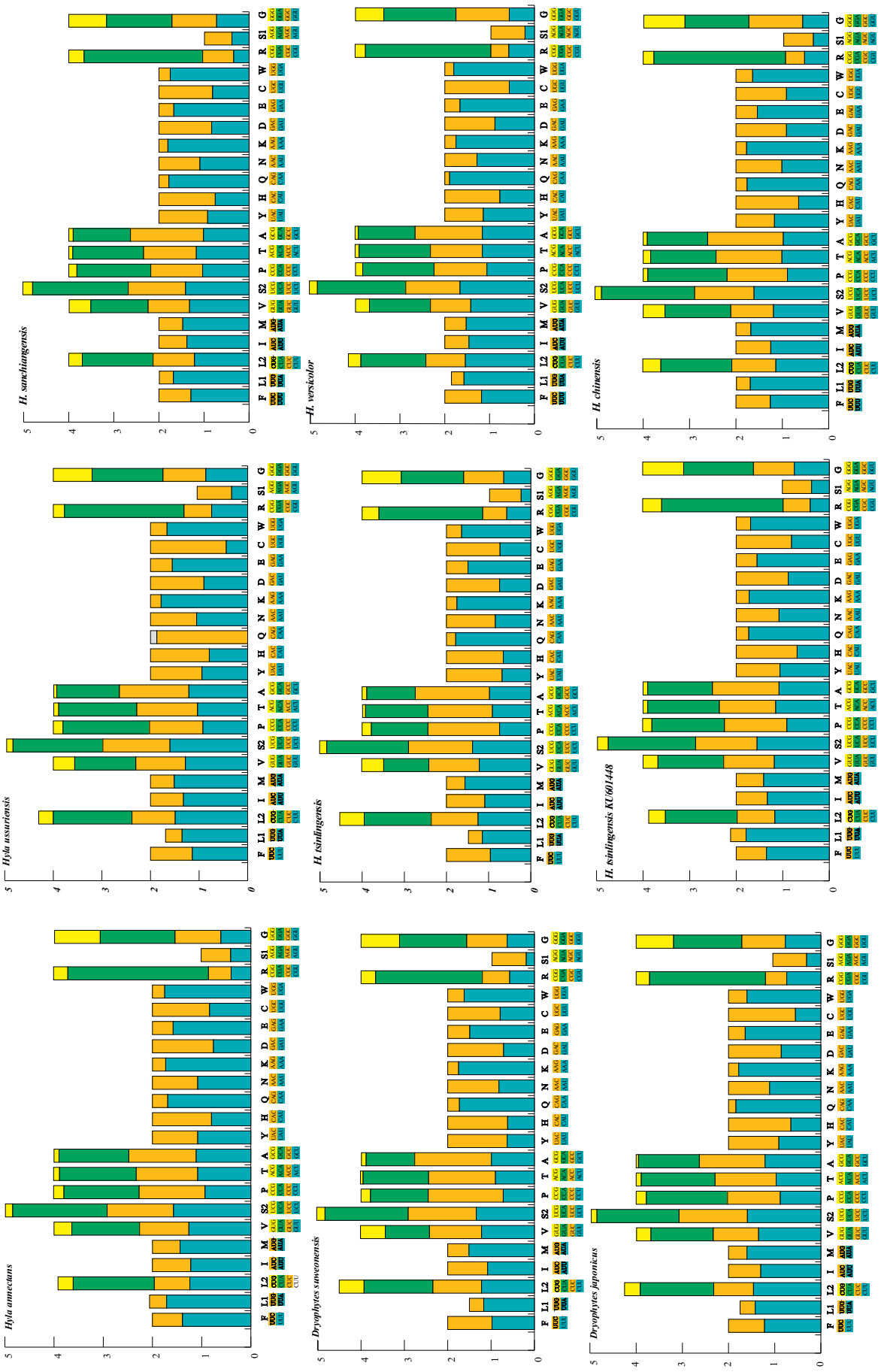

Fig. 2. The relative synonymous codon usage (RSCU) in nine *Hyla* mitogenomes.

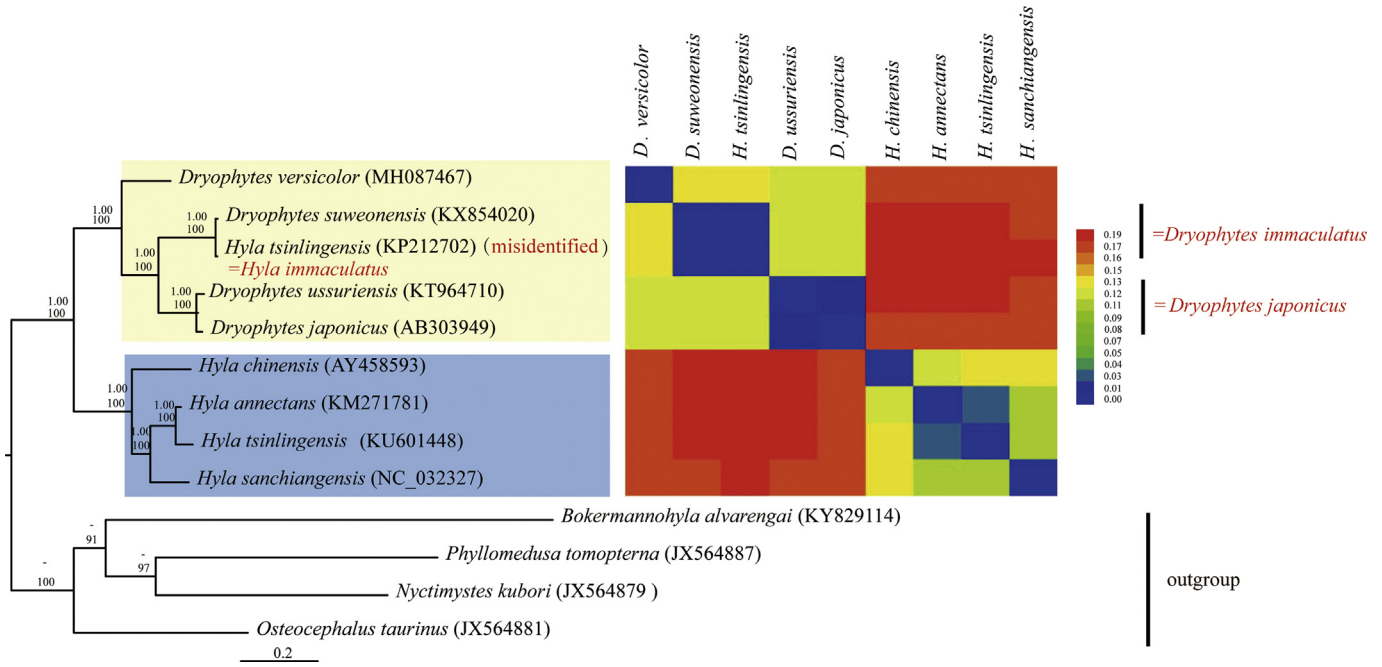

Fig. 3. Phylogenetic relationships inferred from BI and ML analyses. Phylogenetic analyses using nucleotide datasets were carried out for the 13 frogs based on 13 protein-coding genes. Branch lengths and topology are from the BI analysis. The tree was rooted with four out-groups. Numbers above the nodes are the bootstrap values of ML in the bottom and the posterior probabilities of BI in the top. The visualization heatmap of genetic distances is shown to the right of the phylogenetic trees.

3.3. Transcript levels of protein-coding mitochondrial genes

Relative transcript levels of 13 mitochondrial protein-coding genes in skeletal muscle of *D. versicolor* were quantified with RT-qPCR to compare control, 24 h frozen, and 24 h anoxic conditions. Skeletal muscle expression of *COX2* transcripts increased 2.40 ± 0.23 fold in response to anoxia (Fig. 4) but did not change with exposure to freezing (Fig. 5). In addition, skeletal muscle *ND3* transcript levels decreased in response to anoxia to 0.51 ± 0.06 of control levels but remained constant during freezing. By contrast, *COX1* transcript levels decreased to 0.63 ± 0.02 of control levels with exposure to freezing conditions but did not change with anoxia. The relative transcript levels of the remaining 10 protein-coding mitochondrial genes did not change in response to either freezing or anoxia exposure.

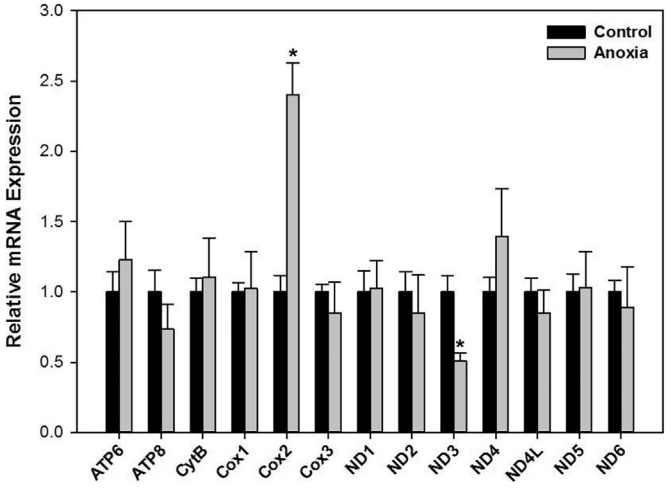

Fig. 4. Relative mRNA expression levels of protein-coding mitochondrial genes in skeletal muscle of control versus 24 h anoxia-exposed *D. versicolor*. Relative mRNA levels were determined with RT-qPCR with $n = 4$ independent biological replicates and results are shown as mean ± SE. Mean values for controls were set to 1.0 and values for anoxic frogs are expressed relative to controls. Relative transcript levels were standardized against *β-actin* transcript levels as a reference gene. Statistical significance was assessed with a two-tailed Student's *t*-test, where * denotes a significant difference from the corresponding control, $p < 0.05$.

4. Discussion

4.1. Species delimitation

The mitochondrial genome of *H. tsinlingensis* (KP212702) was reanalyzed by sequence alignment using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and AmphibiaChina (http://www.amphibiachina.org/). It was deduced that *H. tsinlingensis* (KP212702) was misidentified because the sequence similarity between *H. tsinlingensis* (KP212702) and *Dryophytes immaculata* was very similar (99%), and as a result,

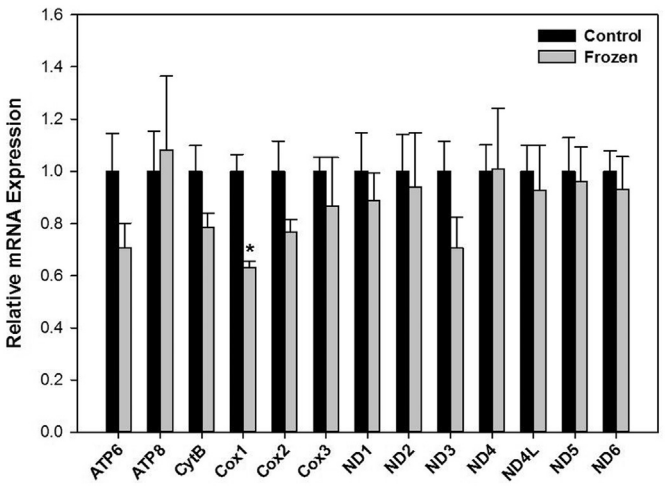

Fig. 5. Relative mRNA expression levels of protein-coding mitochondrial genes in skeletal muscle of control versus 24 h frozen *D. versicolor*. Other information as in Fig. 4.

should be considered as *D. immaculata*. The genetic distances between *D. suweonensis* and *D. immaculata* (misidentified as *H. tsinlingensis* (KP212702)) (0.9%), and between *D. ussuriensis* and *D. japonica* (2.7%) support the results presented previously by Frost [2] which suggested that *D. suweonensis* was synonymous to *D. immaculata*, and *D. ussuriensis* as a synonym of *D. japonica*.

4.2. Phylogenetic analyses

According to geographical distributions and phylogenetic relationships, Duellman et al. [1] suggested that the *Hyla* genus should be divided into *Hyla* (restricted to the Old World) and *Dryophytes* (restricted to the New World but with three species, *D. immaculatus*, *D. japonica*, *D. suweonensis* in Asia) genera. Faivovich et al. [65] also considered the resurrection of *Dryophytes*. Considering that current evidence indicates a misidentification of *H. tsinlingensis* (KP212702) (=*D. immaculata*), the present study supports the monophyly of *Hyla* and *Dryophytes*, respectively. Thus, genetic distance and phylogenetic analysis from the present study recommend that the *Hyla* genus should be divided into the *Dryophytes* and *Hyla* clades.

Based on the geographic distribution of *Dryophytes*, Hua et al. [66] suggested that the *D. japonica* group be included in a clade that includes three Asiatic species: *D. immaculata*, *D. japonica*, and *D. suweonensis*. Furthermore, geographic distribution, deep molecular divergence, and phylogenetic analyses as done by Li et al. [67] are in favor of a *H. chinensis* group for all eastern Asiatic species: *H. annectans*, *H. chinensis*, *H. sanchiangensis*, *H. simplex*, *H. tsinlingensis*, and *H. zhaopingensis*. Considering the phylogenetic relationships of *D. suweonensis* as a sister clade to *D. immaculata*, and *D. ussuriensis* as a sister clade to *D. japonica*, along with the genetic distances between *D. suweonensis* and *D. immaculata* between *D. ussuriensis* and *D. japonica*, the evidence suggests that *D. suweonensis* is synonymous with *D. immaculata*, and *D. ussuriensis* is a synonym of *D. japonica*, as previously mentioned by Frost [2].

4.3. Transcript levels of protein-coding mitochondrial genes

In this study, mitochondrial genomic data was used to investigate the regulation of 13 protein-coding mitochondrial genes in the skeletal muscle of *D. versicolor* exposed to freezing or anoxia conditions. Relative transcript levels of *COX1* decreased with freezing conditions, but *COX2* increased under anoxia exposure (Figs. 1 and 2). This finding parallels an earlier study that characterized *COX1* and *ND5* in multiple tissues of anoxia-tolerant red-eared sliders (*Trachemys scripta elegans*), and freeze-tolerant turtles *Chrysemys picta marginata* [42]. Similar to the response in anoxic treefrogs, *COX1* and *ND5* transcript levels were unchanged in white skeletal muscle of anoxic red-eared sliders (however transcripts of both genes were significantly elevated in red skeletal muscle) [42]. Furthermore, *COX1* and *ND5* skeletal muscle mRNA levels decreased when *C. p. marginata* were exposed to freezing conditions [42]. Furthermore, another study documented that skeletal muscle *ND4* and *CYTB* transcript levels were unaffected from anoxia exposure in *T. s. elegans* [43]. Together, the results demonstrate that mitochondrial gene expression not only depends on skeletal muscle tissue type, but also on the anoxia/freeze-tolerance strategies used by different animals. To our knowledge, other mitochondrial protein-coding genes, such as *ND3*, have not been investigated in other anoxia/freeze-tolerant animals. The present study also observed an anoxia-induced suppression in *ND3* transcript levels (Fig. 1). The observed suppression in *ND3* of *D. versicolor* may highlight a part of a broader response that facilitates global metabolic depression during anoxia. Ultimately, these results suggest that modulations of protein-coding mitochondrial genes *D. versicolor* may play a role in the big picture of structural maintenance and metabolic regulation of skeletal muscle that accompanies tolerance to extreme freezing and anoxia conditions.

5. Conclusion

The total length of the *D. versicolor* mitogenome was 18,800 bp, which was the longest known to date among the available family members of Hylidae. Both BI and ML analyses strongly supported *D. versicolor* as a sister clade to (*D. japonica*+ *D. ussuriensis*) + (*D. suweonensis* + *D. immaculata* (KP212702)), and indicated that *Dryophytes* is monophyletic. In mitochondrial gene expression, skeletal muscle expression of *COX1*, *COX2*, *ND3* transcripts in response to anoxia showed different gene expression levels compared to freezing exposure. These findings suggest that modulations of protein-coding mitochondrial genes of *D. versicolor* are part of a broader response to freezing and anoxic conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.03.220.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

JY Zhang thanks the China Scholarship Council and Zhejiang Department of Education for generous support for a visiting scholar position at Carleton University.

Funding

This research was supported by grants from the National Natural Science Foundation of China (Nos. 31801963) and the Natural Sciences and Engineering Research Council of Canada (No. 6793) for the study design, data collection and analyses.

Ethics approval

All animal care protocols were previously approved by the Animal Care Committee (protocol # 106935) at Carleton University in accordance to guidelines provided by the Canadian Council on Animal Care.

Author contributions

JYZ, BEL and KBS conceived and designed the study. JYZ, BEL, DNY, LPZ, RA and KBS collected and analyzed the data. JYZ, BEL, DNY, LPZ, RA and KBS wrote the paper.

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