Journal of Genetics (2020)99:67
https://doi.org/10.1007/s12041-020-01221-x

RESEARCH ARTICLE

Sequencing and characterization of complete mitogenome DNA of *Rasbora tornieri* (Cypriniformes: Cyprinidae: Rasbora) and its evolutionary significance

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Received 8 October 2019; revised 12 January 2020; accepted 27 April 2020

Abstract. The yellowtail rasbora (*Rasbora tornieri*) is a miniature ray-finned fish categorized under the genus *Rasbora* in the family of Cyprinidae. In this study, a complete mitogenome sequence of *R. tornieri* was sequenced using four primers targeting two halves of the mitogenome with overlapping flanking regions. The size of mitogenome was 16,573 bp, housing 22 transfer RNA genes, 13 protein-coding genes, two ribosomal RNA genes and a putative control region. Identical gene organization was detected between this species and other members of *Rasbora* genus. The heavy strand encompassed 28 genes while the light strand accommodated the other nine genes. Most protein-coding genes execute ATG as start codon, excluding COI and ND3 genes, which utilized GTG instead. The central conserved sequence blocks (CSB-E, CSB-F and CSB-D), variable sequence blocks (CSB-1, CSB-3 and CSB-2) as well as the terminal associated sequence (TAS) were conserved within the control region. The maximum likelihood phylogenetic family tree revealed the divergence of *R. tornieri* from the basal region of the Rasbora clade, where its evolutionary relationships with other Rasbora members are poorly resolved as indicated by the low bootstrap values. This work acts as window for further population genetics and molecular evolution studies of *Rasbora* genus in future.

Keywords. mitogenome; gene arrangement; light strand origin; phylogeny; *Rasbora tornieri*.

Introduction

The yellowtail rasbora (*Rasbora tornieri*) is categorized under the genus *Rasbora* within the family Cyprinidae. As its name suggests, this freshwater ray-finned fish is uniquely different from other Rasbora fishes in terms of the striking yellow colouration with broad black margins at its caudal fin (Kottelat *et al*. 1993). With regard to length, the maximum growth of this fish species can reach up to 17 cm, and they feed on exogenous insects, often spawn in ponds and rivers (Kottelat *et al*. 1993). It is commonly found in canals and streams of the lowlands and are distributed across Indonesia, Indochina as well as Malaysia (Kottelat *et al*. 1993).

Cyprinidae family accommodates 11 subfamilies, namely Barbinae, Leuciscinae, Labeoninae, Danioninae (where the yellowtail rasbora resides), Acheilognathinae, Cyprinninae, Cultrinae, Squaliobarbinae, Gobioninae, Tincinae and Xenocypridinae (Liao *et al*. 2010). The Rasbora group is well-known as 'catch-all' group due to the taxonomical complications known to exist since their discovery contributed by their closely resembled morphological characters (Brittan 1954; Kottelat and Vidthayanon 1993; Siebert and Guiry 1996; Kottelat 2005; Liao *et al*. 2010; Tang *et al*. 2010). The most widely known and accepted Rasbora characterization is of Brittan (1954), where the Rasbora *sensu lato* concept is applied, categorizing them into eight species complexes: *pauciperforata*, *einthovenii*, *sumatrana-elegans*, *lateristriata*, *argyrotaenia*, *trisfasciata*, *caudimaculata* and *daniconius* (Brittan 1954).

Published online: 06 August 2020

The research advancement of the Rasbora genus is in its infancy stages rendering the occurrence of cryptic diversity and taxonomical impediments (Liao *et al*. 2010; Kusuma *et al*. 2016). With the recent research limelight shining on *R. sarawakensis* and *R. caverii* (Wijeyaratne and Pathiratne 2006; Lim *et al*. 2018), the main stage is now open to encompass more Rasbora species, especially with the help of the next-generation sequencing. Thus, a total of 18 Rasbora mitogenome has been sequenced including the one in this study, *R. tornieri*, as well as *R. myersi*, *R. heteromorpha*, *R. sarawakensis*, *R. borapetensis*, *R. lateristriata*, *R. hobelmani*, *R. aprotaenia*, *R. argyrotaenia*, *R. pauciperforata*, *R. maculata*, *R. vaterifloris*, *R. espei*, *R. steineri*, *R. cephalotaenia*, *R. sumatrana*, *R. daniconius* and *R. trilineata* (Miya 2009; Tang *et al*. 2010; Chang *et al*. 2012; Ho *et al*. 2014; Zhang *et al*. 2014; Kusuma and Kumazawa 2015; Kusuma *et al*. 2017; Chung *et al*. 2020b). With the addition of the yellowtail rasbora, the discovered Rasbora mito genome had just crossed the 20% milestone, taking up 20.7% of the total 87 Rasbora fish species discovered to date (Eschmeyer 2015). The mitogenome has the great potential to bridge molecular evolution studies to population genetics, thus providing the link between genotypes and phenotypes. Therefore, this study aimed to unveil the mitogenome contents of *R. tornieri* as well as its evolutionary significance across the other 17 Rasbora genus counterparts.

Materials and methods

Sampling and mitogenome extraction from total tissue

The yellowtail *R. tornieri* fishes were captured from Kanowit River, Sarawak, Malaysia (2°01′36.7″ N, 110°24′41.3″ E) with the permit granted by the Sarawak Forestry Department (permit number: NCCD.94047(Jld13)-178). The fishes were Tricane humanely euthanized using the protocols established by the Animal Ethics Committee, Universiti Malaysia Sarawak (reference number: UNIMAS/TNC(PI)-04.01/06-09(17)). The mitogenome extraction was conducted using the muscle tissues from the total tissue of *R. tornieri* via CTAB method as followed by Chung (2018). The remaining parts of the fish body was preserved in 95% ethanol for long-term storage.

PCR and sequencing

The primers targeting the desired mitogenome regions were designed based on conserved motifs across four closely related Rasbora species (*R. aprotaenia*, *R. agryrotaenia*, *R. trilineata* and *R. sumatrana*) (table 1). The two primer pairs must cover the two halves of the mitogenome with 2000-bp flanking overlapping regions to ensure good sequencing reads. The removal of these overlapping regions will reveal the complete mitogenome sequences.

Table 1. Primers utilized in the amplification of the *R. tornieri* mitogenome.

Primer name	Primer sequence	T_m (°C)	Amplification length (bp)
SF1	GTGCTTCCTCTACACCAC	55.3	8923
SR1	TGATGTTGAGAAGGCTAC		
LF1	CCTATCTTACCGAGAAAG	48.6	9990
LR1	GAGGCCTTCCCATCTAGA		

The polymerase chain reaction (PCR) was executed utilizing the T-100 Thermal Cycler (BioRad, USA) in a 20 μL total reaction volume containing 2.5 mM dNTP, 10 μM forward and reverse primer each, 2.5 U high-fidelity *Taq* polymerase, 2.0 μL 10x PCR buffer (with Mg^{2+}), 14.6 μL nuclease-free water and 25 ng genomic DNA extract. The thermal cycler parameters are defined as follow: one cycle of predenaturation at 94°C for 2 min, followed by 35 cycles of denaturation, annealing and extension at 94°C (30 s), primer-specific temperature (30 s) and 72°C (5 min) respectively and a final extension cycle at 72°C for 5 min. The agarose gel electrophoresis was run following the PCR reaction to size separate the PCR products before visualizing them under UV light. High purity and quality amplicons were subjected to Illumina HiSeq 400 system pair-ended sequencing reactions with short reads. Quality checks were done on all the sequencing reads prior to adaptor trimming employing cutadapt (Martin 2011). The assembly was conducted using *de novo* assembler SPAdes v. 3.12.0 (Bankevich *et al*. 2012).

Mitogenome characterization and gene analysis

The construction and visualization of the mitogenome map of *R. tornieri* was done using MitoFish (Iwasaki *et al*. 2013) with gene annotations. All protein-coding genes were closely inspected via translation of nucleotide sequences into amino acid sequences to ensure full functionalities whereby the truncated and premature stop codons were edited using MEGA 7.0 (Kumar *et al*. 2016). The tRNA-scan SE v. 2.0 (Lowe and Chan 2016) was exercised to detect anti-codons of tRNA genes via default search mode. The L-strand origin (O_L) was predicted and visualized in terms of its secondary structure via RNAstructure 6.0 (Reuter and Mathews 2010) with considerations of sequence homology. NCBI Bankit was utilized to deposit the entire mitogenome sequences into the GenBank database.

Phylogeny

A total of 18 Rasbora mitogenomes (inclusive of *R. tornieri*) were selected for the building of phylogenetic family tree in

Table 2. Features of the complete *R. tornieri* mitogenome.

Gene	Position		Length (bp)	Anti-codon	Codon		Intergenic nucleotide[b]	Strand
	Start	End			Start	Stop[a]		
$tRNA^{Phe}$	1	69	69	GAA			0	H
12S rRNA	70	1025	956				0	H
$tRNA^{Val}$	1026	1096	71	TAC			0	H
16S rRNA	1097	2784	1688				0	H
$tRNA^{Leu\ (UAA)}$	2785	2859	75	TAA			+ 1	H
ND1	2861	3835	975		ATG	TAA	+ 4	H
$tRNA^{Ile}$	3840	3911	72	GAT			− 2	H
$tRNA^{Gln}$	3980	3910	71	TTG			1	L
$tRNA^{Met}$	3982	4050	69	CAT			0	H
ND2	4051	5095	1045		ATG	T–	0	H
$tRNA^{Trp}$	5096	5167	72	TCA			+ 2	H
$tRNA^{Ala}$	5237	5170	68	TGC			+ 1	L
$tRNA^{Asn}$	5311	5239	73	GTT			+ 37	L
$tRNA^{Cys}$	5414	5349	66	GCA			+ 1	L
$tRNA^{Tyr}$	5485	5416	70	GTA			+ 1	L
COI	5487	7037	1551		GTG	TAA	0	H
$tRNA^{Ser\ (UGA)}$	7108	7038	71	TGA			+ 1	L
$tRNA^{Asp}$	7110	7180	71	GTG			+ 7	H
COII	7188	7872	685		ATG	T–	0	H
$tRNA^{Lys}$	7873	7947	75	TTT			+ 4	H
ATP8	7952	8116	165		ATG	TAA	− 7	H
ATP6	8110	8792	683		ATG	TA-	0	H
COIII	8793	9577	785		ATG	TA-	0	H
$tRNA^{Gly}$	9578	9648	71	TCC			0	H
ND3	9649	9997	349		GTG	T–	0	H
$tRNA^{Arg}$	9998	10067	70	TCG			0	H
ND4L	10068	10364	297		ATG	TAA	− 7	H
ND4	10358	11739	1382		ATG	TA-	0	H
$tRNA^{His}$	11740	11808	69	GTG			0	H
$tRNA^{Ser\ (GCU)}$	11809	11876	68	GCT			+ 2	H
$tRNA^{Leu\ (UAG)}$	11879	11951	73	TAG			0	H
ND5	11952	13781	1830		ATG	TAA	− 4	H
ND6	14299	13778	522		ATG	TAG	0	L
$tRNA^{Glu}$	14368	14300	69	TTC			+ 6	L
Cytb	14375	15511	1137		ATG	TAA	+ 5	H
$tRNA^{Thr}$	15517	15587	71	TGT			+ 14	H
$tRNA^{Pro}$	15671	15602	70	TGG			0	L
D-loop	15672	16573	902				–	–

[a]TA- and T– indicate incomplete stop codons.
[b]Positive numbers indicate interspaced nucleotides and negative numbers indicate overlapping nucleotides. H, H-strand; L, L-strand.

which 13 of the sequences are publicly available on GenBank whereas the other five (*R. pauciperforata*, *R. hobelmani*, *R. sarawakensis*, *R. myersi* and *R. tornieri* (from this study) were from our laboratory associates prior to the GenBank release dates. The *Acheilognathus typus* and *Danio rerio* were the two selected outgroups in this study. A sum of 12 protein-coding genes were extracted from the entire Rasbora mitogenomes selected for tree building without the inclusion of ND6 due to its high heterogeneity property (Miya and Nishida 2000). The general time reversible model (GTR+G model with gamma distributed rates among sites) was the best model selected from the model test performed using MEGA 7.0. The maximum likelihood phylogenetic tree was constructed with the parameter of 1000-bootstrap replications employing MEGA 7.0.

Results and discussion

Mitogenome structure

The complete mitogenome sequences of *R. tornieri* was submitted to GenBank for deposition with the given accession number MK955877. This mitogenome covers up to the length of 16,573 bp, housing a total of one control region, two rRNA genes, 13 protein-coding genes as well as 22

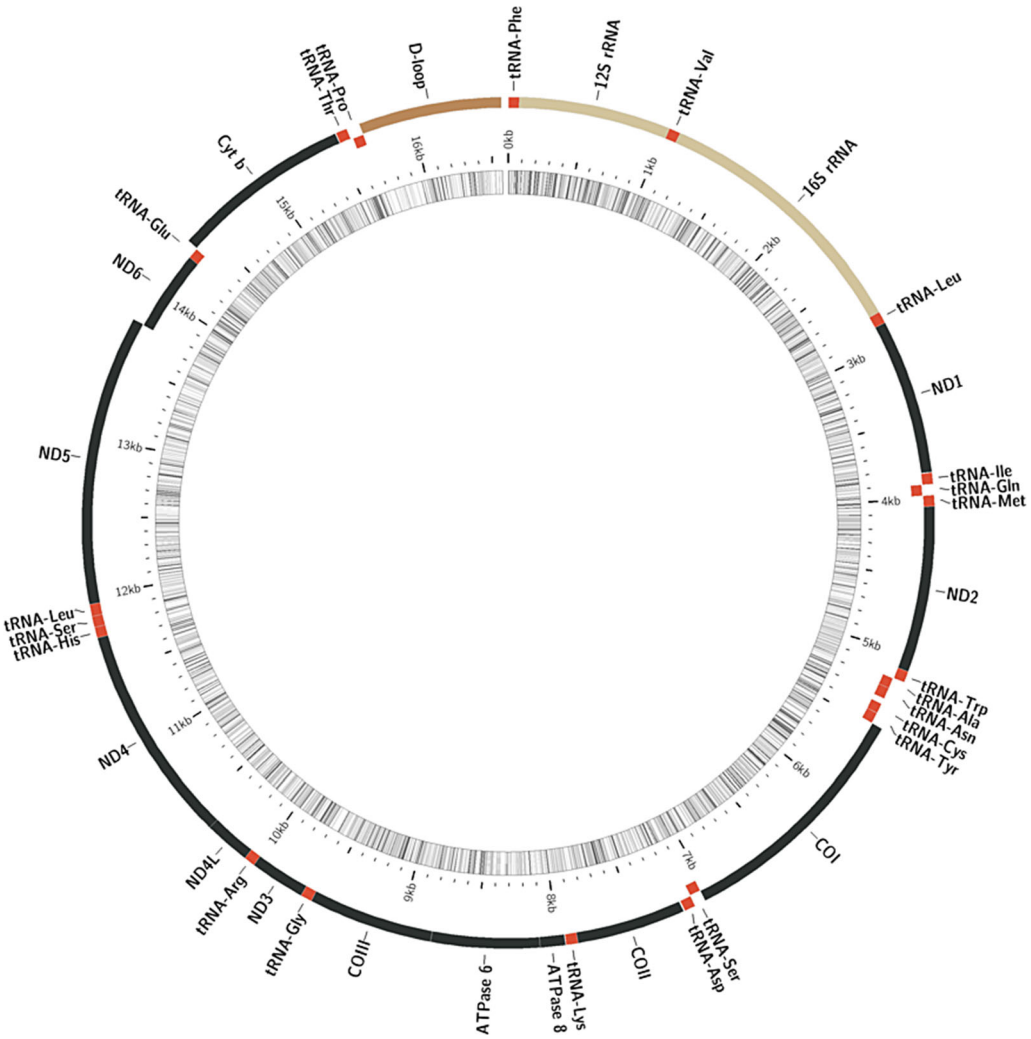

Figure 1. Circular genome map of *R. tornieri*. Genes that encoded on H-strand and L-strand are portrayed in outer and inner circles, respectively. The inner ring displays the GC% every 5 bp where the darker lines represent higher GC percent. The size of the complete mitogenome of *R. tornieri* is 16,573 bp with the inclusion of 22 tRNA genes, 13 protein-coding genes, two rRNA genes and a control region.

tRNA genes (table 2). Majority (28 genes) of them are situated on the heavy strand (H-strand) whereas the remaining are planted on the light strand (L-strand) (figure 1). There are four overlaps detected and all are from the H-strand. The largest overlap is equally shared between ATP8 and ATP6, as well as between ND4L and ND4 genes, scoring a 7 bp overlap. The largest intergenic spacer recorded in this mitogenome is 37 bp, where it is sandwiched between $tRNA^{Asn}$ and $tRNA^{Cys}$.

Overall, the *R. tornieri* mitogenome has lower G + C content (40.8%) than A + T content (59.2%) in which it mirrored *R. myersi* in terms of percentage (Lim *et al.* 2019). For the rough comparison, such percentage is common across *Cobitis lutheri* (Cui *et al.* 2013) and other Rasbora species such as *R. borapetensis*, *R. sarawakensis* and *R. steineri* (Chang *et al.* 2012; Zhang *et al.* 2014). The overall protein-coding genes of *R. tornieri* has 9.7% lower A + T content than the control region (69.2%) (table 3). A similar gap (10%) can be observed between the overall genome and overall protein-coding genes in terms of A + T content in the *R. tornieri* mitogenome. Breaking down to their individual nucleotide composition, the overall protein-coding genes and the overall genome has very similar adenine (A) nucleotide composition (only differed by 0.5%), whereas for other nucleotides the nucleotide composition differences ranged between 2.6% (G) to 9.5% (T).

Protein-coding gene feature

About 69.2% of the mitogenome is identified as protein-coding genes, covering up to a total length of 11,476 bp. The ND5 is no doubt the largest gene in the entire mitogenome and among protein-coding genes, spanning a length of 1830 bp. The shortest gene is only 165 bp in length and is known

Table 3. The nucleotide base composition of different regions in the *R. tornieri* mitogenome.

Region	Base composition (%)				A + T content (%)
	T	C	A	G	
Protein-coding gene					
ND1	26.1	27.5	33.4	13	59.5
ND2	21.7	30.4	37.6	10.2	59.3
COI	29.5	25	28.4	17.2	57.9
COII	28.6	24.8	30.8	15.8	59.4
ATP8	29.1	27.9	35.8	7.3	64.9
ATP6	31.2	26.6	31.5	10.7	62.7
COIII	25.5	28.9	29.6	16.1	55.1
ND3	29.2	27.8	29.5	16.1	58.7
ND4L	28.3	27.9	30	13.8	58.3
ND4	26.2	26.9	34.2	12.7	60.4
ND5	26.2	26.8	35.5	11.5	61.7
ND6	13.6	31.8	43.1	11.5	56.7
Cytb	27.6	27.8	34.1	11.9	61.7
Overall protein-coding gene	26.4	27.4	33.1	13.2	59.5
tRNA gene					
$tRNA^{Phe}$	14.5	26.1	37.7	21.7	52.2
$tRNA^{Val}$	19.7	28.2	29.6	22.5	49.3
$tRNA^{Leu\ (UUA)}$	22.7	28.2	29.6	22.5	52.3
$tRNA^{Ile}$	26.4	23.6	26.4	23.6	52.8
$tRNA^{Gln}$	23.9	26.8	35.2	14.1	59.1
$tRNA^{Met}$	20.3	31.9	30.4	17.4	50.7
$tRNA^{Trp}$	22.2	22.2	33.3	22.2	55.5
$tRNA^{Ala}$	29.4	25	35.3	10.3	64.7
$tRNA^{Asn}$	19.2	19.2	30.1	32.9	49.3
$tRNA^{Cys}$	24.2	28.8	31.8	15.2	56
$tRNA^{Tyr}$	21.4	31.4	30	17.1	51.4
$tRNA^{Ser\ (UCA)}$	23.9	29.6	25.4	21.1	49.3
$tRNA^{Asp}$	28.2	21.1	35.2	15.5	63.4
$tRNA^{Lys}$	24	22.7	36	17.3	60
$tRNA^{Gly}$	29.6	21.1	33.8	15.5	63.4
$tRNA^{Arg}$	24.3	27.1	28.6	20	52.9
$tRNA^{His}$	30.4	21.7	34.8	13	65.2
$tRNA^{Ser\ (AGC)}$	26.5	17.6	39.7	16.2	66.2
$tRNA^{Leu\ (CUA)}$	27.4	17.8	34.2	20.5	61.6
$tRNA^{Glu}$	27.5	17.8	39.7	16.2	67.2
$tRNA^{Thr}$	25.4	26.8	25.4	22.5	50.8
$tRNA^{Pro}$	22.9	28.6	35.7	12.9	58.6
Overall tRNA gene	24.3	25.1	32.6	18.0	56.9
rRNA gene					
12S rRNA	19.1	25.9	34.8	20.1	53.9
16S rRNA	20.2	23.6	37.9	18.4	58.1
Overall rRNA gene	19.8	24.4	36.8	19.0	56.6
Control region	35.1	18.8	34.1	11.9	69.2
Overall genome	25.6	26.3	33.6	14.5	59.2

as the ATP8 gene. The ND6 is the only protein-coding gene found located on the L-strand of the *R. tornieri* mitogenome.

The one special feature of the *R. tornieri* mitogenome which differs itself from other Rasbora genus members is that the codon usage GTG is found in both COI and ND3 gene. The TAA stop codon was found in genes such as ND5, ND4L, ATP8, COI and ND1 whereas the TAG termination codon was discovered exclusively on ND6 gene, all the

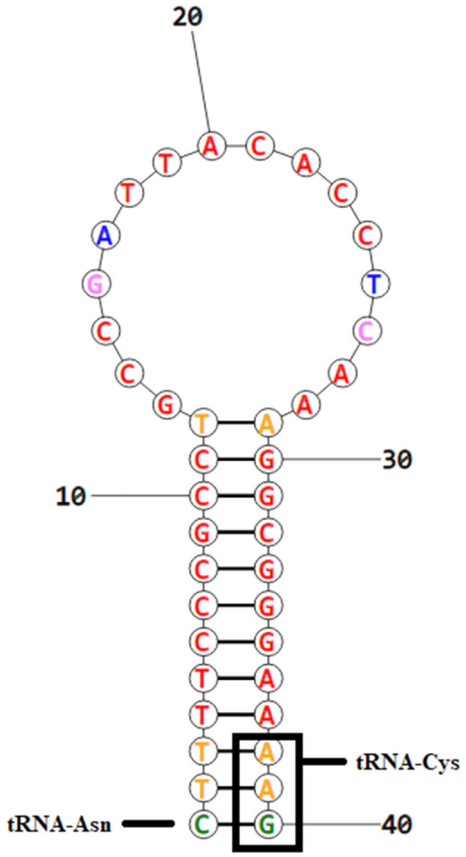

Figure 2. The predicted secondary structure of L-strand origin is positioned between $tRNA^{Asn}$ and $tRNA^{Cys}$ genes of *R. tornieri*. The part of the $tRNA^{Cys}$ gene sequence is in the black box.

other genes which are not mentioned earlier used incomplete stop codons, a condition that is commonly found in *R. myersi*, *R. steineri* and *R. pauciperforata* (Chang *et al*. 2012; Lim *et al*. 2019; Chung *et al*. 2020a) but differs from that of *R. sarawakensis*. The differences in termination codons is one typical phenomenon across vertebrate mitogenomes (Ojala *et al*. 1981).

Transfer and ribosomal RNA gene features

Around 9% of the tRNA genes make up the entire *R. tornieri* mitogenome, contributed by a total of 1484 bp of nucleotides. Only 0.8% of marginal nucleotide differences was observed between the T (24.3%) nucleotide and C (25.1%) nucleotide of overall tRNA genes. The trio, $tRNA^{Val}$, $tRNA^{Ser(UCA)}$ and $tRNA^{Asn}$ bottomed the list in terms of A + T content with only a percentage of 49.3% whereas $tRNA^{Glu}$ topped the list with 67.2% A + T content. Around 88.9% of the L-strand are occupied by members of this tRNA gene group. Majority of the anti-codons of tRNA genes in *R. torneiri* mitogenome are highly conserved across other Rasbora species except for the $tRNA^{Asp}$ where the GTG anti-codon was found instead of the usual GTC anti-codon found in other Rasboras like the *R. sarawakensis*,

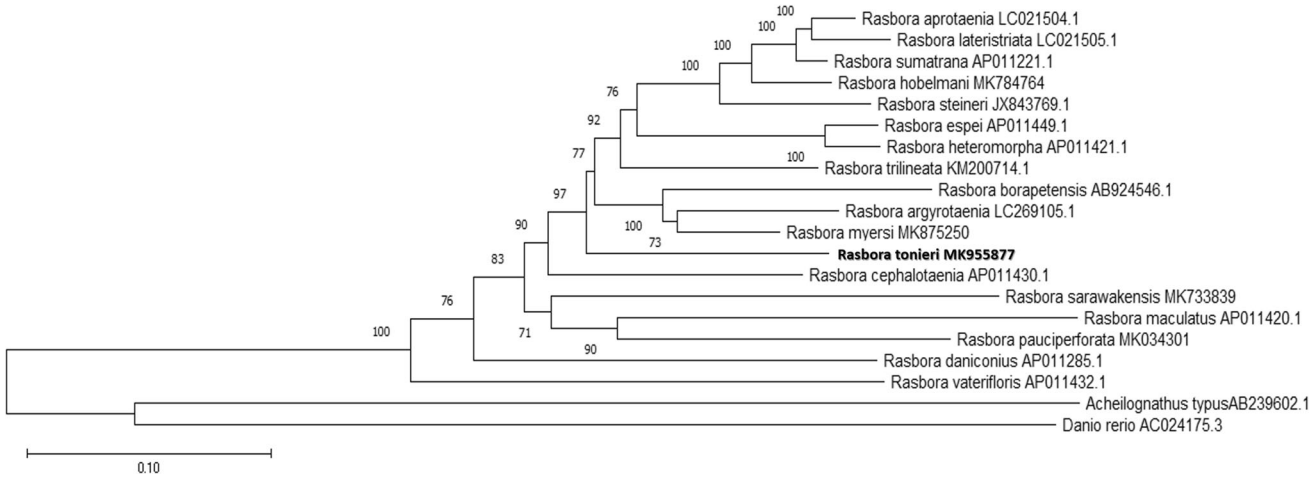

Figure 3. Phylogenetic tree of *R. tornieri* with other *Rasbora* members and outgroups, based on 12 protein-coding genes via the GTR+G ML analysis with bootstrap of 1000 replicates. The tree was rooted by the outgroups *Acheilognathus typus* and *Danio rerio.*

R. pauciperforata and *R. borapetensis* (Zhang *et al*. 2014; Chung *et al*. 2020a).

The rRNA genes make up to 15.9% of the entire *R. tornieri* mitogenome with 2644 nucleotides, entirely on the H-strand. The two rRNA genes, namely 12S rRNA and 16S rRNA were only 71 bp apart from each other, where they flank both ends of the $tRNA^{Val}$ gene. The 12S rRNA is 43.4% (732 bp) smaller than its 16S rRNA counterpart but their nucleotide compositions did not differ significantly, ranging from 1.1% (T) to 3.1% (A), contributing to the overall 56.6% A + T content which is slightly lower than that of the overall genome. When compared with that of the overall protein-coding genes and control region, the tRNA gene group are still lacking in terms of A + T content by 2.9% and 12.6%, respectively.

Noncoding region

There is a total of 13 intergenic spacers, a L-strand origin as well as a control region included in the noncoding gene group of *R. tornieri* mitogenome with their sizes ranging from 1 to 902 bp. The L-strand origin of *R. tornieri* is a 40 bp noncoding region sandwiched between $tRNA^{Asn}$ and $tRNA^{Cys}$. The secondary structure of the L-strand origin is made up of a loop conformation (from 16 nucleotides) and a secondary stem loop (from 12 complementary nucleotide pairs) (figure 2).

The largest member of this gene group is no qualms, the control region (D-loop) spanning a nucleotide size of 902 bp in the *R. tornieri* mitogenome. The smallest difference in the individual nucleotide compositions between overall genome and the control region is in A nucleotide with only 0.5% difference whereas the largest difference was found in the T nucleotide with 9.5%. Further, the central conserved sequence blocks (CSB-E, CSB-F and CSB-D), variable sequence blocks (CSB-2, CSB-3 and CSB-1) and all the terminal associated sequence (TAS) were identified within the control region of *R. tornieri* mitogenome.

Phylogeny

The maximum-likelihood phylogenetic family tree had revealed the evolutionary relationship of *R. tornieri* with the other 17 Rasbora genus members (figure 3). The *R. tornieri* was observed to diverge from the Rasbora basal clade where its evolutionary relationship with other Rasbora members like *R. myersi*, *R. steineri*, *R. trilineata* and *R. argyrotaenia* was resolved poorly as indicated by the low bootstrap value. A huge clade was observed across five Rasbora species, namely *R. hobelmani*, *R. lateristriata*, *R. steineri*, *R. aprotaenia* and *R. sumatrana* with bootstrap value of 100. Two smaller strong clades were discovered between *R. espei* and *R. heteromorpha* (bootstrap value of 100) across the *R. myersi*, *R. borapetensis* and *R. argyrotaenia* trio (bootstrap value of 100); as well as between *R. pauciperforata* and *R. maculatus* (bootstrap value of 90), similar observation was reported previously by Kusuma and Kumazawa (2015), Kusuma *et al*. (2017) and Lim *et al*. (2019), respectively.

In conclusion, the complete mitogenome sequences of *R. tornieri* has been fully unravelled following the detailed gene level characterizations. Further, the study had uncovered the unpublished evolutionary relationship of *R. tornieri* within the *Rasbora* genus along with other 17 Rasbora members. This information will provide insights for evolutionary and population studies in future.

Acknowledgement

This research was supported by Fundamental Research Grant Scheme with Grant Number F07/FRGS/1872/2019 awarded to H. H. Chung by The Ministry of Education Malaysia.

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Corresponding editor: H. A. Ranganath