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MOLECULAR CHARACTERISATION OF *PARADIPLOZOON BARBI* (REICHENBACH-KLINE, 1951) FROM RIVER PERAK RESERVOIRS, MALAYSIA

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ABSTRACT
The paper presents the first molecular analysis of Paradiplozoon barbi from the gills of Cyprinid fishes in Bersia, Chenderoh and Temengor Reservoirs, Perak. The ITS2 rDNA gene of P. barbi was amplified using PCR to obtain the ITS2 rDNA sequence. The 778bp (ITS2 rDNA) sequence were phylogenetically compared with related species in GenBank database by maximum likelihood method(ML) and pairwise comparisons using Kimura-2 parameter model. The ITS2 rDNA of P. barbi showed no identical sequence with closely related species. The new sequence was deposited in GenBank with accession number MN688771.

Keywords: Monogenean DNA, Perak River, Malaysia

INTRODUCTION

Paradiplozoon species are monogeneans that belong to the Diplozoidea family which are common ectoparasites on the gills of Cyprinid freshwater fishes in Africa, Europe, and Asia (Khotenovsky,1985; Pugachev *et al*, 2010). These ectoparasites have a direct life cycle, with an oncomiracidia which is free-swimming, diporpa (larval stage) and adult. In the adult stage, two diporpae (larvae) fuse permanently into sexually matured adult in an X-shaped (Smyth & Halton, 1983). The digestive organ and vitellaria are in the anterior position of the organism, while in the posterior part of the body, the male and female reproductive system, end of the gut and clamps are situated. The adult has one pair of small central hooks and four pairs of clamps located on the ventral side of the opisthaptors of each monogenean. Oncomiracidia used the central hooks for attachment to the fish host, while larvae and adult used clamps as attachment apparatus (Khotenovsky, 1985).

Morphological structures and metrical differences in the size of the clamps, body and central hooks are mostly used for the identification of diplozoids species (Matejusova *et al*, 2004). These structures vary widely within the monogeneans species and depend on the size of the fish host and stage of the *Paradiplozoon* species development (Matejusova *et al*, 2001; Mine and Avenant-Olderwage, 2012), making identification of diplozoids to species level difficult.

More recently, molecular markers have been developed based on species-specific variable in the ribosomal DNA region for accurate identification of diplozoids species (Simkova *et al.*, 2006). The second internal transcribed spacer of ribosomal DNA (ITS-2brDNA) have been used for the precise identification of *Paradiplozoon* species (Jirsova *et al*, 2018).

River Perak is in Perak State, Malaysia, with a total length of 427 km approximately, making it the second-longest river in the Peninsular Malaysia (Hashim *et al.*, 2012). The river water source is Perak-Kelantan-Thailand mountainous border. Its water catchment areas include the Belum-Temengor Forest Reserve (Salam *et al*., 2019). The river flow from a watershed north to south along the state borders of Perak with Thailand, the state of Kelantan, and Kedah. The river is situated at a lowland with primary and secondary forest types of river systems.

The depth of the river is about 0.9 meter to 2.4 meter, it is 100 m at the widest point. The major towns along the river are Grik and Kuala Kangsar (Salam *et al*., 2019). Temengor reservoir is located on the upper most of the river follows by Bersia reservoir; located at about 20km downstream of Temengor reservoir. Next, Kenering reservoir; located at about 45 km downstream to Bersia reservoir. Chenderoh reservoir is the last reservoir (Salam *et al*., 2019).

However, there is no published molecular characterisation of monogeneans in Perak Reservoirs and none of the *Paradiplozoon* species genome have been described. The present research reports the results of molecular characterization of the second internal transcribed spacer (ITS2) of ribosomal DNA of *P. barbi* using Polymerase chain reaction (PCR) and nucleotide sequencing.

MATERIALS AND METHODS

Fish sampling and identification

Live freshwater fishes were sampled from fishermen and from fish farm from April 2017 to March 2019 from Chenderoh Reservoir (5.02º N, 100.97 º E), Bersia Reservoir (5.02 º N, 101.22 º E) and Temengor Reservoir (5.55 º N, 101.34 º E) (Figure 1) along Perak River.

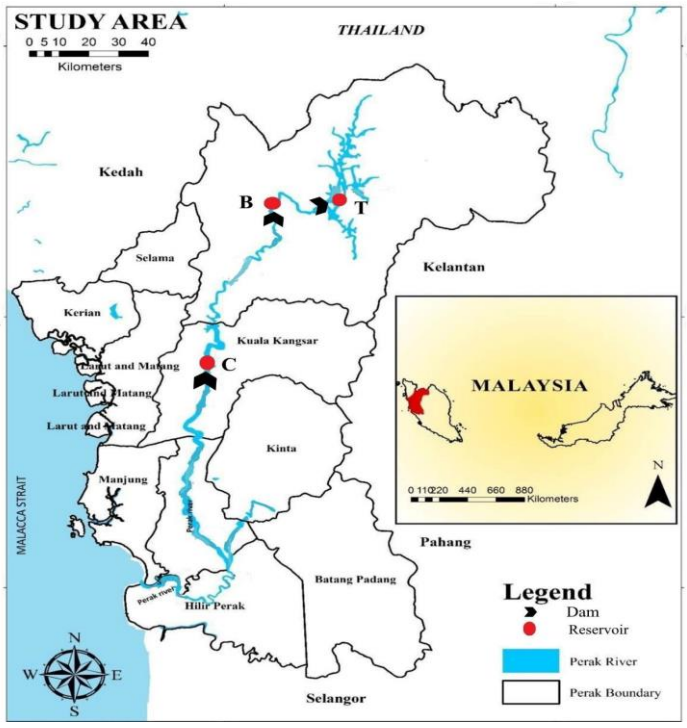

Figure 1: Map showing the study area along Perak River: Temengor Reservoir (T), Bersia Reservoir (B) and Chenderoh Reservoir (C). Source: Salam *et al*., (2019).

The fish were transported in battery-power aerated cooler with the local water to the laboratory. The fish were identified using keys prepared by Froese and Pauly (2019).

Parasites collection and identification

Fish were euthanized, and the gills were dissected. Diplozoids were isolated from the gills and preserved in 70 % ethanol. The parasites were mounted in 5 % Sodium Dodecyl Sulphate (Wong *et al*, 2006) and molten glycerine-jelly on a microscope slide, covered with cover slip and sealed with colourless nail vanish. *Paradiplozoon barbi* was morphologically identified (Figure 2) under the microscope after Gussev (1985) and Khotenovsky (1985).

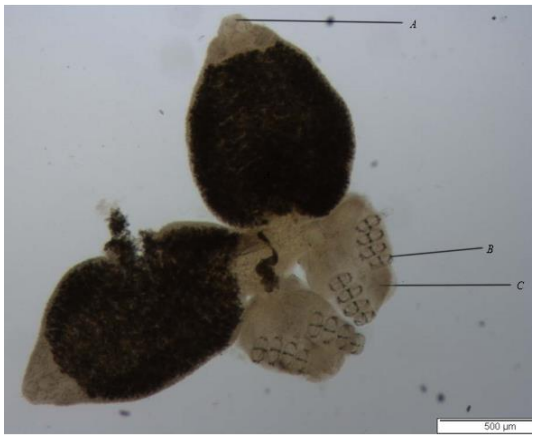

Figure 2: *Paradiplozoon barbi,* magnification 50×. A: oral sucker; B: clamp; C:haptor.

Molecular characterization of *P. barbi*

Individual parasite of *P. barbi* was put in a microcentrifuge tube and the genomic DNA was extracted using DNeasy blood and tissues kit (Qiagen, USA) following the manufacturer's instructions. Five microlitres of extracted DNA was used as a template in the PCR reaction to amplify the second internal transcribed spacer (ITS2 rDNA) of *P. barbi* using primers D(5'-GGCTYRYGGNGTCGATGAAGA CCAG -3') and B1(5'GCCGGATCCGAATCCTGGTTAGT TTCTTTTCCT-3') (Bachellerie & Qu, 1993). The PCR reaction (50 µL) was carried out by combining 7.5 µL of 0.3M of each PCR primer, 25 µL of Taq PCR Master Mix (Qiagen), 5 µL of genomic DNA. The reaction was processed in PCR Machine (BIO-RAD, USA) as summarized in table 1.

Table 1: Condition for PCR amplification of ITS2 rDNA gene of *P. barbi*

Step	Temperature (^{o}C)	Time (min)	cycle
Initial denaturation	94	3	1
Denaturation	94	1	
Annealing	55	1	35
Extension	72	1	
Final extension	72	10	1
Hold	4		

The PCR was visualized on 1 % Gel Red (Bioline, UK) stained agarose gel. The amplified PCR product was purified using QIA quick PCR purification kit (Qiagen, USA) according to manufacturer's instructions. The purified PCR product sequenced using the same primers used in PCR amplification in both direction by MyTACG Bioscience Enterprise, Malaysia.

Nucleotide BLAST (blastn) search was conducted to identify the *P. barbi* in GenBank database and similar diplozoids sequences submitted to the National Center for Biotechnology Information (USA) nucleotide sequence database. Clustal W (BioEdit Software Version 7.2.5) (Hall, 1999) was used for sequence alignment. MEGA 7.0 Software (Kimura *et al*, 2015) was also used in the analysis of maximum likelihood (ML) and pairwise genetic distance. The new sequence was submitted to NCBI database.

RESULTS

The agarose gel electrophoresis of the PCR product length of *P. barbi* was 778bp (Figure 3) as referred to molecular marker 100bp DNA ladder (Thomas Scientific).

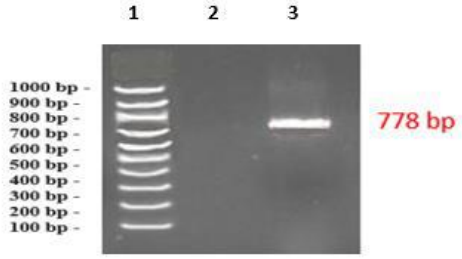

Figure 3: Gel electrophoresis of ITS2 rDNA partial sequences. (1- molecular marker 100 bp plus DNA ladder, 2- negative control, 3- *Paradiplozoon. barbi*)

According to nucleotide BLAST searched, the ITS2 rDNA sequence of *P. barbi* showed 86.64 % - 87.72 % resemblance with closely related species of *Paradiplozoon* in the GenBank database. The 778bp sequence of *P. barbi* was deposited in the NCBI GenBank database with accession number MN688771. Species of *Paradiplozoon* that were closely related to *P. barbi* used in this research are shown in Table 2.

Table 2. Reference sequences from GenBank used in this research, their country of origin and accession numbers. *Dactylogyrus macracanthus* is the outgroup sequence

Species	**Country**	**GenBank accession number**
Paradiplozoon barbi	Malaysia	MN688771
Paradiplozoon hemiculteri	China	DQ098892
Paradiplozoon jiangxiensis	China	DQ098885
Paradiplozoon opsariichthydis	China	DQ098890
Paradiplozoon parabramisi	China	DQ098886
Paradiplozoon parapeleci	China	DQ098882
Dactylogyrus macracanthus	China	KJ605447

Table 3 shows the result of the pairwise comparison in the ITS2 rDNA sequences among the five nucleotide sequences and *P. barbi* using Kimura-2 parameter model. *Paradiplozoon barbi* have the lowest divergence value and highest BLAST score.

Table 3: Pairwise genetic distance (using Kimura 2-parameter model in % difference) for the ITS2 rDNA sequence of *P. barbi* and *Paradiplozoon* species.

Species	**1**	**2**	**3**	**4**	**5**	**6**
Paradiplozoon barbi						
Paradiplozoon hemiculteri	0.131					
Paradiplozoon opsariichthydis	0.131	0.004				
Paradiplozoon parabramisi	0.131	0.004	0			
Paradiplozoon parapeleci	0.131	0.005	0.001	0.003		
Paradiplozoon jiangxiensis	0.133	0.005	0.001	0.001	0.003	

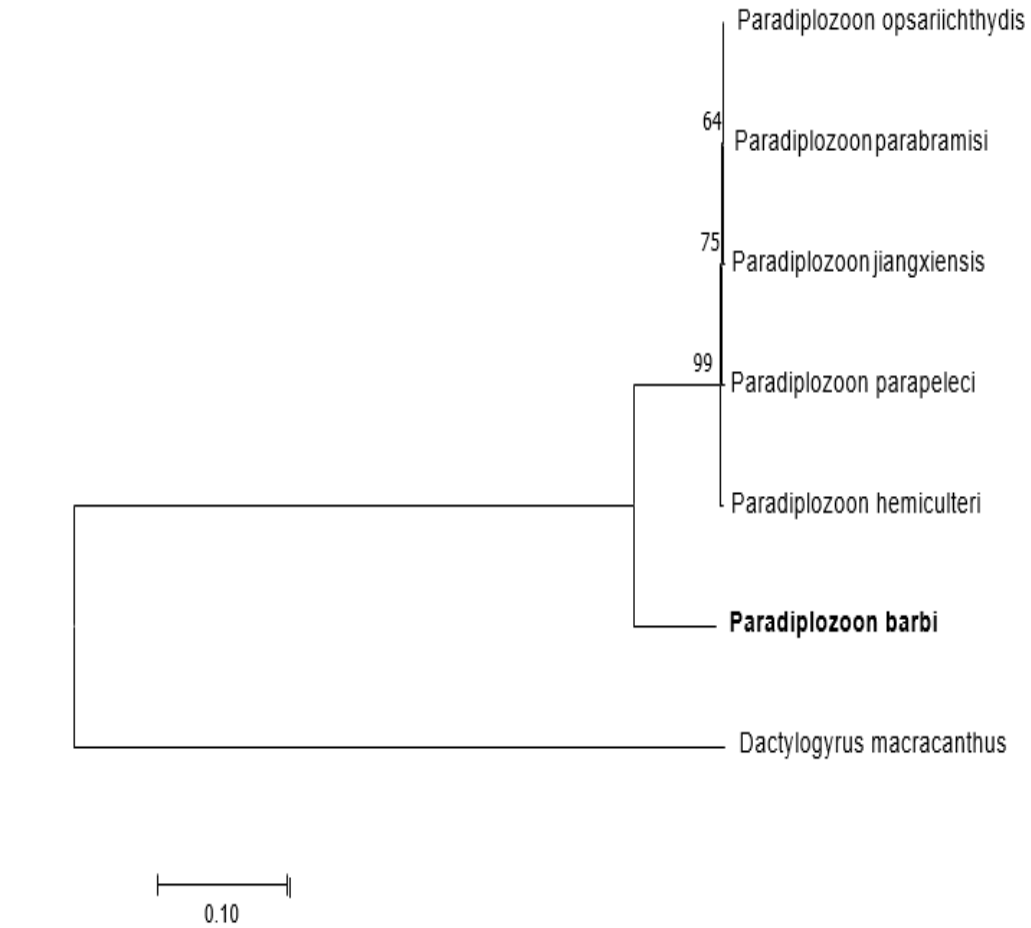

Figure 4: Phylogenetic tree generated by maximum likelihood method (1000 bootstrap) analysis based on ITS2 rDNA of selected different species of *Paradiplozoon*, with *Dactylogyrus macrcanthus* as the outgroup. The scale bar indicates the proportion of sites changing along each branch. Newly sequenced species in this research is in bold.

Figure 4 shows the phylogenetic reconstruction of ITS-2 sequences with *Dactylogyrus macranthus* as an outgroup by maximum likelihood method. The result shows that *P. barbi* cluster 99 % (1000 bootstrap) with *P. parapeleci* in the ML tree. The ITS2 rDNA sequences of *P.barbi* and 5 *Paradiplozoon* species were aligned as shown in Figure 5, and these showed clear differences among the nucleotide sequences of the diplozoids.


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P. barbi                TGT-AA-TATTGGTGAATTGC-AACTGCCTTGAACATCGACTTCTTGAACGCTAATTGCG  57
P. hemiculteri          ...G..T....-.........A.............................A.......  93
P. opsariichthydis      ...G..T....-.........A.............................A.......  93
P. parabramisi          ...G..T....-.........A.............................A.......  93
P. parapeleci           ...G..T....-.........A.............................A.......  93
P. jiangxiensis         ...G..T....-.........A.............................A.......  93

P. barbi                ACATTAGGCCATGCCTGATGCCACGCCTATCCGAGAGTCGGCATTTTATTAATCGCGACG  117
P. hemiculteri          G.....................................AG.............  153
P. opsariichthydis      G.....................................AG.............  153
P. parabramisi          G.....................................AG.............  153
P. parapeleci           G.....................................AG.............  153
P. jiangxiensis         G.....................................AG.............  153

P. barbi                CTGAATTGGTCGTGGATTGGTTTGTTGTCAGCCGTCGTGTTTGTCTTTTCAACGTGTTGC  177
P. hemiculteri          ...........C.................A.............A......AC.....  213
P. opsariichthydis      ...........C.................A.............A......AC.....  213
P. parabramisi          ...........C.................A.............A......AC.....  213
P. parapeleci           ...........C.................A.............A......AC.....  213
P. jiangxiensis         ...........C.................A.............A......AC.....  213

P. barbi                AAGTTGATAAGACGGCGGAGTATGTGACGCTTACCTAATTTATTGGAGAGTACGTGTATA  237
P. hemiculteri          .T..................G..............CC...........G........  273
P. opsariichthydis      .T..................G..............CC...........G........  273
P. parabramisi          .T..................G..............CC...........G........  273
P. parapeleci           .T..................G..............CC...........G........  273
P. jiangxiensis         .T..................G..............CC...........G........  273
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Figure 5: Multiple alignment of the ITS2 rDNA sequences of *Paradiplozoon* species: nucleotide identical to *P. barbi* showed by dots, dashes are inferred insertion- deletion events.

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P. barbi                TGTGCATACTCTTCCAGTAGCATTTCTCGTCGGTCGAGCTTTTACACATCTGTATTGAAT 297
P. hemiculteri          .C......G...G.......C.........A.....................AA.... 333
P. opsariichthydis      .C......G...G.......C.........A.....................C.A..... 333
P. parabramisi          .C......G...G.......C.........A.....................C.A..... 333
P. parapeleci           .C......G...G.......C.........A.....................C.A..... 333
P. jiangxiensis         .C......G...G.......C.........A.....................C.A..... 333

P. barbi                TTTCTTGATAGCCTTAGATGTGGATAGCGGCTATGACGTTGACTGTGCGTGTTTTCTGGT 357
P. hemiculteri          .............A..............A.T.............................. 393
P. opsariichthydis      .............G..............A.T.............................. 393
P. parabramisi          .............G..............A.T.............................. 393
P. parapeleci           .............G..............A.T.............................. 393
P. jiangxiensis         .............G....C.........A.T.............................. 393

P. barbi                TGGCATTCACTAGCAAGGATGTGGGTCTTTTAATATATCGTTTATGACTGCAATGCGTTG 417
P. hemiculteri          ..A..........TT...T......-........T.....................T.A.. 452
P. opsariichthydis      ..A..........TT...T......-........T.....................T.A.. 452
P. parabramisi          ..A..........TT...T......-........T.....................T.A.. 452
P. parapeleci           ..A..........TT...T......-........T.....................T.A.. 452
P. jiangxiensis         ..A..........TT...T......-........T.....................T.A.. 452

P. barbi                CTTTCCATAAATGCGGTTCTCCTGTCGCCTATCGGCTTCTGGTTGTGCAGTCGACCAGTG 477
P. hemiculteri          ....T.....................-......AA...C.T.............-........ 510
P. opsariichthydis      ....T.....................-......AA...C.T.............-........ 510
P. parabramisi          ....T.....................-......AA...C.T.............-........ 510
P. parapeleci           ....T.....................-......AA...C.T.............-........ 510
P. jiangxiensis         ....T.....................-......AA...C.T.............-........ 510

P. barbi                TTTCTTAAGTGCGTGCACTTGCGTGCCAGGTCGCAGCATAATGTGTTTGCGATGCCTTTT 537
P. hemiculteri          ...........T..............A.........................C..TG.C. 570
P. opsariichthydi       ...........T..............A.........................C..TG.C. 570
P. parabramisi          ...........T..............A.........................C..TG.C. 570
P. parapeleci           ...........T..............A.........................C..TG.C. 570
P. jiangxiensis         ...........T..............A.........................C..TG.C. 570

P. barbi                GCGCTTGTGTGATGCACTTGATTTGTGTTGCTGCCGCACGCCCAAGAGTGCATTTGTAAT 597
P. hemiculteri          AA........A..T..T..A..C.............TTG....G..G...A......... 630
P. opsariichthydis      AA........A..T..T..A..C.............TTG....G..G...A......... 630
P. parabramisi          AA........A..T..T..A..C.............TTG....G..G...A......... 630
P. parapeleci           AA........A..T..T..A..C.............TTG....G..G...A......... 630
P. jiangxiensis         AA........A..T..T..A..C.............TTG....G..G...A......... 630
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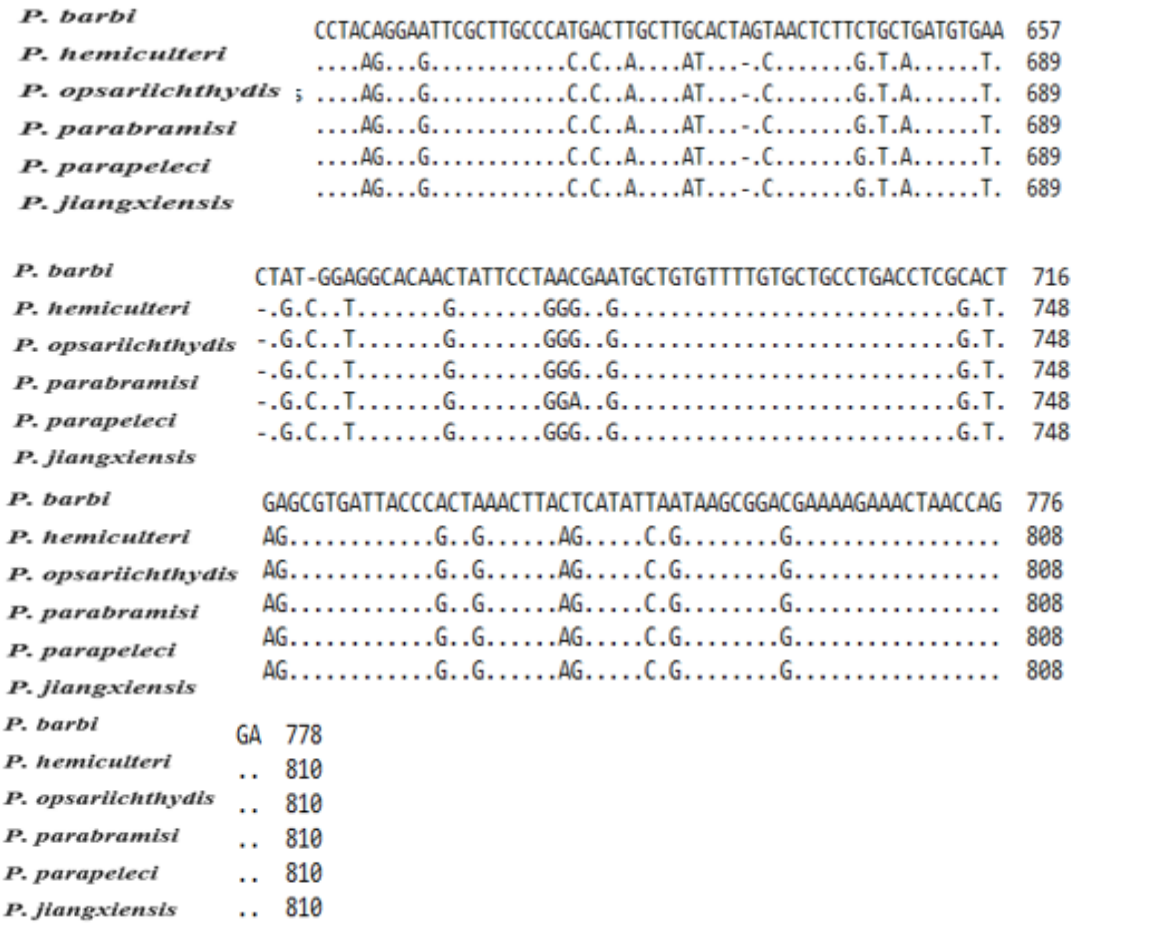

DISCUSSION

The Diplozoinae are divided into five genera using the dichotomic keys developed by Khotenovsky (Mutejusova *et al*., 2001). These genera are differentiated based on the presence-absence of dilatation in the middle part of the haptor, the shape and length of lateral branches departing from the intestinal caecum in the posterior end of the body, presence and size of plicae, location of uterine pore, and presence of glandular structures before the suckers (Mutejusova *et al.*, 2001). Paradiplozoon is the most diverse genus of Diplozoinae and is distinguished from other genera by the absence of a pronounced dilatation in the posterior region of the prehaptor (Dos Santos & Avenant-Oldewage, 2015). The posterior part of the specimens in the present study is without tegumental ridges or folds and was not cup-shaped or saucer-shaped. The specimens herein are ascribed to this genus based on the absence of these characters, which is typical of Paradiplozoon.

Based on the morphological features as described by Reichenbach-Kline (Khetenovsky,1985), *P. barbi* identified from this research shared similar features like, the same total length which ranged from 1mm to 1.3 mm, the haptor shape is nearly rectangular, the posterior body part is shorter and halve in length than the anterior part (Khetenovsky, 1985) and non-parallel arrangement of the set of clamps row (Khetenovsky,1985). Based on this morphological characters, it could be concluded the diplozoid is *P. barbi.*

Analysis of the ITS2 region following sequencing clearly allowed us discrimination at the species level and produced the same results as species identification made by using morphological structures. During the present study it was observed that the alignment of nucleotide sequences with those of other *Paradiplozoon* species (Mutejusova *et al*., 2001), clearly revealed the boundaries of the 5.8S and 28S rDNA genes. As noted in comparison of ITS2 sequences of Monogenean species, the first part of the ITS2 is also highly conserved, with only 6 variable sites in the first 65 nucleotides of the diplozoid sequences. Kimura 2-parameter distances between sequences of the ITS2 fragment of *P. barbi* and five *Paradiplozoon* species ranged from 0.131 to 0.133.

The molecular analysis of the ITS-2 gene sequence of *P. barbi* shows the novelty of the gene sequence. Nucleotide BLAST search for *P. barbi* gene sequence in the GenBank database shows no identical sequence. Multiple alignment of closely related ITS2 rDNA gene sequences of *Paradiplozoon* species with *P. barbi* showed 86.64% - 87.72% resemblance with closely related species. Furthermore, maximum likelihood method was used to phylogenetically analyzed closely related species to *P. barbi*. The phylogenetic tree reconstruction of closely related species to *P. barbi* shows a well-supported monophyletic clade comprising of *P. parapeleci, P. hemiculteri, P. parabramisi, P. jiangxiensis and P. opsariichthydis* all from China are closely related with 99% bootstrap support, while *P. barbi* (Malaysia) from this study cluster alone as shown by the topology of the phylogenetic tree.

CONCLUSION AND RECOMMENDATION

The molecular method was used for the accurate identification of *P. barbi*. Surprisingly, this is the first molecular identification of these monogeneans. The new DNA sequence of *P. barbi* GenBank accession number MN688771 were deposited in National Center for Biotechnology Information (U.S.A) GenBank database. The DNA sequence of monogeneans and other freshwater fish parasites in the reservoirs along Perak River have not been studied before and identifying these parasites by genomic may prove a better way for the accurate identification of these numerous monogeneans.

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