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The complete mitochondrial genome of *Gyrodactylus pseudorasborae* (Platyhelminthes: Monogenea) with a phylogeny of Gyrodactylidae parasites

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Abstract

Background *Gyrodactylus* von Nordmann, 1832, a genus of viviparous parasites within the family Gyrodactylidae, contains one of the largest nominal species in the world. *Gyrodactylus pseudorasborae* Ondračková, Seifertová & Tkachenko, 2023 widely distributed in Europe and China, although its mitochondrial genome remains unclear. This study aims to sequence the mitogenome of *G.pseudorasborae* and clarify its phylogenetic relationship within the Gyrodactylidea. The mitochondrial genome of *G. pseudorasborae* was amplified in six parts from a single parasite, sequenced using primer walking, annotated and analyzed using bioinformatic tools.

Results The mitochondrial genome of *G. pseudorasborae* is 14,189 bp in length, containing 12 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (rRNAs), and two major non-coding regions (NCR: NC1 and NC2). The overall A + T content of the mitogenome is 73.1%, a medium content compared with all reported mitochondrial genomes of monogeneans. The mitogenome of *G. pseudorasborae* presents a clear bias in nucleotide composition with a negative AT skew and a positive GC skew except for NCR. All tRNAs have the typical cloverleaf secondary structure except for $tRNA^{Cys}$, $tRNA^{Ser1}$, and $tRNA^{Ser2}$, which lack the dihydrouridine (DHU) arm. Furthermore, one repetitive non-coding region of 32 bp repeats occurred in the NC1 region with poly-T stretch, stem-loop structure, and TAn motif. The gene order is identical to the mitochondrial genomes reported from other *Gyrodactylus* species except *Gyrodactylus nyanzae* Paperna, 1973 and *Gyrodactylus* sp. FZ-2021. Phylogenetic analyses show that *G. pseudorasborae* and *Gyrodactylus parvae* You, Easy & Cone, 2008 cluster together with high nodal support based on 12 PCGs sequences and amino acid sequences, Gyrodactylidae forms independent monophyletic clade within Gyrodactylidea.

Conclusion Both the mitochondrial genome and phylogenetic analyses support *G. pseudorasborae* is a member of the genus *Gyrodactylus* and Gyrodactylidae forms an independent monophyletic clade within Gyrodactylidea.

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Furthermore, the mitochondrial genome of *G. pseudorasborae* is the shortest in the Gyrodactylidea species compared with size differences in NCR.

Keywords *Gyrodactylus pseudorasborae*, Mitochondrial genome, Gyrodactylidea, *Pseudorasbora parva*, China

Background

The topmouth gudgeon, *Pseudorasbora parva* (Temminck & Schlegel, 1846) is widely distributed in East Asia [1, 2] and is introduced the Yunnan-Guizhou Plateau [3], Qinghai-Tibet Plateau [4], and European countries [5]. It become an ideal species for studying parasite diversity for the fish, such as *Gyrodactylus parvae* You, Easy & Cone, 2008 [6] and *Gyrodactylus pseudorasborae* Ondračková, Seifertová & Tkachenko [7, 8]. *Gyrodactylus pseudorasborae* has been shown to have been co-introduced to Europe with human-translocated fish in Europe [7, 8]. However, the parasite *G. parvae* from the same host never occurred in Europe with limited regions [7], therefore, a larger range sampling in Europe and other non-natives is needed to confirm its existence or non-existence in future research.

Recently, nine *Gyrodactylus* species were recorded on *P. parva* in the world, including *Gyrodactylus cyprini* Diarova, 1964, *Gyrodactylus gobioninum* Gusev, 1955, *Gyrodactylus kathariner* Malmberg, 1964, *G. parvae*, *Gyrodactylus prostae* Ergens, 1963, *G. pseudorasborae* and three unnamed novel species [6–10], however, there was mere mitogenome of *G. parvae* obtained in China [11].

Gyrodactylidea is a hyperdiverse monogenean order with more than 600 described species [12–14]. However, merely thirteen mitogenomes of Gyrodactylidea parasites were sequenced, including twelve species of Gyrodactylidae and one species of Oogyrodactylidae [11, 15–21]. These studies found the genes arrangement and tandem repeat units of Gyrodactylidea species, including parasites *Gyrodactylus nyanzae* Paperna, 1973 and *Macrogyrodactylus karibae* Douëllou & Chishawa, 1995 in Africa [21], *Gyrodactylus* sp. FZ-2021 (unpublished) and *Paragyrodactylus variegatus* You, King, Ye & Cone, 2014 in Asia [17], and *Aglaiogyrodactylus forficulatus* Kritsky, Vianna & Boeger, 2007 in South America [20]. Recently, the entire mitogenome of *G. parvae* was sequenced from *P. parva*, while the *G. parvae* is only distributed in China [11]. The A + T content of whole mitogenomes and its elements within the order Gyrodactylidea ranged from 62.5% (*Gyrodactylus salaris* Malmberg, 1957) [15] to 80.1% (*G. nyanzae*) [21], while the mitogenome of *Paratetraonchoides inermis* Bychowsky, Gussev & Nagibina, 1965 was the highest A + T content (82.6%) among the monogenean [22]. Although there were morphological and phylogenetic researches based on nuclear gene about this parasite *G. pseudorasborae* from the same host in China and Europe [7, 8], its mitogenome is unreported, so, it is interesting to compare their differences in morphological characteristics and test gene arrangement and tandem repeat units of mitogenomes in *G. pseudorasborae*. Therefore, we aimed to sequence and obtain the entire mitogenome of *G. pseudorasborae* using the Sanger sequencing method. The findings will compare the interspecific difference between *G. pseudorasborae* and *G. parvae* from the same host in different environments and enrich molecular database within the Gyrodactylidea. Furthermore, we analyzed the phylogenetic relationships among twelve complete mitogenomes in Gyrodactylidea to elucidate the evolutionary relationship of Gyrodactylidae.

Methods

Ethical approval

The study was approved by the Animal Care and Use Committee of Guilin Medical University (Accession number: GLMC202103005). In addition, the method of euthanasia on fish and parasites was performed in accordance with the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals (2020). Ethanol have been used as euthanasia methods for some fish species and aquatic invertebrates with low concentrations in AVMA guidelines for euthanasia. Each fish was rapidly euthanized by a blow to the head and immersed in 30% ethanol within 30 min to render the fish unconscious. The topmouth gudgeon *P. parva* is listed as Least Concern in the International Union for Conservation of Nature (IUCN) red list status (https://www.iucnredlist.org) and not endangered or protected in China. Fish sampling was permitted by the local level authority in scientific research.

Sample collection and DNA extraction

In this study, the gyrodactylid was collected on *P. parva* from the Huixian karst wetland in Guilin urban South China (25.06° N, 110.11° E). Five specimens of *G. pseudorasborae* were deposited at the Museum of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan City, Hubei Province, China (http://www.ihb.ac.cn/) under the voucher numbers SM-GP 202301-05 based on previous study [8]. Previous studies, based on the morphometric identification and sequencing of internal transcribed spacer (ITS) rDNA, that the *G. pseudorasborae* if the only species found on the host in the studied area [8]. Total genomic DNA was extracted from the body of one specimen following the operation instructions of the TIANamp Micro DNA Kit (Tiangen Biotech, Beijing,

China). The degrees of concentration and purity of DNA were evaluated using a Thermo Scientific NanoDrop 2000. The sequence of total mitogenomic DNA of *G. pseudorasborae* was obtained using long PCR and primer walking method of DNA sequencing according to a previous study [11]. Finally, the specimens of host and *G. pseudorasborae* were stored in 95% ethanol, respectively.

Mitochondrial genome assembly and annotation

Contiguous sequences were assembled using Staden Package v1.7.0 [23]. The MITOS model was used to predict the locations of protein-coding genes (PCGs) and rRNA genes (RNAs) [24]. The initiation and termination codons were identified using an open reading frame (ORF) finder and Blastn of NCBI, according to their alignment with other related species. Moreover, tRNAs and their structures were identified by combining the results of ARWEN [25] and MITOS [24]. The tRNAs, which were not detected, were obtained by comparing the sequence to *Gyrodactylus* species [20]. OGDRAW (version 1.3.1) was applied to generate a graphical diagram of the complete mitogenome [26]. The base composition, codon usage, and relative synonymous codon usage (RSCU) values were calculated with MEGA v7.0.21 [27]. The formula of AT skew = (A – T)/(A + T) and GC skew = (G – C)/(G + C) was used to analyze the base composition [28]. Sliding window analysis was carried out in DnaSP v5 [29]: a sliding window of 200 bp and a step size of 20 bp were applied to estimate the nucleotide divergence Pi between the mitogenomes of *G. pseudorasborae* and other Gyrodactylidea species. Evolutionary rate analyses among the 12 PCGs of the mitogenomes of *G. pseudorasborae* and other Gyrodactylidea species were conducted using KaKs_Calculator v. 2.0 with the MA method [30]. Tandem repeats in non-coding regions were identified with Tandem Repeats Finder [31], and their secondary structures were predicted by UNAFold software [32]. The genomic synteny of the 13 mitogenomes (Table 1) was analyzed with Mauve v2.4.0 [33]. Finally, the complete mitogenome sequence was deposited to the GenBank database with accession number PP808686.

Phylogenetic analyses

Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) methods based on 12 PCGs sequences and amino acid sequences of the complete mitogenome of *G. pseudorasborae* and eleven other Gyrodactylidea species from hosts of Cyprinidae, Nemacheilidae, and Cobitidae within Cypriniformes, Salmonidae within Salmoniformes, Cichlidae

Table 1 The information of protein-coding genes (PCGs) sequences of twelve Gyrodactylidea species used for phylogenetic analyses

Species	GenBank number	Host	Host family	Host order	Locality	Parasitic site	Reference
Gyrodactylus pseudorasborae	PP808686	*Pseudorasbora parva*	Cyprinidae	Cypriniformes	Huixian wetland, Guilin City, China	Fins	This study
Gyrodactylus derjavinoides	EU293891	*Oncorhynchus mykiss*	Salmonidae	Salmoniformes	Danish freshwater rainbow trout farm (Refsgaard, Jutland)	Laboratory culture	[16]
Gyrodactylus nyanzae	MG970256	*Oreochromis niloticus*	Cichlidae	Cichliformes	Democratic Republic of the Congo: INERA station Kipopo	Gills	[21]
Gyrodactylus gurleyi	KU659806	*Carassius auratus*	Cyprinidae	Cypriniformes	Wuhan, China	Fins and gills	[18]
Gyrodactylus parvae	KP780992	*Pseudorasbora parva*	Cyprinidae	Cypriniformes	Cold-water streams in Lantian County Lantian Counties, China	Skin	[11]
Gyrodactylus brachymystacis	KT277549	*Oncorhynchus mykiss*	Salmonidae	Salmoniformes	Fish farm located in Niubeiliang Nature Reserve Lantian Counties, China	Skin	[11]
Gyrodactylus kobayashii	KU057942	*Carassius auratus*	Cyprinidae	Cypriniformes	Wuhan, China	Fins and gills	[19]
Gyrodactylus salaris	DQ988931	*Salmo salar*	Salmonidae	Salmoniformes	River Signal-dalselva, North Norway	undescribed	[15]
Gyrodactylus sp. FY-2015	KP780991	*Homatula variegata*	Nemacheilidae	Cypriniformes	Qinling Mountain region of central China	Skin and fins	unpublished
Gyrodactylus sp. FZ-2021	MW464989	*Misgurnus anguillicaudatus*	Cobitidae	Cypriniformes	Lancang River in Jinghong city, China	Gills	unpublished
Paragyrodactylus variegatus	KM067269	*Homatula variegata*	Nemacheilidae	Cypriniformes	Qinling Mountain region of central China	Skin and fins	[17]
Aglaiogyrodactylus forficulatus	KU679421	*Kronichthys lacerta*	Loricariidae	Siluriformes	Brazil: Parana, Rio Morato, Microbacia Garaquecaba basin	External surfaces	[20]

within Cichliformes, and Loricariidae within Siluriformes [15–21] (Table 1). The oviparous flatworm *A. forficulatus* (KU679421) from loricarid host *Kronichthys lacerta* Nichols, 1919 was used as an outgroup [20]. The best-fit models were determined using the Bayesian information criterion (BIC) in jModelTest v2.1.10 [34] and ProtTest v3.4.2 [35]. The GTR + I + G and MtArt + G + F were chosen as the best-fitting models for BI and ML analyses. BI analysis was performed using MrBayes v3.2.7 [36], and one set of four chains was allowed to run simultaneously for 50,000,000 generations. ML analysis was conducted using RAxML v8.2.10 [37], with bootstrap analysis performed with 1,000 replicates.

Results

Features of the mitogenome and gene arrangement

The circular mitogenome of *G. pseudorasborae* (GenBank accession number PP808686) was 14,189 bp in length (Fig. 1) and contained 12 protein-coding genes (PCGs, lacking *Atp8*), 22 tRNA genes (tRNAs), two rRNA genes (rRNAs), and two major non-coding regions (NCR: NC1 and NC2) (Table 2; Fig. 1). All the genes were transcribed from the same strand. The base composition of the whole mitogenome was 41.3% T, 10.9% C, 31.8% A, and 16.1% G (Table 3). The overall A + T content was up to 73.1%, which was approximate to the *G. parvae* from the same host (Table 3 and Table S1). The AT skew values of *G.*

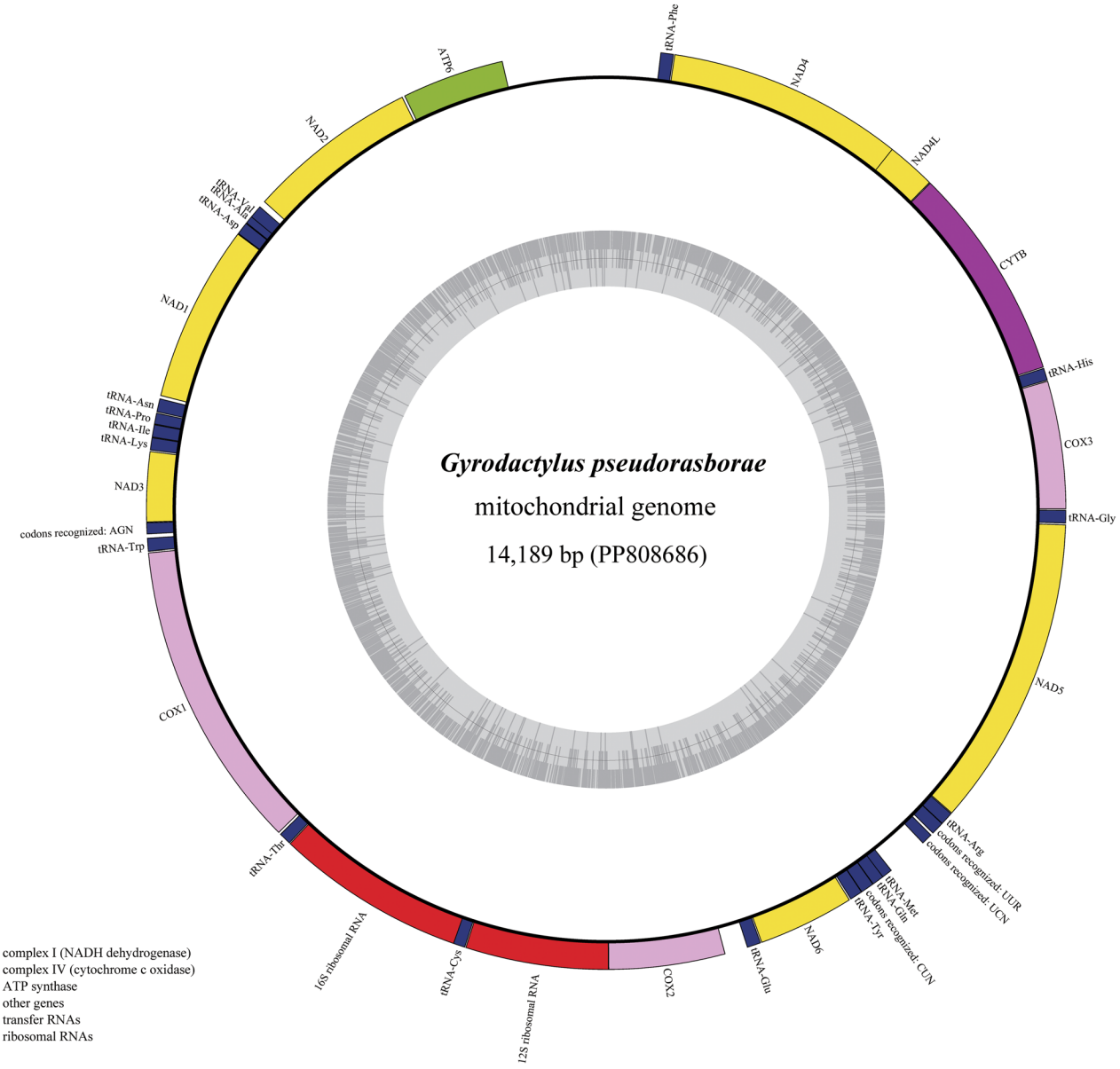

Fig. 1 Graphical map of the mitogenome of *Gyrodactylus pseudorasbora*. The yellow, pink, and green segments represent protein-coding genes (PCGs); the blue and red segments represent tRNA genes (tRNAs) and rRNA genes (RNAs), respectively. Genes for tRNAs are abbreviated using three letters

Table 2 Organization of the mitochondrial genome of *Gyrodactylus pseudorasbora*

Gene/reginon	Position		Size	Intergenic nucleotides	Codon		Anti-codon
	From	End			Start	Stop	
Cox3	1	639	639		ATG	TAA	
tRNAHis	642	705	64	2			GTG
Cytb	709	1782	1074	3	ATG	TAG	
Nad4l	1784	2032	249	1	ATG	TAA	
Nad4	2005	3210	1206	-28	ATG	TAA	
tRNAPhe	3214	3279	66	3			GAA
NC1	3280	4060	781				
Atp6	4061	4573	513		ATG	TAA	
Nad2	4584	5441	858	10	ATG	TAA	
tRNAVal	5446	5510	65	4			TAC
tRNAAla	5510	5577	68	-1			TGC
tRNAAsp	5578	5643	66				GTC
Nad1	5644	6531	888		ATG	TAG	
tRNAAsn	6545	6613	69	13			GTT
tRNAPro	6617	6674	58	3			TGG
tRNAIle	6675	6739	65				GAT
tRNALys	6740	6803	64				CTT
Nad3	6808	7158	351	4	ATG	TAG	
tRNASer(AGN)(S1)	7159	7217	59				GCT
tRNATrp	7235	7301	65	17			TCA
Cox1	7306	8853	1548	4	ATG	TAA	
tRNAThr	8863	8926	64	8			TGT
rrnL	8929	9899	971	2			
tRNACys	9876	9937	62	-24			GCA
rrnS	9940	10 654	715	2			
Cox2	10 653	11 234	582		ATG	TAA	
tRNAGlu	11 346	11 416	71	111			TTC
Nad6	11 420	11 902	483	3	ATG	TAA	
tRNATyr	11 908	11 973	66	5			GTA
tRNALeu(CUN)(L1)	11 972	12 041	70	-2			TAG
tRNAGln	12 040	12 102	63	-2			TTG
tRNAMet	12 106	12 169	64	3			CTT
NC2	12 170	12 356	187				
tRNASer(UCN)(S2)	12 357	12 415	59				TGA
tRNALeu(UUR)(L2)	12 426	12 493	68	10			TAA
tRNAArg	12 494	12 560	67				TCG
Nad5	12 561	14 114	1554		ATG	TAG	
tRNAGly	14 120	14 186	67	5			TCC
	14 187	14 189		3			

pseudorasborae were negative except for the NC1 and NC2 regions, while the GC skew values of *G. pseudorasborae* were positive except for the NC2 region (Table 3). *G. pseudorasborae* exhibit obvious bases T and G bias. The Gyrodactylidea species exhibit obvious bases T and G bias in whole mitogenomes and PCGs (Tables S1-S2).

The gene order of *G. pseudorasborae* was identical to that of *G. parvae* from the same host in different environments and matched exactly with that of most other *Gyrodactylus* species (Table 2; Fig. 2a), and the rearrangements of tRNAs (red rectangle) and PCGs (blue rectangle) occurred in five gyrodactylids, including *G. nyanzae*, *Gyrodactylus* sp. FZ-2021, *P. variegatus*, *M. karibae*, and *A. forficulatus* (Fig. 2a). Gene synteny analysis revealed three homologous regions (A-C) in 13 Gyrodactylidea mitogenomes (Fig. 2b). The relative positions and sizes of regions *G. pseudorasborae* and the other 10 species were highly conserved, while *M. karibae*, and *A. forficulatus* were rearrangements from one to two homologous regions (A and B) corresponding one to two PCGs genes, the mitogenome sequence of *Gyrodactylus* sp. FZ-2021 was considerably longer than that of other mitogenomes, with the largest non-coding region (Fig. 2b).

Table 3 Nucleotide composition and skewness comparison of different elements of the mitochondrial genome of *Gyrodactylus pseudorasbora*

Regions	Size (bp)	T(U) (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)	AT skew	GC skew
Complete genome	14189	41.3	10.9	31.8	16.1	73.1	26.9	-0.131	0.192
PCGs	9945	43.4	10.6	29.6	16.4	73.0	27.0	-0.189	0.213
1st codon position	3315	37.3	10.5	32.4	19.8	69.6	30.4	-0.070	0.307
2st codon position	3315	48.7	12.6	19.7	19.1	68.4	31.6	-0.424	0.205
3st codon position	3315	44.3	8.7	36.7	10.2	81.1	18.9	-0.093	0.076
Atp6	513	44.4	13.8	26.1	15.6	70.6	29.4	-0.260	0.060
Cox1	1548	41.9	12.1	28.0	18.0	69.9	30.1	-0.200	0.197
Cox2	582	40.4	12.0	29.7	17.9	70.1	29.9	-0.152	0.195
Cox3	639	43.5	11.1	28.6	16.7	72.1	27.9	-0.206	0.202
Cytb	1074	40.8	11.9	29.3	18.0	70.1	29.9	-0.163	0.202
Nad1	888	45.3	10.7	26.7	17.3	72.0	28.0	-0.258	0.237
Nad2	858	44.8	7.2	33.4	14.6	78.2	21.8	-0.145	0.337
Nad3	351	49.9	7.7	27.1	15.4	76.9	23.1	-0.296	0.333
Nad4	1206	43.5	9.1	31.3	16.0	74.9	25.1	-0.163	0.274
Nad4L	249	49.0	8.8	30.9	11.2	79.9	20.1	-0.226	0.120
Nad5	1554	42.5	10.9	31.1	15.5	73.6	26.4	-0.155	0.173
Nad6	483	45.8	8.9	30.8	14.5	76.6	23.4	-0.195	0.239
rrnL	971	39.6	10.9	35.5	13.9	75.2	24.8	-0.055	0.120
rrnS	715	36.8	12.2	35.1	15.9	71.9	28.1	-0.023	0.134
rRNAs	1686	38.4	11.4	35.3	14.8	73.8	26.2	-0.042	0.127
tRNAs	1432	37.5	10.8	35.3	16.3	72.8	27.2	-0.030	0.203
NC1	781	32.5	13.3	37.1	17.0	69.7	30.3	0.066	0.122
NC2	187	30.5	13.9	43.3	12.3	73.8	26.2	0.174	-0.061

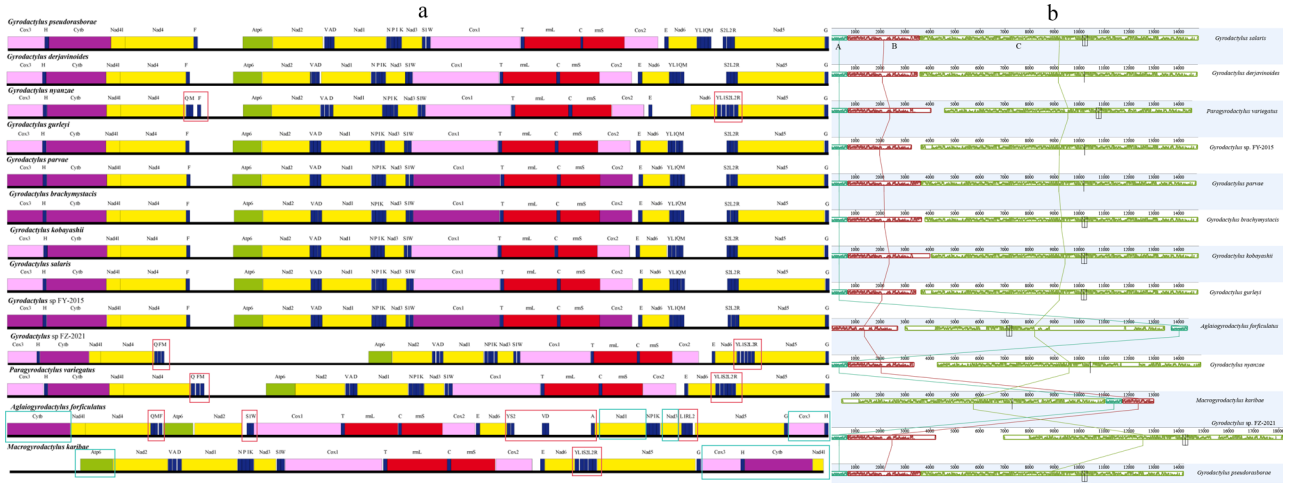

Fig. 2 Gene orders (**a**) and gene synteny analysis (**b**) are shown among 13 mitogenomes of Gyrodactylidea species. The rearrangements of tRNAs (red rectangle) and PCGs (blue rectangle) occurred in five gyrodactylids, including *G. nyanzae*, *Gyrodactylus* sp. FZ-2021, *P. variegatus*, *M. karibae*, and *A. forficulatus* (**a**). Gene synteny analysis revealed three homologous regions (A-C) (**b**)

The length of the 22 tRNA genes varied from 58 bp ($tRNA^{Pro}$) to 71 bp ($tRNA^{Glu}$) (Table 2). All tRNAs could fold into the conventional secondary structure, except for three unorthodox tRNAs, $tRNA^{Ser(AGN)}$, $tRNA^{Ser(UCN)}$, and $tRNA^{Cys}$ lacked the dihydrouridine (DHU) arms (Fig. 3). The sizes of *rrnL* and *rrnS* were 971 bp with 75.2% and 715 bp with 71.9% A + T content, respectively (Table 3), they were separated by $tRNA^{Cys}$ (Table 2). Seven *Gyrodactylus* species (*G. pseudorasborae*, *G. derjavinoides*, *G. parvae*, *G. brachymystacis*, *G. salaris*, *Gyrodactylus* sp. FY-2015, and *P. variegatus*) had positive AT skew values for the rRNAs and NCR, respectively (Tables S3-S4).

All PCGs used the canonical ATG as the start codon and the canonical stop codons TAA and TAG (Table 2). The length of 12 PCGs was 9,945 bp, with 73.0% A + T

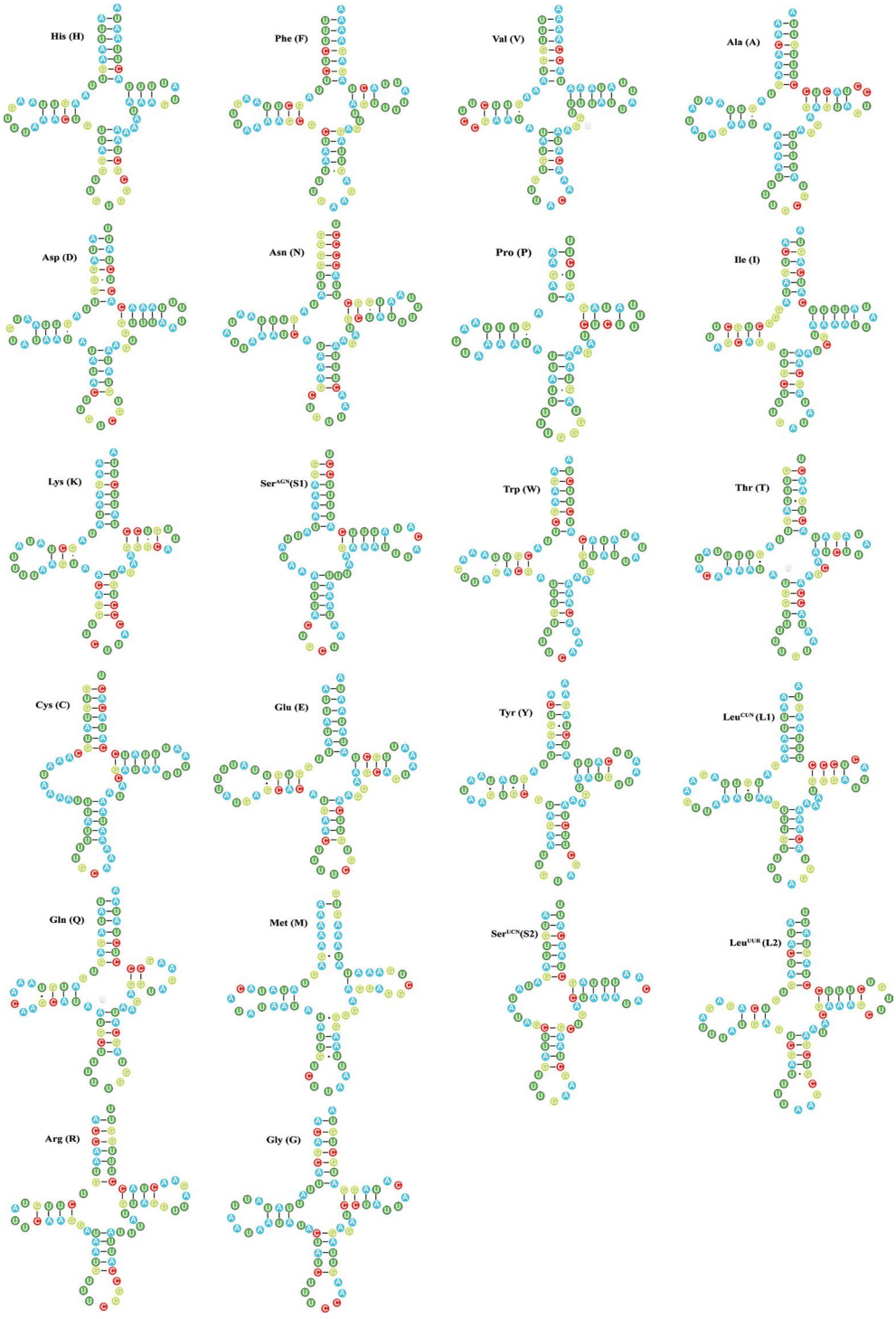

Fig. 3 Secondary structures of tRNAs in the mitochondrial genome of *Gyrodactylus pseudorasbora*

content (Table 3). Furthermore, the incomplete stop codon TA was observed in gyrodactylid *G. brachymystacis* (Table S5). The codon usage and RSCU values are summarized (Fig. 4). The most RSCU values of amino acids in the PCGs of *G. pseudorasborae* are as follows: Serine (Ser) (8.01), Leucine (Leu) (6.00%), Threonine (Thr) (4.01), the lowest in Methionine (Met) and Lysine (Lys) (1.00) (Fig. 4). In addition, there were five cases of overlapping regions within the mitogenome. The overlap between *Nad4L* and *Nad4* was common in metazoan mtDNAs. There were 21 cases of short intergenic areas ranging from 1 bp to 111 bp (Table 2).

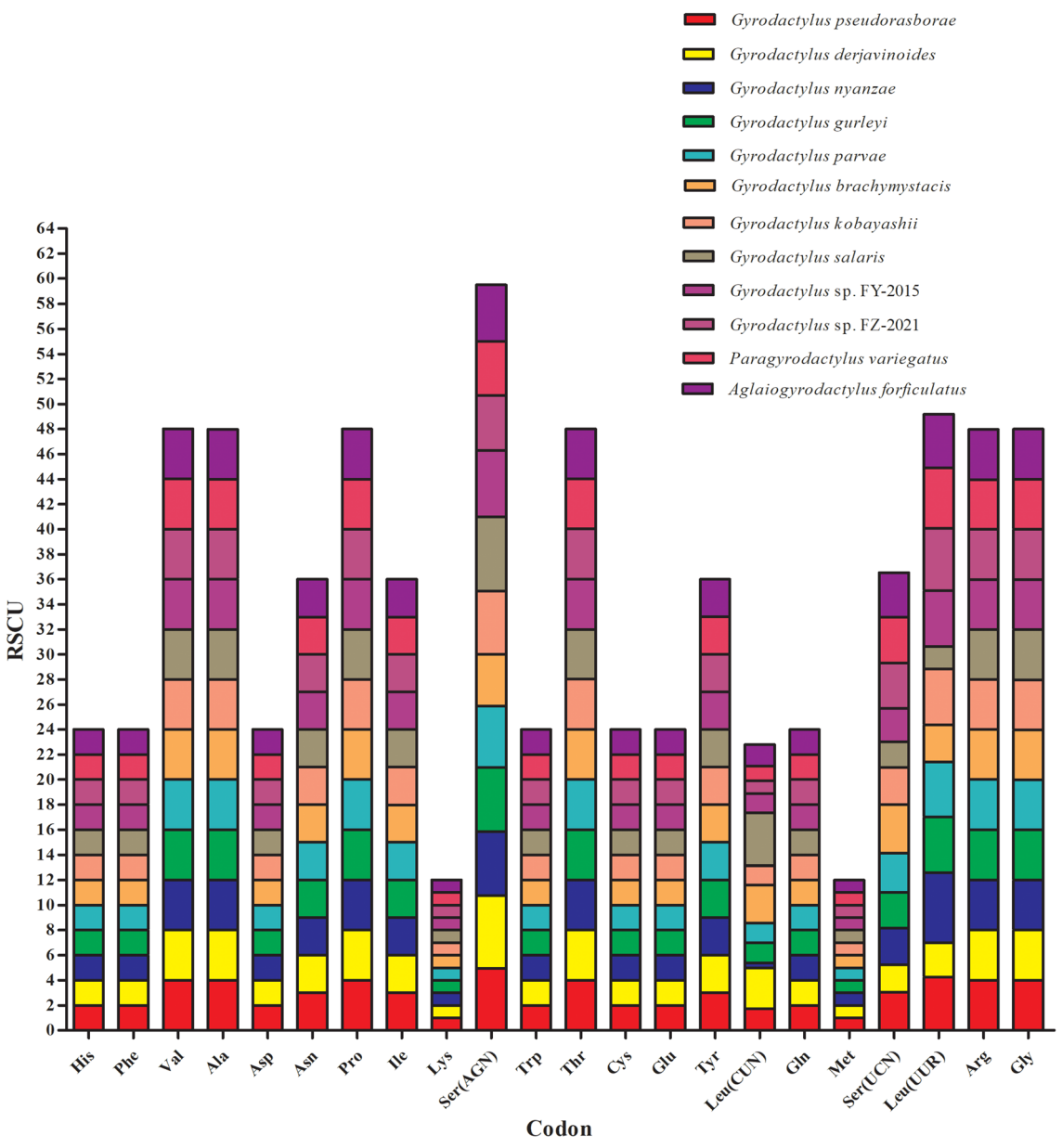

Fig. 4 Codon and relative synonymous codon usage (RSCU) values of the protein-coding genes (PCGs) in the mitochondrial genomes of Gyrodactylidea are shown in different colors. Grey-colored codon indicates that codon is not present in the genome. A total of 3311, 3279, 3319, 3311, 3309, 3225, 3310, 3274, 3304, 3322, 3329, and 3307 codons for *Gyrodactylus pseudorasborae*, *Gyrodactylus derjavinoides*, *Gyrodactylus nyanzae*, *Gyrodactylus gurleyi*, *Gyrodactylus parvae*, *Gyrodactylus brachymystacis*, *Gyrodactylus kobayashii*, *Gyrodactylus salaris*, *Gyrodactylus* sp. FY-2015, *Gyrodactylus* sp. FZ-2021, *Paragyrodactylus variegatus*, and *Aglaiogyrodactylus forficulatus* were analyzed, respectively, excluding stop codons

Non-coding regions

The two long non-coding regions, NC1 (between $tRNA^{Phe}$ and *Atp6*) and NC2 (between $tRNA^{Met}$ and $tRNA^{Ser(UCN)}$) were 781 bp and 187 bp long (Table 2; Fig. 1), with 69.7% and 73.8% A + T content, respectively (Table 3). There was one repetitive region with a consensus pattern (32 bp) in the NC1 region, and it could form a stem-loop structure with poly-T stretch, stem-loop structure, and TAn motif (Fig. 5).

Nucleotide diversity and evolutionary rate

The plot of sequence variation ratio exhibited highly variable nucleotide diversity, with Pi values for the 200 bp windows ranging from 0.174 to 0.503 (Fig. 6a). Genes with comparatively high sequence variability were *Nad2* (0.408), *Nad5* (0.390), *Nad4* (0.374), and *Nad6* (0.360), whereas *Cox1* (0.229) and *Cytb* (0.251) had a comparatively low sequence variability. The non-synonymous/synonymous (dN/dS) ratio analysis showed that all PCGs

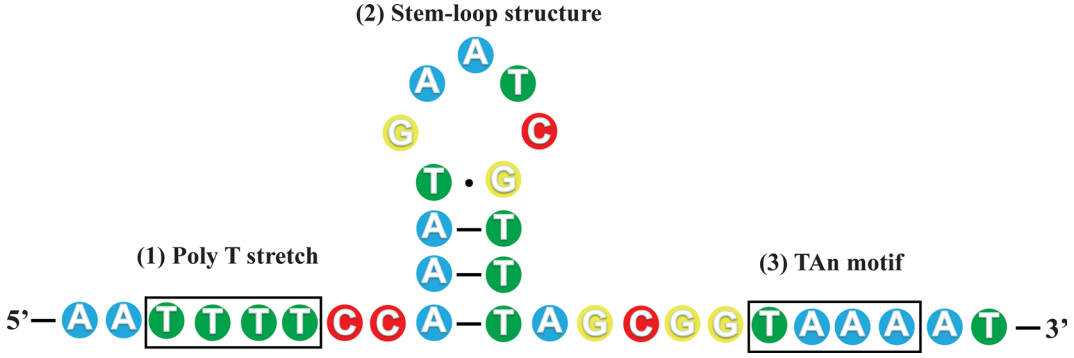

Fig. 5 Stem-loop structural elements of one consensus repeat pattern for the mitochondrial major non-coding region 1 (NC1) of *Gyrodactylus pseudorasbora*

are under purifying selection and evolving under comparatively relaxed mutational constraints (Fig. 6b).

Phylogeny based on mitogenomes

BI and ML analyses based on the PCGs sequences and amino acid sequences produced phylogenetic trees (Fig. 7a and b) with identical branch topologies and only minor differences in Bayesian posterior probabilities and bootstrap values for some nodes (Fig. 7). The results support the phylogenetic affinity of *G. pseudorasborae* and *G. parvae* with maximum support (both BI = 1, both ML = 100) and the Gyrodactylidae formed an independent clade. Furthermore, parasites grouped in different clusters were associated to fish host families Cyprinidae, Nemacheilidae, and Cobitidae within Cypriniformes, Salmonidae within Salmoniformes, Cichlidae within Cichliformes.

Discussion

Mitogenome characteristics and evolution

The complete mitochondrial genome of *G. pseudorasborae* contains all 36 common flatworm mitochondrial genes [38], including twelve protein-encoding genes (PCGs; *Atp8* is absent), two rRNA genes (rRNAs), and 22 tRNA genes (tRNAs). The mitogenome of *G. pseudorasborae* exhibits a moderate A + T content (73.1%) in the whole mitogenomes and its elements within the order Gyrodactylidea, ranging from 62.5% (*G. salaris*) [15] to 80.1% (*G. nyanzae*) [21] in the whole mitogenome (Tables S1-S4), while the mitogenome of *Paratetraonchoides inermis* Bychowsky, Gussev & Nagibina, 1965 was the highest A + T content (82.6%) among the monogenean [22]. In the monogenean, TTG was also used as start codon for *Tetraonchus monenteron* (Wagener, 1857) Diesing, 1858 [39], *G. nyanzae* and *M. karibae* [21], and *P. variegatus* [17] (Table S5). It was also proposed as an alternative start codon for flatworm mitogenomes [40]. The second position of the PCGs exhibited a strong preference for base T of *G. pseudorasborae* and was consistent with the Gyrodactylidea species without *Gyrodactylus* sp. FZ-2021 (MW464989) and *P. variegatus* [17]. This is also the argument that codons for hydrophobic amino acid residues, which are functionally preferred for conformational stability of mitochondrial proteins, mostly have T in the second codon position [41]. The third position of the PCGs exhibited a strong preference for T of *Gyrodactylus* sp. FZ-2021 (MW464989) and *P. variegatus* [17], the third position affects translation speed and the folding of the nascent protein [42, 43].

Present study confirms the same trends of the RSCU values with the highest in Serine (Ser), Leucine (Leu), and Threonine (Thr), the lowest in Methionine (Met) and Lysine (Lys) were also observed within the order Gyrodactylidea (Fig. 4) [11, 15–22]. The dN/dS values for all mitochondrial PCGs fall well below 1 (Fig. 6b) between Gyrodactylidea species, indicating purifying selection and confirming earlier mitogenomic work on monogeneans [11, 15–21, 39]. Overall purifying selection acting on mitochondrial genes has also been observed in a range of monogeneans [11, 15–21, 39]. All tRNAs could fold into the conventional secondary structure, except for three unorthodox tRNAs, $tRNA^{Ser(AGN)}$, $tRNA^{Ser(UCN)}$, and $tRNA^{Cys}$ lacked the DHU arms within the Order Gyrodactylidea [11, 15–21], and $tRNA^{Pro}$ lacked the TΨC arm in *G. salaris* [15]. In addition, $tRNA^{Ser(AGN)}$ and $tRNA^{Cys}$ also lacked DHU arms in *P. inermis* (Tetraonchoididae) [22]. The arrangement of *rrnL* and *rrnS* was the same as in the monopisthocotyleans [15–22, 39].

Similar repetitive region and stem-loop structures formed by their tandem repeats were also found in NCR located in different intergenic regions within Gyrodactylidea, including parasites *G. gurleyi* [18], *Gyrodactylus* sp. FZ-2021 [unpublished], *G. nyanzae* [21], *P. variegatus* [17], and *(A) forficulatus* [20]. The presence of tandem repeats forming stable stem-loop structure is usually associated with replication origin in mitochondria [17, 18, 20, 21, 44, 45], which suggests that NCR may be involved in the initiation of replication of the mitogenome of *G. pseudorasborae*. Furthermore, the longest NCR is located between $tRNA^{Met}$ and *Atp6* genes in the

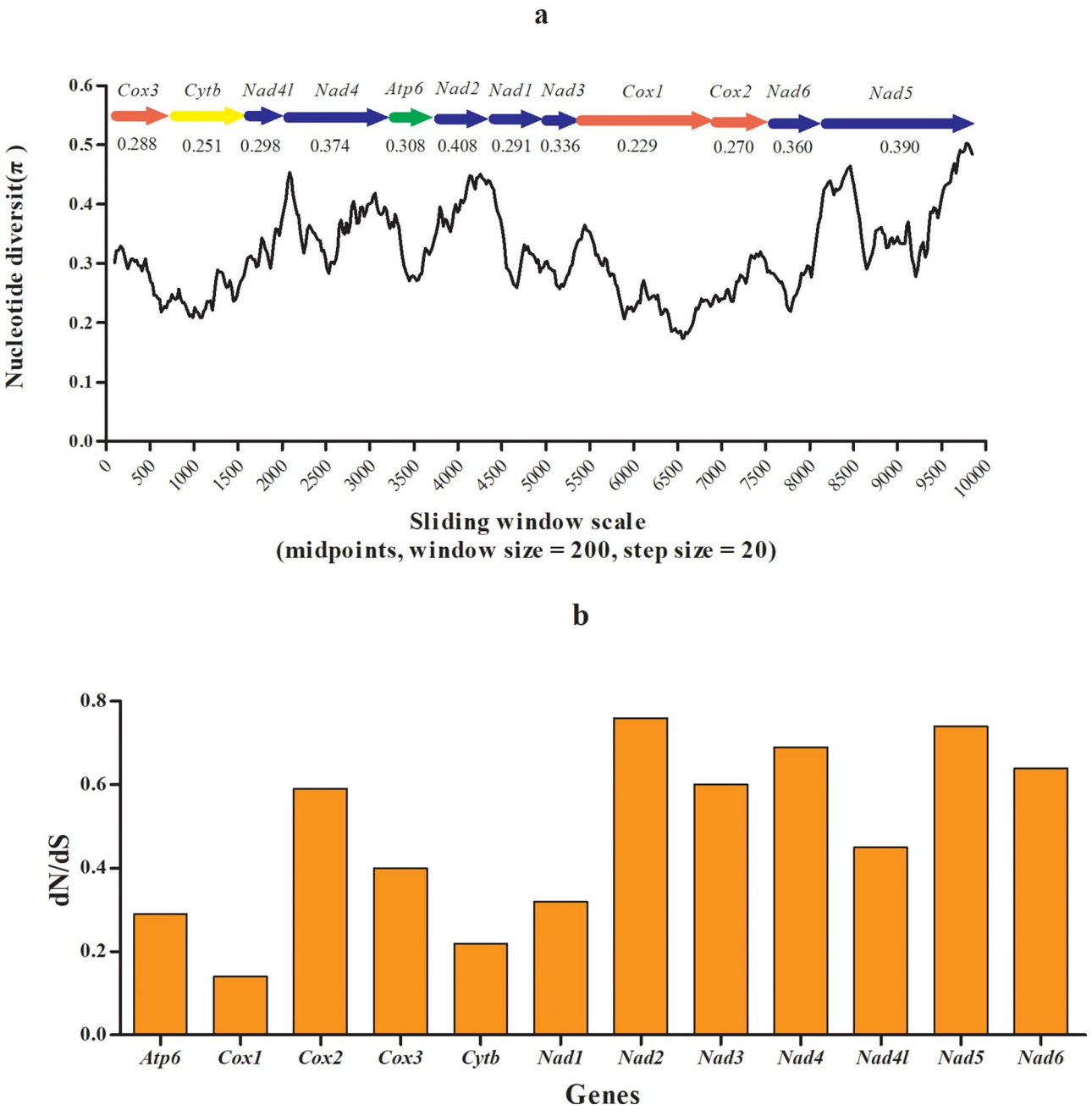

Fig. 6 Sliding window and evolutionary rate analyses of the PCGs of mitogenomes among twelve Gyrodactylidea species. (**a**) Sliding window analysis was conducted on concatenated alignments of 12 PCGs. The black line represents the value of nucleotide diversity (window size = 200 bp, step size = 20 bp, with the value inserted at its mid-point). Gene names, boundaries, and average nucleotide diversity values are indicated above the graph. (**b**) Ratios of non-synonymous (dN) to synonymous (dS) substitution rates calculated for protein-coding genes (PCGs)

mitogenome of *Gyrodactylus* sp. FZ-2021 (MW464989). The overlap between *Nad4L* and *Nad4* was common in metazoan mtDNAs [46], except *Benedenia hoshinai* Ogawa, 1984 and *(B) seriolae* (Yamaguti, 1934) Meserve, 1938 [47].

The sliding window and evolutionary rate analyses produced similar results (Fig. 6). They consistently showed that the *Cox1* gene is the slowest-evolving and least variable gene and is often used in population genetics studies of monogeneans [11, 17, 21, 39, 48–50]. On the contrary, the faster-evolving *Nad2* and *Nad5* genes may be better molecular markers for species and population-level genetics studies of the Gyrodactylidea, as rapidly evolving genes are capable of providing higher resolution for phylogenetically closely related taxa/populations [11, 17, 22, 39, 51].

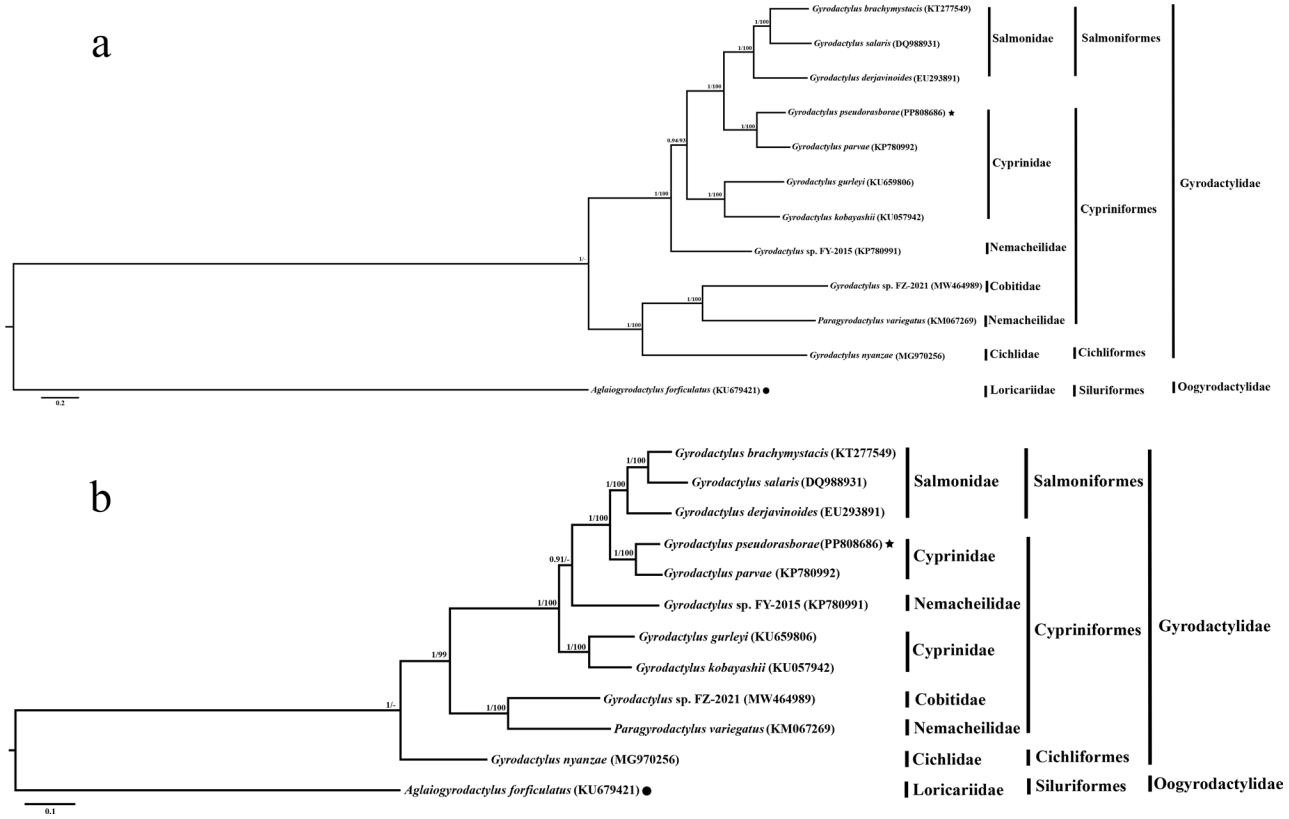

Fig. 7 Phylogenetic relationships between *Gyrodactylus pseudorasborae* and related species of Gyrodactylidea based on the concatenated protein-coding genes (PCGs) sequences (**a**) and amino acid sequences (**b**) were analyzed with Bayesian inference (BI) and maximum likelihood (ML) methods. The GTR + I + G and MtArt + G + F were selected as the best-fitting models according to the BIC criterion for BI and ML analyses. The scale bar represents the estimated number of substitutions per site. Node numbers represent the Bayesian posterior probabilities and bootstrap values (BI/ML), respectively. The study gyrodactylid and outgroup species are highlighted by a pentagram and a circle, respectively

Gene arrangement

Although the result shows that the gene order (Fig. 2a) and gene synteny (Fig. 2b) of *G. pseudorasborae* were the same with most Gyrodactylidea species (Fig. 2), extensive rearrangements of tRNAs exhibited in Gyrodactylidea (Fig. 2a) and Tetraonchidea species, including *Gyrodactylus* sp. FZ-2021, *G. nyanzae*, *M. karibae* with incomplete mitogenome, *P. variegatus*, *A. forficulatus*, *P. inermis*, and *T. monenteron* [17, 20–22, 39]. Minor rearrangements of PCGs exhibited in Gyrodactylidea (Fig. 2), including viviparous *M. karibae* in Africa [21, 52] and oviparous *A. forficulatus* [20, 53]. The unique gene orders (Q-M-F) and (Y-L1-S2-L2) are found in other monogeneans, including *Gyrodactylus* sp. FZ-2021, *G. nyanzae*, *P. variegatus*, *A. forficulatus*, *P. inermis*, and *T. monenteron* [17, 20–22, 39]. The most extensive rearrangements of tRNAs and PCGs occurred in an oviparous *A. forficulatus* (Fig. 2a) [20]. The mitogenome sequence of *Gyrodactylus* sp. FZ-2021 was considerably longer than that of other mitogenomes, with the largest NCR (Fig. 2b). The gene order of *G. pseudorasborae* was identical to *G. parvae* from the same host in different habitats with slight morphological characteristics in the size of the marginal hook and shape of the dorsal bar [8, 11]. At the same time, the main difference was in the length and base composition of the NCR of the mitogenome [11].

The rearranged may be evolving at a relatively fast rate in the Gyrodactylidea. It also confirmed the inference that the evolution of mitogenomic gene order arrangements is discontinuous in monogeneans [11, 15–22, 39, 54], as there is an increasing number of orders in non-conserved genes in this group of animals. Although gene order can be used for inferring phylogenetic relationships [55, 56], this discontinuity in rearrangements of gene order in monogeneans might produce misleading evolutionary signals and cause long-branch attraction artifacts. Thus, the gene order may not be suitable for inferring the monogenean phylogeny. Future studies should establish whether these arrangements are synapomorphic for the Gyrodactylidea and sequence the gene order of more genera species in the Gyrodactylidea to compare rearrangements.

Phylogeny

The phylogenetic results support the *G. pseudorasborae* is close relationship to *G. parvae* from same host in

China and belongs to the genus *Gyrodactylus* (Fig. 7) [7, 8]. *Gyrodactylus pseudorasborae* is originally an Asian parasite [8] and anthropogenically introduced to Europe [7], however, the parasite *G. parvae* never occurred in Europe with limited regions [7], therefore, a larger range sampling in Europe and other non-natives is needed to confirm its existence or non-existence in future research. Phylogenetic analyses also indicated that Gyrodactylidae formed an independent monophyletic clade within Gyrodactylidea, which is consistent with previous studies based on several mitogenomes, single mitochondrial genes, and nuclear genes [20, 21, 39, 57], and the genus *Gyrodactylus* is not monophyletic [22, 36, 58]. Furthermore, parasites grouped in different clusters are associated with fish host families Cyprinidae, Nemacheilidae, Cobitidae, Salmonidae, and Cichlidae (Fig. 7; Table 1), showing high host specificity with limited species [59, 60]. It would be of benefit to motivate researchers to porduce more mitogenome data as a base to infer Gyrodactylidea phylogeny.

Conclusions

In comparison to the Gyrodactylidea mitogenomes, *G. pseudorasborae* possessed a medium A + T content, the same gene order as most *Gyrodactylus* species, $tRNA^{Ser(AGN)}$, $tRNA^{Ser(UCN)}$, and $tRNA^{Cys}$ without the DHU arms, and one tandem repeat sequence within the NC1 formed a stem-loop structure. The gene order of *G. pseudorasborae* was identical to *G. parvae* from the same host in different habitats except for slight morphological characteristics in the size of the marginal hook and shape of the dorsal bar. At the same time, the main difference was in the length and base composition of the NCR of the mitogenome. Phylogenetic analyses based on PCGs consistently support the *G. pseudorasborae* is a member of the genus *Gyrodactylus* and Gyrodactylidae forms an independent monophyletic clade within Gyrodactylidea. As the limited resolution of our datasets, it is necessary to sequence more molecular data, such as mitogenomes, transcriptomes, and multiple nuclear genes, to resolve the deep phylogeny of Gyrodactylidea.

Abbreviations

PCGs	protein-coding genes
rRNAs	ribosomal RNA genes
tRNAs	transfer RNA genes
NCR	Non-coding regions
ITS	internal transcribed spacer
BI	Bayesian inference
ML	maximum likelihood
BIC	Bayesian information criterion
RSCU	relative synonymous codon usage
bp	Base pairs
DHU	dihydrouridine
AVMA	American Veterinary Medical Association
IUCN	International Union for Conservation of Nature
ORF	open reading frame
MA	model averaging
Ser	Serine
Leu	Leucine
Thr	Threonine
Met	Methionine
Lys	Lysine

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions

TC designed the study. XYZ. YLa. YLb. YYC. YJL. and MWS performed the research. YLa and YLb analyzed the data. TC wrote the manuscript.

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Data availability

The data and materials are available in the manuscript and its supplementary material. The complete mitochondrial genome of Gyrodactylus pseudorasborae was deposited to the GenBank of NCBI (https://www.ncbi.nlm.nih.gov) and obtained the accession number (GenBank accession no. PP808686).

Declarations

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee of Guilin Medical University (Accession number: GLMC202103005). We consent for the publication of identifying images that compromise anonymity.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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