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The Complete Mitogenome of Amazonian *Hyphessobrycon heterorhabdus* (Characiformes: Characidae) as a Valuable Resource for Phylogenetic Analyses of Characidae

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Abstract: *Hyphessobrycon heterorhabdus* (Ulrey, 1894), popularly known as 'Flag Tetra' in English speaking countries, belongs to the genus *Hyphessobrycon* of the family Characidae, and is widely present in the eastern Amazon basin. Here, using Illumina sequencing, we report the complete mitogenome sequence of *H. heterorhabdus*. Overall, the mitogenome has 17,021 bp, containing 13 protein-coding, 22 tRNA, and 2 rRNA genes. Non-ambiguous nucleotide compositions of the *H. heterorhabdus* mitogenome are A: 29.2%, T: 29.4%, G: 15.6%, and C: 25.8%. As recently indicated, the phylogenetic analyses did not support four separate genera (*Hemigrammus*, *Hyphessobrycon*, *Moenkhausia*, and *Psalidodon*) of Characidae. Understanding the *H. heterorhabdus* mitogenome is important for taxonomic purposes as well as for the molecular characterization of environmental pollutants. Thus, the mitogenome described here will be a valuable resource for studies on environmental changes, evolutionary genetics, species delimitation, and phylogenetic analyses in Characidae.

Keywords: mitochondrial genome; fish DNA; mitochondrial DNA; heteroplasmy; Characidae; phylogeny; Amazon

Key Contribution: In this study, the whole mitogenome sequence of *H. heterorhabdus* was completed. In addition, the phylogenetic relationship within the family Characidae was investigated.

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1. Introduction

Hyphessobrycon heterorhabdus (Ulrey, 1894), commonly known as 'Flag Tetra' in English-speaking countries, is considered one of the most common fishes in the highland streams of the eastern Amazon (e.g., Ref. [1]) and occurs in Brazil in the coastal drainages from Pará to the Curuá-Una River basin and the lower Tapajós River [2]. It is a nektonic species with body morphology adapted to foraging in the water column and at the surface [3,4], an omnivore with a tendency to eat insects [5], and a popular freshwater ornamental fish [6].

The genus *Hyphessobrycon* includes about 160 species [2] and is polyphyletic. Although polyphyly has been demonstrated by molecular phylogenies and total evidence

approaches [7–10], others have proposed a monophyletic species group within *Hyphessobrycon* using morphological taxonomy [11,12] and integrative taxonomy [2]. Thus, the phylogeny of this group is still controversial.

In addition, *H. heterorhabdus* is considered an important species for the assessment of environmental conditions in eastern Amazonian rivers, as it frequently occurs in areas with diverse quality conditions [5,13]. Toxins in the environment may have the potential to affect cellular functions, such as homeostasis promoted by mitochondria, a cytoplasmic organelle [14]. These organelles have their own genome, known as the mitochondrial genome or mitogenome. Therefore, characterizing the mitogenome of *H. heterorhabdus* is important not only for elucidating taxonomic discussions, but also to provide potential biological markers for environmental contaminants. The complete mitogenome described here will therefore be a valuable resource for research on environmental changes, evolutionary genetics, species delimitation, and phylogenetic analyses in Characidae.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

Samples were collected in the Capim–Guamá basin, state of Pará, Brazil (1°46′ S, 47°15′ W) (Figure 1). Total genomic DNA was extracted from the caudal fin of adults using the Wizard Genomic DNA Purification Kit extraction (Promega, Madison, WI, USA) following supplier's instructions. Quantification was performed using NanoDrop 1000 spectrophotometer and Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The voucher specimens were deposited in the Natural History Collection of the Museology Department (RTM) of the Universidade Federal do Pará (UFPA), Belém, Brazil. Both the voucher specimens (code: MitoFish01male and MitoFish02female) and other specimens collected in the same stream were identified to species level by author LFAM from this study. The genome sequence data supporting the results of this study are freely available in NCBI's GenBank (https://www.ncbi.nlm.nih.gov/) under accession number OQ857750.

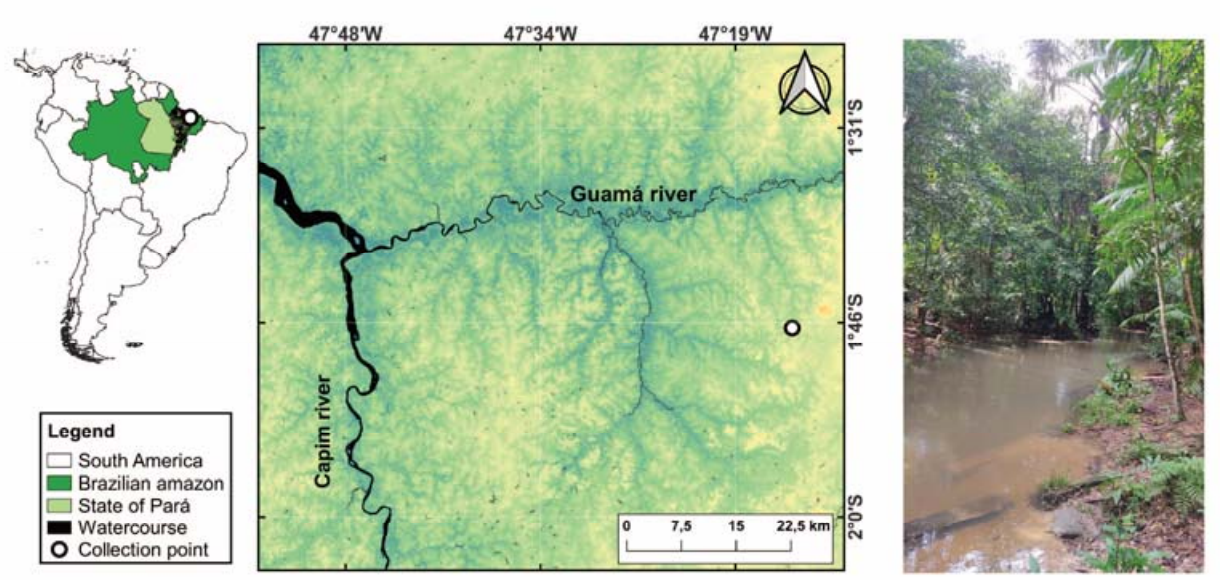

Figure 1. Samples were collected in the Capim–Guamá basin (Site 1), state of Pará, Brazil. Photo by G. Palheta.

2.2. Assembly and Annotation of the Complete Mitochondrial Genome

Two genomic libraries (one from a male and one from a female) were constructed using Illumina DNA Prep kit (Illumina, San Diego, CA, USA) with a short-insert size of 500 bp following manufacturer's instructions. Libraries were sequenced on Illumina NextSeq550 platform using a paired-end High Output Kit v2 (300 cycles).

Raw sequencing data were filtered in order to trim adapter and low-quality sequences, which yielded approximately 14 Gb. Genome assembly was carried out with MEGAHIT [15] and SOAPdenovo [16]. Locations of protein-coding genes (PCGs), ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs) were predicted using MiFish pipeline [17,18] and identified by alignment with other mitogenomes of *Hyphessobrycon*. tRNA predictions were confirmed using tRNAscan-SE [19], and R2DT [20] was used to predict and visualize secondary structures. Gene arrangement and structure were compared to seven mitogenomes from the Characidae family (including *Hyphessobrycon* species). Detection of mitochondrial heteroplasmy and nuclear mitochondrial pseudogenes (NUMTs) was performed using NOVOPlasty ver. 4.3.1 [21]. Mitochondrial DNA heteroplasmy above a level of 2% was considered possibly real (see [22]).

2.3. Phylogenetic Analysis

Phylogenetic analysis was performed using the same genomes reported previously [9]. We created a concatenated set of base sequences from 35 species to examine the phylogenetic relationships of the Characidae (see Supplementary Table S1; [10,23–37]). Geneious® software (version 9.0.5) was used to generate the alignments [38] and *Lebiasina astrigata* (Regan, 1903) was used as an outgroup. The 13 PCGs of each species were aligned separately using the algorithm MAFFT v. 7.017 [39] with the strategy L- INS -I and the default parameters. A dataset containing all 3 codon positions of the 13 PCGs was prepared for phylogenetic analyses on the IQ-TREE web server [40] using an ultrafast bootstrap algorithm with 10,000 repetitions and automatic model selection (GTR + I + G).

3. Results

3.1. Mitochondrial Genome Structure

The complete circular mitogenome of *H. heterorhabdus* is 17,021 bp long containing 13 PCG, 22 tRNA, and 2 rRNA genes (Figure 2), with a non-ambiguous nucleotide composition as follows, A: 29.2%, T: 29.4%, G: 15.6%, C: 25.8%. The A + T content (58.5%) is higher than the C + G content (41.5%), showing that the mitogenome is biased toward AT. Among the genes, 28 are encoded in the H-strand (heavy strand), and the other 9 are encoded in the L-strand (light strand), as shown in Figure 2 and Table 1.

Table 1. Mitochondrial genome organization and gene content of *H. heterorhabdus* (Capim–Guamá basin) with a detailed description of gene boundaries, gene length (in bp), as well as start and stop codons for protein-coding genes and anticodons for tRNA genes.

Name	Type	Strand	Start	Stop	Length	Anticodon and Start Codon/Stop Codon	Intergenic Nucleotides
tRNA-Phe	tRNA	H	1	69	69	GAA	0
12S rRNA	rRNA	H	70	1023	954	-	0
tRNA-Val	tRNA	H	1024	1095	72	TAC	0
16S rRNA	rRNA	H	1096	2754	1659	-	0
tRNA-Leu	tRNA	H	2755	2828	74	TAA	0
ND1	Gene	H	2829	3797	969	ATG/TAA	7
tRNA-Ile	tRNA	H	3805	3876	72	GAT	12
tRNA-Gln	tRNA	L	3889	3959	71	TTG	4
tRNA-Met	tRNA	H	3964	4032	69	CAT	1
ND2	Gene	H	4034	5101	1068	ATG/TAA	12
tRNA-Trp	tRNA	H	5114	5183	70	TCA	7
tRNA-Ala	tRNA	L	5191	5259	69	TGC	1
tRNA-Asn	tRNA	L	5261	5333	73	GTT	31
tRNA-Cys	tRNA	L	5365	5430	66	GCA	−1

Table 1. *Cont.*

Name	Type	Strand	Start	Stop	Length	Anticodon and Start Codon/Stop Codon	Intergenic Nucleotides
tRNA-Tyr	tRNA	L	5430	5500	71	GTA	1
COX1	Gene	H	5502	7061	1560	ATG/AGG	−13
tRNA-Ser	tRNA	L	7049	7120	72	TGA	5
tRNA-Asp	tRNA	H	7126	7193	68	GTC	16
COX2	Gene	H	7210	7900	691	ATG/T-	0
tRNA-Lys	tRNA	H	7901	7973	73	TTT	1
ATP8	Gene	H	7975	8142	168	ATG/TAG	−10
ATP6	Gene	H	8133	8814	682	TTG/T-	0
COX3	Gene	H	8815	9598	784	ATG/T-	0
tRNA-Gly	tRNA	H	9599	9670	72	TCC	0
ND3	Gene	H	9671	10,019	349	ATG/T-	0
tRNA-Arg	tRNA	H	10,020	10,088	69	TCG	0
ND4L	Gene	H	10,089	10,385	297	ATG/TAA	−7
ND4	Gene	H	10,379	11,759	1381	ATG/T-	0
tRNA-His	tRNA	H	11,760	11,828	69	GTG	0
tRNA-Ser	tRNA	H	11,829	11,896	68	GCT	1
tRNA-Leu	tRNA	H	11,898	11,970	73	TAG	0
ND5	Gene	H	11,971	13,809	1839	ATG/TAA	−4
ND6	Gene	L	13,806	14,321	516	ATG/TAA	0
tRNA-Glu	tRNA	L	14,322	14,389	68	TTC	5
CYTB	Gene	H	14,395	15,531	1137	ATG/TAA	4
tRNA-Thr	tRNA	H	15,536	15,608	73	TGT	−2
tRNA-Pro	tRNA	L	15,607	15,676	70	TGG	0
D-loop		H	15,677	17,020	1344	-	1

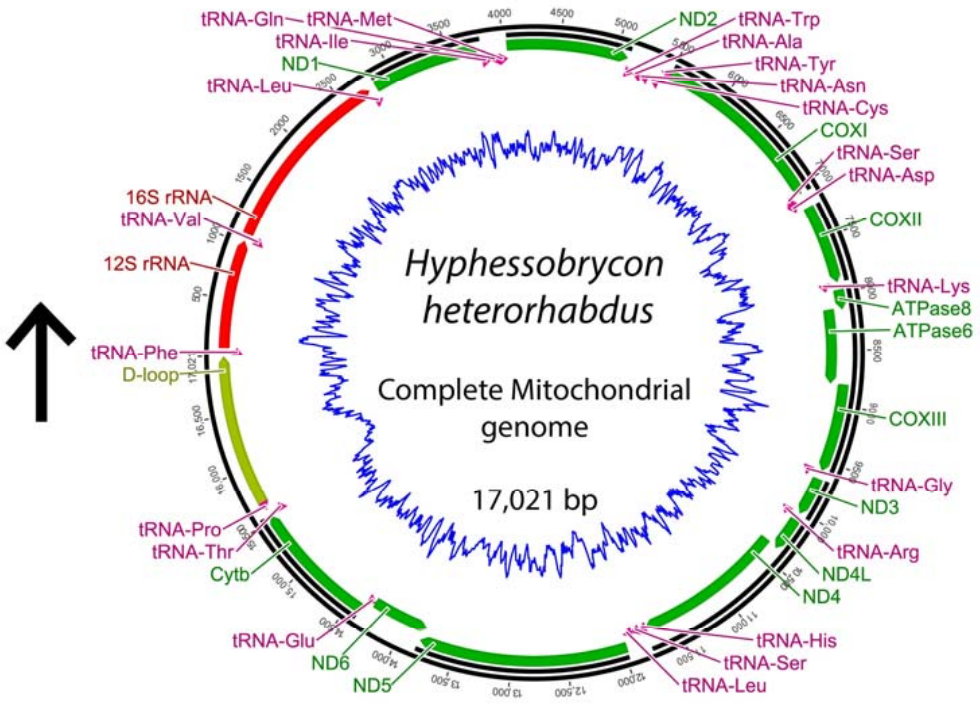

Figure 2. Graphical representation of the circular mitogenome map of *H. heterorhabdus* (Capim–Guamá basin). Different colors indicate groups of genes: 13 protein-coding genes (in green), 22 tRNAs (in pink), 2 rRNAs (in red), and control region (D-loop; in yellow). The blue ring represents the GC content.

The *H. heterorhabdus* mitogenome is very similar to that of other species of Characidae, such as the four previously described mitochondrial mitogenomes [30] and *Hyphessobrycon amandae* (Géry and Uj, 1987) [24] (Figure 3).

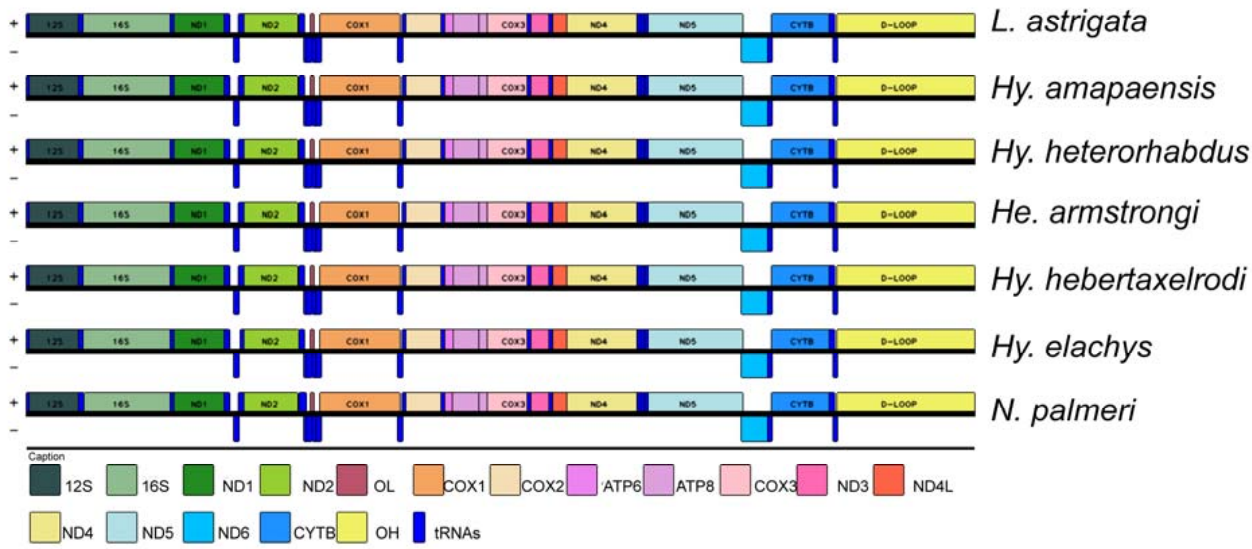

Figure 3. Arrangement of genes encoding RNAs and proteins in four species from the *Hyphessobrycon* genus and three outgroups (*Lebiasina astrigata*, *Hyphessobrycon amapensis*, *Hy. heterorhabdus*, *Hemigrammus armstrongi*, *Hy. herbetaxelrodi*, *Nematobycon palmeri*, and *Hy. elachys*).

3.2. Protein-Coding Genes

The overall length of the PCGs in the *H. heterorhabdus* mitogenome was 11,441 bp, ranging from 168 bp (ATP8) to 1839 bp (ND5). The average A + T content was 58.1%. Most PCGs used the conventional start codon ATG and ended with the codon TAN or an incomplete codon (T−−), except for the COX1 gene, which was terminated with AGG. The PCGs ND1-ND6, ND4L, COX1, COX2, COX3, ATP8, ATP6, and CYTB were observed in other teleost fishes and vertebrates (e.g., [41]).

3.3. Transfer and Ribosomal RNA Genes and Control Region

The mitogenome of *H. heterorhabdus* has 2 rRNAs and 22 typical tRNAs. The 16S rRNA and 12S rRNA were 954 and 1659 bp long, respectively, and the A + T contents of rRNA were 58.2%. Compared with other mitogenomes of characids, the tRNA genes of *H. heterorhabdus* are well conserved (see [42]). Among them, 14 tRNAs were encoded on the H-strand, and the remaining 8 were encoded on the L-strand. As shown in Figure 4, the 22 tRNAs have a typical cloverleaf secondary structure, with sizes ranging from 66 bp (tRNA-Cys) to 74 bp (tRNA-Leu); the total length of the 22 tRNAs was 1551 bp.

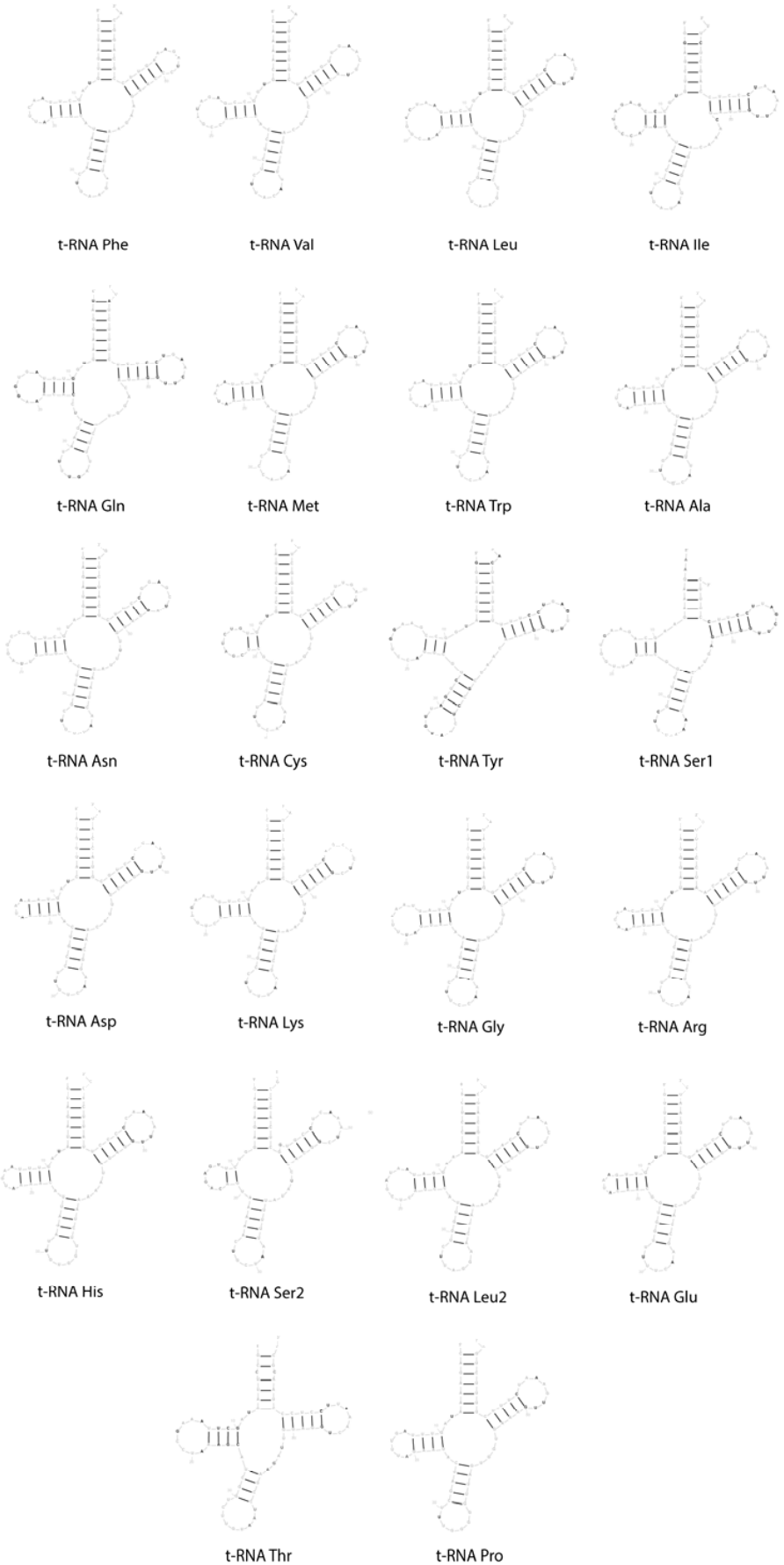

Figure 4. Predicted secondary structures of 22 inferred tRNAs from the *H. heterorhabdus* mitochondrial genome.

As shown in Figure 5, the relative usage of synonymous codons (RSCU) was biased for most amino acids. The two most commonly used codons were consistently AUU (15.0%) and CUU (13.6%). The comparative summaries of the RSCU of the mitogenomes for species of *Hyphessobrycon* show that they are very similar, as seen in Figure 5. In addition, synonymous codon preferences were conserved for all seven species, which can be attributed to their close relationship within the genus and family. Like other fish mitogenomes, the Control Region (D-loop) was located between the tRNA-Pro and tRNA-Phe.

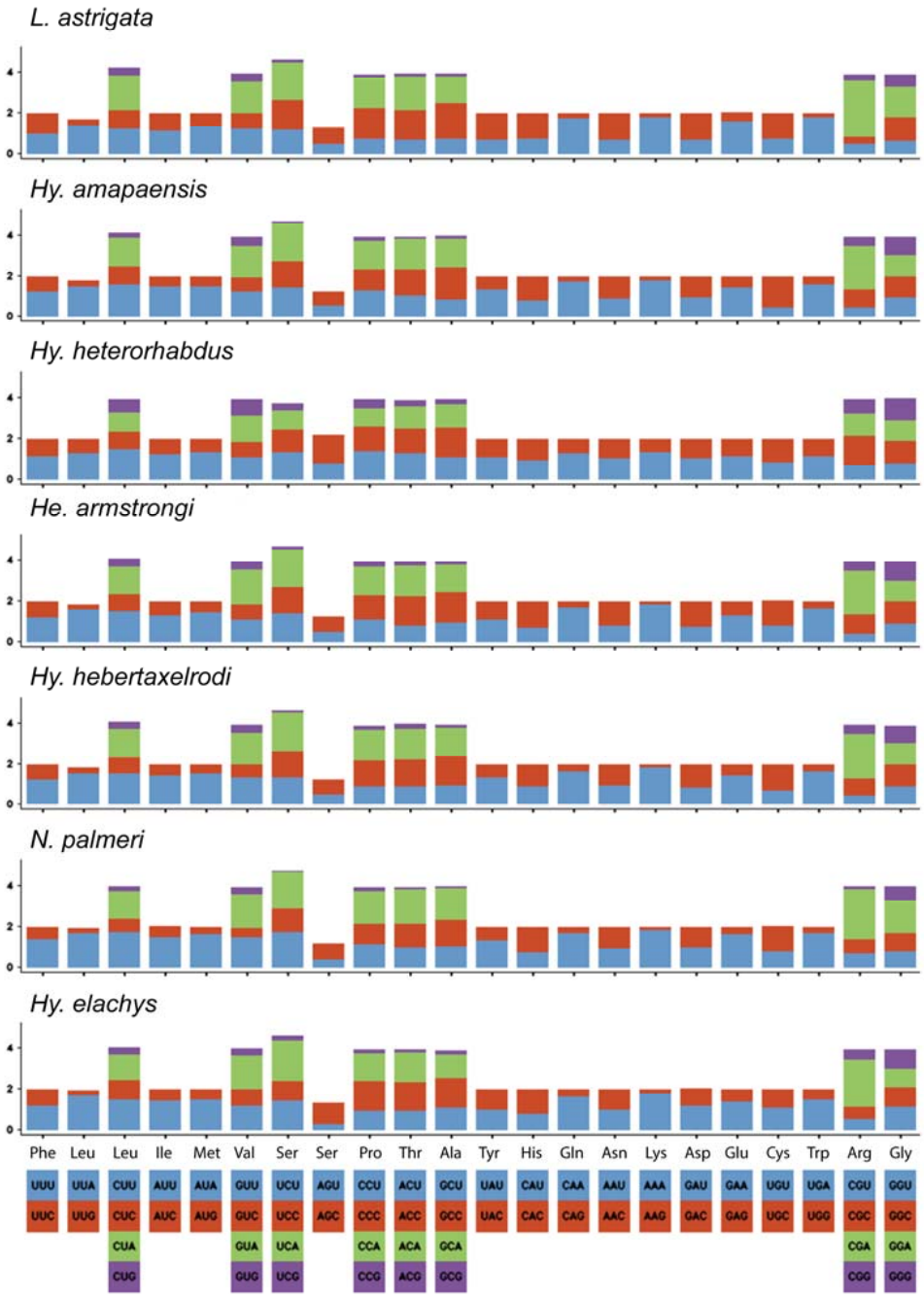

Figure 5. Relative synonymous codon usage (RSCU) of protein-coding genes of four species from the *Hyphessobrycon* genus and three outgroups (*Lebiasina astrigata*, *Hyphessobrycon amapensis*, *Hy. heterorhabdus*, *Hemigrammus armstrongi*, *Hy. herbetaxelrodi*, *Nematobycon palmeri* and *Hy. elachys*).

3.4. Mitogenomic Heteroplasmy and NUMTs Analysis

In the analysis of heteroplasmy and NUMTs, we detected the presence of six heteroplasmic variants in male genomes and two heteroplasmic variants in female genomes (Table 2). Allele frequencies greater than 2%, a value that is usually valid for this detection, were found in the male D loop (locus 16951; C, AF = 0.576 and A, AF = 0.020) and in the female ND4 gene (locus 10803; A, AF = 0.0263). As for the NUMTs, only one possible degenerate sequence of the D-loop was assessed in the construction of the female genome.

Table 2. Divergent alleles associated with male and female mitogenomes of *Hyphessobrycon heterorhabdus* (Ulrey, 1894). REF Alelle: reference allele; ALT allele: alternative allele; AF: allele frequency; DP: depth of coverage at this site for this sample.

Sample	Locus	REF Allele	ALT Allele	AF	DP	Gene Region
Male	2691	C	A	0.0145	137	16S rRNA
Female	10,803	G	A	0.0263	75	ND4
Male	13,940	T	A	0.0122	81	ND6
Male	14,076	CC	C	0.0124	322	ND6
Female	14,192	T	G	0.0162	123	ND6
Male	14,397	G	A	0.0161	123	CYTB
Male	14,691	T	G	0.0128	77	CYTB
Male	16,951	T	C,A	0.576, 0.0205	97	D-Loop

3.5. Phylogenetic Analysis

All species were well separated from the outgroup species, with good bootstrap values in ML. The *H. heterorhabdus* (Capim–Guamá basin) was more phylogenetically close to *Hyphessobrycon amapaensis* (Zarske and Géry, 1998), as seen in Figure 6.

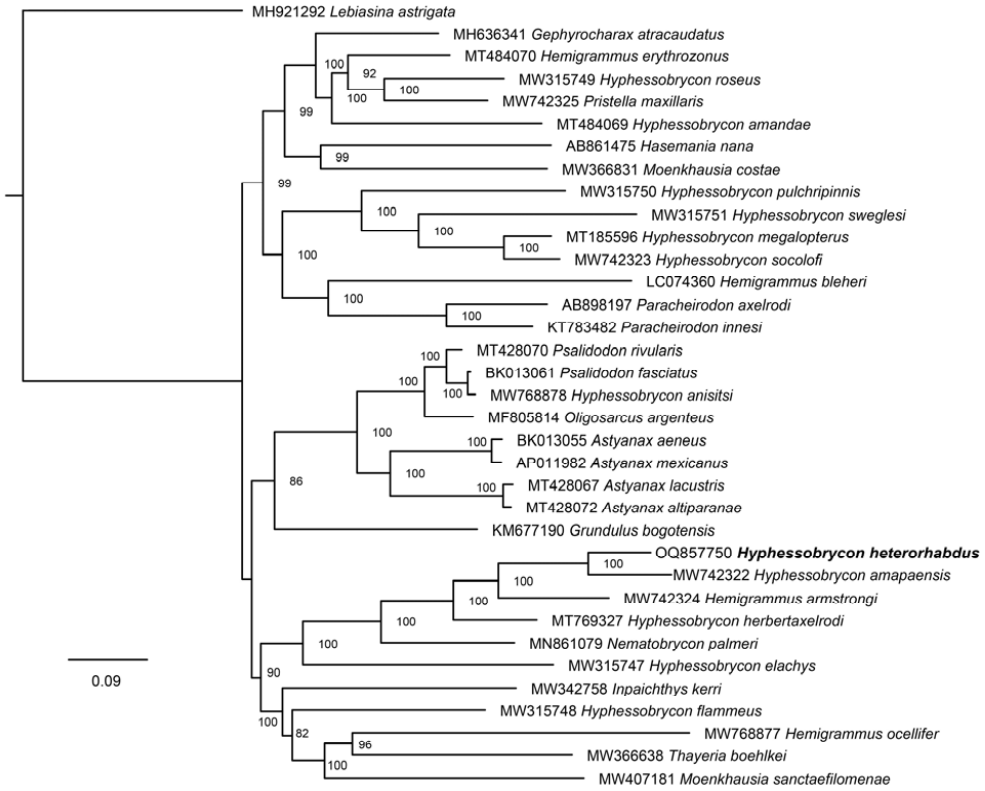

Figure 6. Phylogenetic tree of 35 species of Characidae based on 13 PCGs. Values shown next to nodes are maximum-likelihood ultrafast bootstrap values.

4. Discussion

Hyphessobrycon heterorhabdus is one of the most abundant fishes in upland streams of the eastern Amazon river and is considered an important species for assessing environmental conditions due to its ability to withstand variation in water quality [13]. Considering that mitochondria are cytoplasmic organelles with a crucial role in cellular homeostasis, and that toxic environmental contaminants may impact mitochondrial function and genetics [14], we sequenced the whole mitochondrial genome of *H. heterorhabdus* in order to fully characterize their mtDNA and provide a reference sequence for this species.

We observed that the circular mitogenome of *H. heterorhabdus* is 17,021 bp long and contains 13 PCG, 22 tRNA, and 2 rRNA genes (Figure 2). When comparing the distribution of genes found in this study to those previously reported in the literature, all 37 genes can be observed in other species of Characidae [30,42,43], demonstrating that these genes are conserved within this genus (Figure 3).

PCGs have shared similarities with teleosts, namely, the same start codon (ATG), with the exception of the ATP6 gene, which starts with TTG, a feature found in *Hyphessobrycon megalopterus* (Eigenmann, 1915), *Hyphessobrycon amandae*, and other teleosts [24,30,44,45]. Interestingly, eight PCGs have complete stop codons. TAA, the most frequent stop codon, is used by the genes ND1, ND2, ND4L, ND5, ND6, and CYTB, while other PCGs have the incomplete stop codon T-, seen in the ATP6, COX2, COX3, ND3, and ND4 genes (Table 1). These types of stop codons may be completed by a TAA with the addition of a poly-A tail during the RNA processing [46]. Such findings are frequent among teleosts, and have been discussed in other mitogenomes [24,47].

Regarding rRNAs and tRNAs, we observed that *H. heterorhabdus* have 2 rRNAs and 22 typical tRNAs. Compared to other Characidae mitogenomes, tRNA genes are well conserved [42] and cloverleaf secondary structures are common in many teleost mitogenomes [47,48]. Finally, regarding heteroplasmy and the presence of NUMTs in our mitogenomes, studies using this type of evaluation are still rare. Although our results show the presence of a few conspicuous allelic variations, it should be emphasized that these types of analyses are important for the confidence in genetic information in both evolutionary studies and molecular identification (see more in [49]).

There are approximately 160 species in the *Hyphessobrycon* genus, and the phylogeny of this group is not well established [2]. Since molecular taxonomy usually employs mitochondrial gene sequences to infer phylogenetic relationships [8], we used the sequence of 13 PCGs to improve the understanding of Characidae phylogeny. Our results did not support four genera (*Hemigrammus*, *Hyphessobrycon*, *Moenkhausia*, and *Psalidodon*) (Figure 6), corroborating the findings of the previous studies [10] and indicating that *Hyphessobrycon* is not a monophyletic group.

The polyphyly of the three clades of *Hyphessobrycon* species revealed by our mitochondrial genome analysis can be explained by several biogeographic factors. One possible explanation for why *Hyphessobrycon* is not a monophyletic group is vicariation events, during which a population is divided by a physical barrier, leading to isolation and the formation of two new isolates [50], or also by dispersal with high vagility, mainly by a temporary connection between basins [50]. The history of physical changes and connectivity between the basins that make up the Amazon basin is the most important factor in the diversity and speciation patterns observed for the fish basins found in this location [51].

We also note the phylogenetic proximity of *H. heterorhabdus* to *H. amapaensis*. The latter species is endemic to the state of Amapá, with a record distance of less than 500 km from the sequenced specimens. Previous morphological studies have indicated similarities among these species, such as a well-defined and elongated humeral spot and a spot on the caudal peduncle, aligning them to the same subgroup [2]. Regarding the mitogenome, tRNAs were very similar between species or showed only one or no nucleotide change, as in tRNA-Pro, tRNA-Lys, and t-RNA-Ser. Despite the possibility of analyzing the evolutionary divergence of these species based on molecular dating, it must be noted that the currently known distribution of both species is restricted to areas of repetitive marine influence during the

Tertiary and Quaternary (see [52]), which must have led to changes in the evolution of species associated with this type of environment (e.g., [53]).

5. Conclusions

Here, we reported the first record of the complete mitogenome of *H. heterorhabdus* from the family Characidae. This mitochondrial genome has a length of 17,021 bp, including 13 PCGs, 2 rRNAs, and 22 tRNAs, with a close genomic structure to other mitogenomes of teleosts. Based on the molecular data from the *H. heterorhabdus* mitogenome, our phylogenetic analyses reinforce the recent proposal that *Hemigrammus*, *Hyphessobrycon*, *Moenkhausia*, and *Psalidodon* are not separate genera of Characidae.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8050233/s1, Table S1: Mitogenomes used in phylogenetic analyses.

Author Contributions: L.F.d.A.M., Â.R.-d.-S., T.S.M., R.L. and L.J. designed the study. R.K., J.E.S.d.S., S.J.d.S. and D.H.F.G. performed formal analyses of the manuscript. L.F.d.A.M., R.K., Â.R.-d.-S., L.M., G.C.C., C.S.S., S.G., D.H.F.G., J.E.S.d.S., S.J.d.S., L.B.S., M.D.L.d.L., E.G.P., I.C.d.S.P., T.S.M., R.L. and L.J. wrote the original draft. All authors contributed to writing, reviewing, and editing the manuscript. All authors contributed to the research and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The genome sequence data supporting the results of this study are freely available in NCBI's GenBank (https://www.ncbi.nlm.nih.gov/) under the accession number OQ857750.

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Conflicts of Interest: S.J.S. declares that there is a conflict of interest with the Chief Scientific Officer of DNA GTx Bioinformatics, LTDA, and a shareholder of DNA GTx, Inc., Dubai, United Arab Emirates. The remaining authors declare no commercial or financial relationships that could be construed as potential conflicts of interest.

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