

Genetic identification and gut content of Gerong fish *Osteochilus waandersii* from Larai River

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Abstract. Gerong fish, or *Osteochilus waandersii*, is a popular freshwater fish among the Jakun tribe in Kampung Peta, Johor National Park of Endau-Rompin, particularly for its use in traditional medicine to alleviate dizziness by consuming its raw gut. Despite its cultural significance, the genetic and feeding behavior of Gerong fish remains unclear. This study aimed to amplify the mitochondrial DNA barcode region of Gerong fish for genetic identification and to identify its gut content. The DNA barcodes were produced using polymerase chain reaction and single-pass DNA sequencing of partial COI gene, and the gut content was observed using stereo microscope. Several five partial COI gene sequences were successfully obtained after DNA sequencing. In addition, the results of BLAST analysis with percent identities of 96.24% to 97.20% along with the phylogenetic inference of maximum likelihood analyses suggested the Gerong fish as *Osteochilus waandersii* or the Waanders's hard-lipped barb. The sequences were deposited in GenBank (accession numbers MN223396-MN223400). Microscopic observation of the fish guts revealed undigested worms and digested aquatic mosses, known as lumut batuk. These findings mark a significant step forward in understanding the genetics and feeding behavior of the Gerong fish.

1 Introduction

Osteochilus waandersii, or *Waandersii's hard-lipped barb*, is a freshwater fish in tropical countries such as Malaysia. It is listed under the least concern (LC) species category by the International Union for Conservation of Nature (IUCN; [1]). A species categorized as "least

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concern" by the IUCN indicates that it is not considered a priority for conservation efforts. Regarding its species description, *O. waandersii* is characterized by iridescent orange or red dorsal, caudal, anal, and pelvic fins. Additionally, it exhibits a distinct black stripe along the sides, extending from the gill opening to the termination of the median caudal rays, and possesses 12-13 branched dorsal fin rays [2]. According to [3], the species is typically connected to rocky or gravelly bottoms and clear, swiftly moving waters. *O. waandersii* usually flows into flooded forests next to upland streams during elevated water levels. [4].

Gerong fish, a popular freshwater fish among the Jakun tribe in Kampung Peta, Johor National Park of Endau-Rompin resembles *O. waandersii* morphologically. Traditionally, the fish gut is eaten raw by the Jakun tribe to relieve dizziness. However, no studies have been found to date on the fish gut content analysis and its medicinal properties that can be related to the healing effect. Besides determining the food components or the presence of medicinal properties in the Gerong fish gut, gut content analysis is also required to contribute to developing the fisheries sector, including freshwater fish aquaculture. A gut content study by [5] observed that the intestines of a freshwater tilapia fish included the zooplankton Rotifer and three phytoplankton divisions, i.e., Chlorophyta, Chrysophyta, and Cyanophyta. *Oreochromis niloticus* from Jagir River. Previously, [6] concluded that immature fish of the freshwater cyprinid *Hypselobarbus kurali*, which is indigenous to the Kallada River in Kollam, Kerala, India, primarily ate *Daphnia* sp.. In addition, [7] discovered that planktons, or zooplanktons and phytoplankton, as well as plant and animal items, were present in the stomach of freshwater catfish *Clarias gariepinus*; however, planktons, detritus, plant materials, and animal materials made up the majority of *O. niloticus*'s stomach contents.

This research aimed to establish the species classification of Gerong fish in the Larai River within the Johor National Park of Endau-Rompin, Malaysia, employing sequences of the mitochondrial gene cytochrome c oxidase I (COI). Previously, with accession number JX074190.1, [8] successfully sequenced the mitochondrial gene of cytochrome c oxidase I of *O. waandersii* and deposited the partial sequence in GenBank, the National Center for Biotechnology Information (NCBI), of the United States National Library of Medicine. Nevertheless, no sequence of *O. waandersii* from Malaysian freshwater was available in the GenBank. Moreover, since the gut of the freshwater fish is eaten raw by the Jakun tribe to relieve dizziness, the gut content of the Gerong fish was also examined in this study. The results of this investigation contribute to an enhanced comprehension of the genetic makeup and alimentary content of Gerong fish, establishing a significant correlation with the traditional medicinal practices of the Jakun tribe in Kampung Peta within the Johor National Park of Endau-Rompin.

2 Materials and methods

2.1 Study site and specimen collection

Several five specimens of Gerong fish (F1 – F5, Figure 1) were sampled from Larai River, Johor National Park of Endau-Rompin, Malaysia in March 2019 with an official permit from Johor National Parks Corporation (reference: TNJ 700-2/1/1 dated 21 February 2019). Upon reaching the Molecular Biology and Genetics Laboratory at the Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia, Pagoh Campus, Muar, Johor, Malaysia, via land transportation, the samples remained pristine and freshly preserved. The exterior morphology and the data provided by the Jakun people of Kampung Peta, Johor National Park of Endau-Rompin, were used to identify the morphospecies. Before being transported, a tiny portion of tissue was removed from each specimen using a sterile blade, and it was then put into a 1.5 mL microcentrifuge tube with 1.0 mL of absolute ethanol. Each

specimen was also dissected, and the gut was cut and placed in the microcentrifuge tube with 1.0 mL absolute ethanol. The specimens were soaked in absolute ethanol, packed, and sealed in a plastic bag. The specimens are stored in a laboratory chest freezer set at a temperature of -20 °C for extended-term preservation, accompanied by meticulous cataloging procedures to ensure organized and systematic storage.



Fig. 1. Gerong fish specimens from Larai River, Johor National Park of Endau-Rompin, Malaysia.

2.2 Gut content observation

Approximately 1 cm of each fish gut was put on a glass microscope slide, and a blade was used to dissect it. The gut content was taken out and immediately spread on the microscope slide. The prepared slide was observed under a digital stereo microscope with a camera installed. The images were captured and recorded.

2.3 Polymerase Chain Reaction

The genomic DNA extraction process utilized Favorgen's Tissue Genomic DNA Extraction Mini Kit to isolate the entire genomic DNA. Standard polymerase chain reaction (PCR) procedures were then used to amplify the protein-coding COI mitochondrial gene using the exTEN 2X PCR Master Mix by 1st BASE Biochemicals (~655 bp of fragment length based on [9]). A NanoDrop spectrophotometer was used to determine the quantity and quality of the total genomic DNA extracts.

FishF1 (forward) 5'- TCA ACC AAC CAC AAA GAC ATT GGC AC -3' (26 bases)
FishR1 (reverse) 5'- TAG ACT TCT GGG TGG CCA AAG AAT CA -3' (26 bases)

The PCR cycle conditions were outlined as follows: an initial denaturation phase of 4 minutes at 95°C, followed by denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 45 seconds, with steps 2-4 repeated for a total of 34 cycles. Subsequently, a final extension step was conducted at 72°C for 10 minutes, and the temperature was maintained at 4°C. The estimated yields of PCR products, their quantity, and their quality were then determined using agarose gel electrophoresis on a 1% agarose gel using FloroSafe DNA Stain as the gel stain. The unprocessed PCR specimens submitted to Apical Scientific Sdn. Bhd. located in Seri Kembangan, Selangor Darul Ehsan, Malaysia, specifically for single-pass DNA sequencing and purification of PCR fragments.

2.4 Basic local alignment, phylogenetic analyses, and GenBank submission

The alignment and comparison of each query sequence in this investigation were conducted by matching them against fish gene sequences present in the GenBank database. This was achieved using the online Basic Local Alignment Search Tool program designed for nucleotide searches (blastn). The Molecular Evolutionary Genetics Analysis (MEGA) version 11 program was used to perform phylogenetic analyses [10]. The sequence data was then prepared using the BankIt tool in order to submit them to GenBank and receive the accession numbers.

3 Result and discussion

Successful amplification of COI mitochondrial gene segments encoding proteins ranging from 650 to 700 bp was achieved (Figure 2). Chromas software version 2.6.6 facilitated the effective visualization of DNA sequencing data, specifically covering a segment of 674-687 nucleotide bases within the COI mitochondrial gene [11]. Furthermore, the blastn results demonstrated that, when compared to the corresponding sequences with GenBank accession codes of JX074190.1 and KC631203.1, the specimens of Gerong fish were positively recognized as *O. waandersii*, with high percent identities (Per. Ident) ranging from 96.24% to 97.20% (Table 1). The maximum likelihood phylogenetic tree (Figure 3) also supported the clustering of the specimens of Gerong fish from Larai River, Johor National Park of Endau-Rompin, Malaysia (forming a clade with 100% bootstrap support) and two *O. waandersii* specimens from outside Malaysia (grouping in a cluster with 95% bootstrap support) with 88% bootstrap support suggesting the species status of the morphospecies *O. waandersii* as *Osteochilus waandersii*. In addition, the formation of the two separate clusters of *O. waandersii* as shown in Figure 3 also suggested a genetic difference biogeographically between *O. waandersii* individuals from Johor National Park of Endau-Rompin, Malaysia; and *O. waandersii* individuals from Laos and the unknown region which is outside Malaysia. Additionally, the morphospecies *O. waandersii* query cover scores ranged from 94 to 97%, and the expected value (E value) was zero, indicating the highest significance score and alignment with the relevant sequences. The remaining values ranged from 1088 to 1094, representing the Maximum score and the Total score. Accordingly, the results implied that the Gerong fish specimens from the Larai River in the Endau-Rompin, Malaysia, Johor National Park, were *O. waandersii*. Therefore, the specimens' species designation as *O. waandersii*, previously proposed by their morphological data, was validated by the genetic data obtained from the COI mitochondrial gene.

The partial COI mitochondrial gene sequences registered in the GenBank (Accession No.: MN223396 - MN223400). The availability of DNA sequences of Malaysian Gerong fish or *O. waandersii* in the GenBank DNA database will provide more information and references to other scientists in their ongoing or future research and analyses related to the mitochondrial DNA of freshwater fishes. Indeed, the current findings have enhanced our understanding of the status of the Gerong fish in the Larai River by providing valuable insights into both morphological and molecular techniques. The protein-coding COI mitochondrial gene is commonly used in DNA barcoding due to its informative region for studying species variations [13]. Furthermore, the cytochrome b mtDNA gene and other protein-coding genes, including COI, are frequently utilized in genetic research [14, 15] and useful to confirm the non-protein-coding mitochondrial rRNA gene sequencing results. Even though Gerong fish, or *O. waandersii*, is currently categorized as the least concern species by the IUCN [1], the genetic data will help law enforcement organizations keep an eye on and deal with any problems of species substitution and product mislabelling in Malaysia, specifically concerning goods intended for import and export.

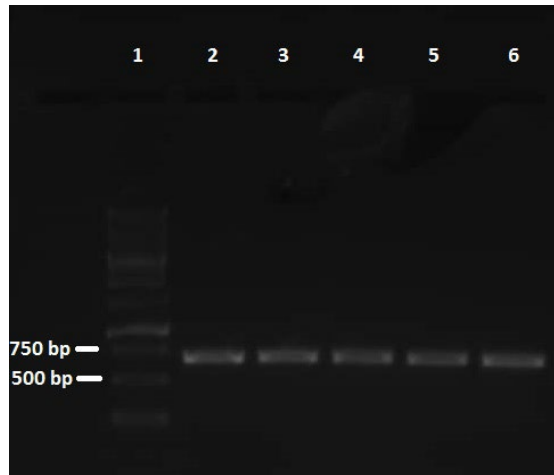


Fig. 2. Positive PCR products of COI mitochondrial gene of Gerong fish from Larai River, Johor National Park of Endau-Rompin, Malaysia. Lane 1 - DM3100 ExcelBand™ 1 KB (0.25-10 kb) DNA Ladder, Lane 2-6 – COI mitochondrial gene PCR products of Gerong fish (i.e. F1-F5).

Table 1. Basic Local Alignment Search Tool (BLAST) results of COI mitochondrial gene sequences of Gerong fish from Larai River, Johor National Park of Endau-Rompin, Malaysia.

Specimen	Max score	Total score	Query coverage (%)	E value	Percent identity (%)	GenBank accession no. of corresponding sequence	Species identity
F1	1094	1094	97	0.0	96.39	JX074190.1	<i>Osteochilus waandersii</i>
F2	1088	1088	96	0.0	96.24	JX074190.1	<i>Osteochilus waandersii</i>
F3	1088	1088	94	0.0	97.20	KC631203.1	<i>Osteochilus waandersii</i>
F4	1090	1090	96	0.0	96.24	JX074190.1	<i>Osteochilus waandersii</i>
F5	1088	1088	95	0.0	97.20	KC631203.1	<i>Osteochilus waandersii</i>

In terms of the gut content, the microscopic observation showed the presence of undigested worms and digested plant materials that could be aquatic mosses locally known as lumut batuk (Figure 4). However, molecular species identification of the undigested worms and the digested plant materials is required in subsequent studies to produce more effective, definitive results in the future. According to the Jakun people, Gerong fish only eat lumut batuk baits and usually ignore worm-based baits. Therefore, the observation of the undigested worms in the fish guts cannot be related to using worm-based baits. Research has indicated that the source of tetrodotoxin (TTX) in the gut contents of juvenile toxic fish, such as pufferfish (*Chelonodon patoca*) and goby (*Yongeichthys criniger*), is the polyclad flatworm *Planocera multitentaculata* whereby this flatworm contains highly concentrated TTX during its larval stage [16], thus suggesting functional properties of a fish gut could originate from the organisms inhabiting the gut or the food the fishes consumed. It is strongly believed that the Lumut batuk could contain medicinal properties that can relieve dizziness. However, further studies need to be done to test the hypothesis.

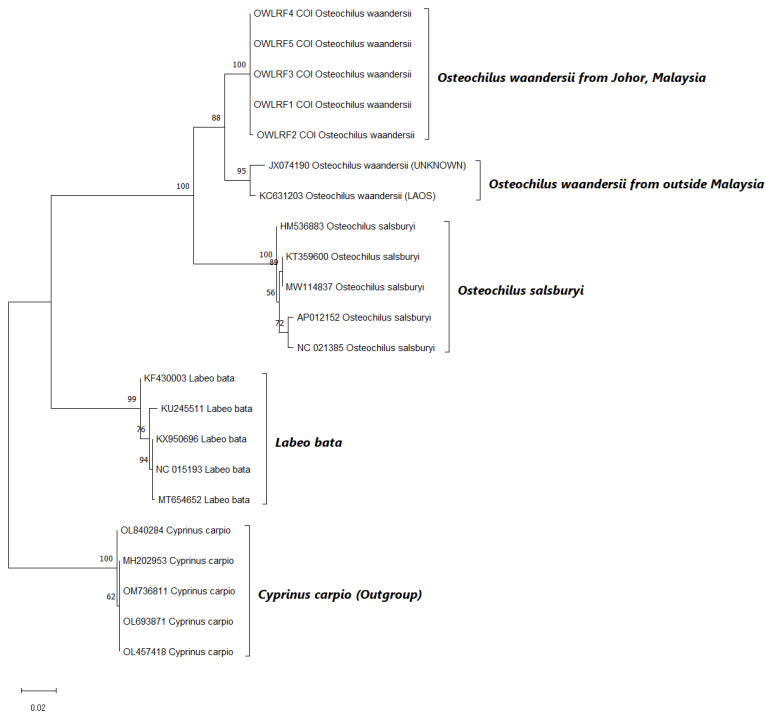


Fig. 3. The Hasegawa-Kishino-Yano model and the Maximum Likelihood approach were incorporated to infer the evolutionary history [12]. There is a display of the tree with the highest log probability (-1645.62). The percentage above branches represents the frequency of related taxa grouping in trees. The initial tree or trees for the heuristic search were automatically created by applying Neighbor-Join and BioNJ algorithms to a pairwise distance matrix derived from Maximum Composite Likelihood (MCL) calculations. The topology with the highest log likelihood was selected, and evolutionary rate variations between sites (in 5 categories with a discrete Gamma distribution, +G, parameter = 0.2937) were incorporated into the model. The branch lengths of the scaled-up tree are expressed in terms of the number of replacements made at each location. There were 100 bootstrap replicates and 22 nucleotide sequences in this research. The final dataset contained 630 locations in total. MEGA11 software was used to perform evolutionary analyses [10].

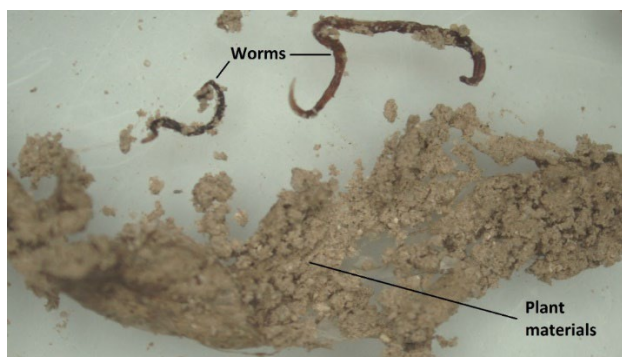


Fig. 4. The presence of undigested worms and digested plant materials that could be the aquatic mosses locally known as lumut batuk through microscopic observation of the Gerong fish gut content.

4 Conclusion

The Gerong fish specimens were identified as *Osteochilus waandersii*, or Waandersii's hard-lipped barb, based on the partial COI mitochondrial gene sequences analyzed using blastn and phylogenetic analysis. Five partial COI mitochondrial gene sequences were submitted to the GenBank for registration (Accession No.: MN223396 - MN223400). In terms of the gut content, the microscopic observation showed the presence of undigested worms and digested plant materials that could be the aquatic mosses locally known as lumut batuk. Moreover, the significance of morphological and molecular methods, as well as the current state of the Gerong fish from the Larai River in the Johor National Park in Endau-Rompin, Malaysia, were better understood due to the recent findings.

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