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Metabarcoding of Fish Larvae in Merbok River Reveals Species Diversity and Distribution Along its Mangrove Environment

Norli Fauzani Mohd Abu Hassan Alshari[1], Siti Zuliana Ahmad[1], Azali Azlan[1], Youn-Ho Lee[3], Ghows Azzam[1,*], and Siti Azizah Mohd Nor[1,2]

[1]*School of Biological Sciences, Universiti Sains Malaysia, 11800, Penang, Malaysia.*
**Correspondence: E-mail: ghows@usm.my (Azzam)*
E-mail: nfauzani0693@gmail.com (Mohd Abu Hassan Alshari); liazuliana11@gmail.com (Ahmad); azlanazali01@gmail.com (Azlan); sazizah@usm.my (Nor)
[2]*Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia. E-mail: s.azizah@umt.edu.my (Nor)*
[3]*Korean Institute of Ocean Science and Technology, Republic of Korea. E-mail: ylee@kiost.ac.kr (Lee)*

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The Merbok River (north-west of Peninsular Malaysia) is a mangrove estuary that provides habitat for over 100 species of fish, which are economically and ecologically important. Threats such as habitat loss and overfishing of fish become a great concern for fisheries conservation and management. The identification of larval fish in this estuarine system is important to complement information on the adults. This is because the data could inform the spawning behaviour, reproductive biology, selection of nursery grounds and the migration route of fish. Such information is invaluable for fisheries and aquatic environmental monitoring and thus for their conservation and management. However, identifying fish larvae is a challenging task based only on morphology and even traditional DNA barcoding. To address this, DNA metabarcoding was utilised to detect the diversity of fish in Merbok River. To complete the study, the fish larvae were collected at six sampling sites of the river. The extracted larval DNA was amplified for the Cytochrome Oxidase subunit 1 (*COI*) and 12S ribosomal RNA (12S rRNA) genes based on the metabarcoding approach using shotgun sequencing on next-generation sequencing (NGS) Illumina MiSeq platform. Eighty-nine species from 65 genera and 41 families were detected with *Oryzias javanicus, Oryzias dancena, Lutjanus argentimaculatus* and *Lutjanus malabaricus* among the most common species. The lower diversity observed from previous morphological studies is suggested to be mainly due to seasonal variation of sampling period between the two methods and limited 12S rRNA sequences in

current databases. The metabarcode data and a validation Sanger sequencing step using 15 species-specific primer pairs detected three species in common; *Oryzias javanicus*, *Decapterus maruadsi* and *Pennahia macrocephalus*. Several discrepancies observed between the two molecular approaches could be attributed to contaminants during sampling and DNA extraction, which could mask the presence of target species, especially when DNA from the contaminants is more abundant than the target organisms. In conclusion, this rapid and cost-effective identification method using DNA metabarcoding allowed the detection of numerous fish species from bulk larval samples in the Merbok River. This method can be applied to other sites and other organisms of interest.

Key words: Fish larvae, Mangrove estuary, Merbok River, DNA metabarcoding, Next-generation sequencing.

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BACKGROUND

In their various stages of life cycles, fish communities provide valuable insights into the ecological conditions of their habitats and furnish information for management of fisheries resources (Moser and Smith 1993; Moser 1996; Kidwai and Amjad 2001). However, a prerequisite for such investigations, is their precise identification. Acknowledging the shortcomings of traditional approach for species identification, molecular techniques are increasingly used to facilitate the identification process (Lewis et al. 2016). The DNA barcoding approach introduced by Hebert et al. (2003) based on species variation at the mitochondrial cytochrome oxidase subunit 1 (*COI*) gene, is regarded as the gold standard for molecular identification. It has been widely successful in discriminating most animal specimens to their species level, including identifying fish species, whether whole or using specific parts of an individual (Ko et al. 2013; Lewis et al. 2016; Azmir et al. 2017; Collet et al. 2018). However, sorting and identifying minute larval ichthyoplankton specimens needed for individual-based DNA barcoding is time- and cost-consuming.

DNA metabarcoding which applies the next generation sequencing (NGS) approach, is a rapid and cost-effective approach in processing bulk samples, damaged and fragmented specimens, and possibly degraded DNA (*e.g.*, ichthyoplankton, soil, water, and feces) (Taberlet et al. 2012) for biodiversity assessment and ecological studies (Coissac et al. 2012; Cristescu 2014, Lobo et al.

2017). DNA metabarcoding could provide an accurate taxonomic and biodiversity assessment of organisms in their native habitats, which are critical for their management. Based on this technique, several studies focussing on bulk ichthyoplankton specimens have successfully assigned ichthyoplankton to species level (Maggia et al. 2017; Mariac et al. 2018; Nobile et al. 2019; Ratcliffe et al. 2020).

Considering the threat of overharvesting and degradation of habitat, more active and stringent steps must be taken to manage the area to support sustainable fisheries for the local community. While regulations are in place to manage the adult fishes, nothing is known on the larvae diversity and its distribution. This information is vital to fisheries managers to understand the species utilizing the area and the locations they inhabit as their nursery grounds. With this knowledge, fisheries managers can take measures to protect the specific sites. Thus, to complement the management efforts on the adult fishes, a more comprehensive and holistic management strategies can be implemented through this study using the DNA metabarcoding method.

This study investigates the diversity and distribution of fish larvae in a mangrove estuarine area in the northern part of Peninsular Malaysia known as Merbok River. The main river connects small rivers or tributaries within the Merbok Permanent Forest Reserve (MPFR). Facing the Strait of Malacca, this ca. 4000 hectare of mangrove area is recognised as one of the world's mangrove species diversity hotspots, harbouring more than half of the global species (Mazlan et al. 2005). Merbok River, similar to other mangrove estuarine areas, is an important ecosystem for fisheries resources, in addition to its highly diverse natural floral resources (Jusoff 2008). Previous studies of the Merbok River have recorded a combined total of 120 fish species through morphological identification of the adult specimens (Mansor et al. 2012a b). The 35 km Merbok River which runs through a gradient of freshwater in the upper reaches to the more saline coastal waters flows through agricultural, aquaculture and residential areas. The land conversion in the MPFR area for these activities, including the infrastructure development, could negatively impact the faunal and floral communities that occupy the mangrove ecosystems, such as reducing fisheries catch (Manson et al. 2005; Jusoff 2008). In addition, based on this study, we hypothesise that the diversity and abundance of fish larvae is higher in the coastal lower reaches of the river than in the upper reaches.

MATERIALS AND METHODS

Study area

The fish larvae samples were collected from a mangrove estuary in the Merbok Permanent Forest Reserve (MPFR) at north-west Peninsular Malaysia. The estuary is locally known as Merbok River. It lies between latitude 100°20'57.33" and longitude 5°40'53.74" facing the Straits of Malacca and between latitude 100°30'24.56" and longitude 5°42'13.46" at the upper reaches (Mansor et al. 2012b). Small tributaries connect the 35 km estuary with freshwater discharged into the estuary from small tributaries, especially at the upper part of the river. The Merbok River has high salinity along the lower zone and decreases up the river, the former due to its proximity to the coastal area, while the upper zone has freshwater inflow. The Merbok River is surrounded by 39 true mangrove species (Ong et al. 2015) dominated by *Rhizophora apiculata* and *Bruguiera parviflora* along the 35 km stretch of the main river (Mansor et al. 2012a). The upper zone of the river is surrounded by mangrove forests near residential areas, fishing villages, agricultural fields, shrimp and oyster farms. The middle zone is surrounded by mangrove forests and some fish aquaculture activities. The lower zone is surrounded by mangrove forests, palm oil plantations, and its shrimp and fish facilities and land development activities for tourist attractions. Thus, the whole area is anthropogenically important with high mangrove diversity.

Sample collection and preservation

Fish larvae samples were collected during the primary wet season in August 2016 at six sampling locations along the tributaries of the Merbok River (Fig. 1). The sampling localities were from the freshwater upper zone (St1: Lalang River and St2: Semeling River), middle (St3.1: Keluang River and St3.2: Teluk Wang River), and lower brackish/marine zone (St4.1: Gelam River and St4.2: Terus River), in concordance to the sites of previous studies which divided the sampling sites according to these three zonations (Mansor et al. 2012b; Fatema et al. 2014). This allows comparisons of biodiversity assessment among the studies based on different approaches (morphological vs. metabarcoding). Furthermore, the decreasing salinity gradient from the upper to lower zone provides an excellent insight into larval diversity based on their salinity tolerance and nursing grounds. The collection of fish larvae samples was standardized by five times scooping at the same (or approximately) spot near the mangrove roots at the riverbank area by using a modified hand scoop net of 500 μm mesh size (radius: 30 cm) (Arshad et al. 2012; Wibowo and Sloterdijk 2015). The samples were kept in separate 50 mL bottles filled with water from the sampling sites and kept cool on ice during transport to the Molecular Ecology Research Laboratory, Universiti Sains Malaysia (USM), Penang. The filtered samples were then rinsed in distilled water and were pooled in five replicate tubes for each site filled with 70% ethanol prior to the DNA extraction process.

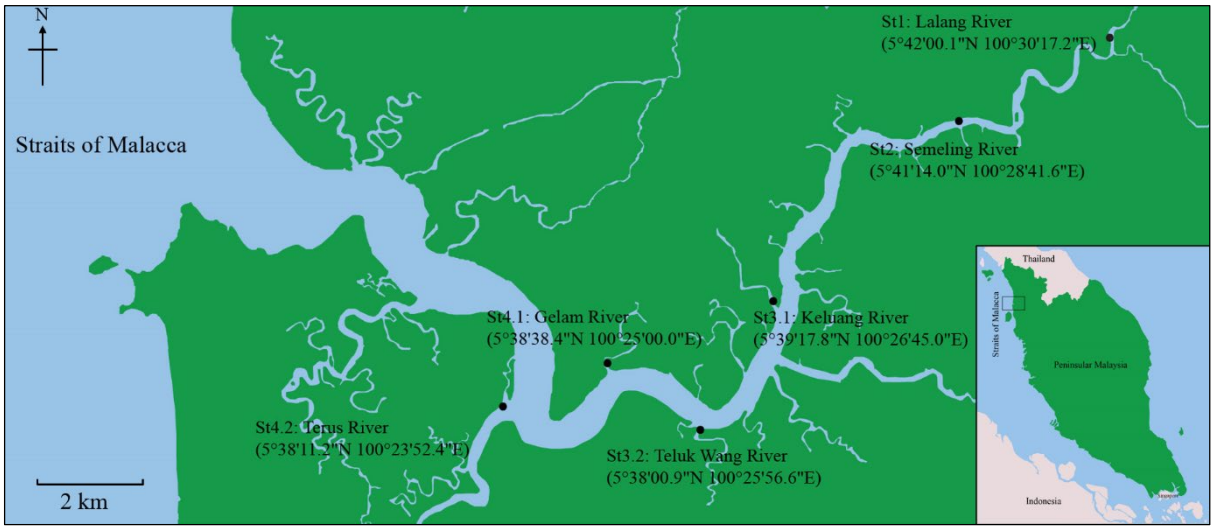

Fig. 1. Merbok River with six sampling stations, divided into three zones: upper [St1: Lalang River (5°42'00.1"N 100°30'17.2"E), St2: Semeling River (5°41'14.0"N 100°28'41.6"E)], middle [St3.1: Keluang River (5°39'17.8"N 100°26'45.0"E) and St3.2: Teluk Wang River (5°38'00.9"N 100°25'56.6"E)] and lower [St4.1: Gelam River (5°38'38.4"N 100°25'00.0"E) and St4.2: Terus River (5°38'11.2"N 100°23'52.4"E)].

Water parameters were recorded for each sampling site to assess the habitat type (FishBase category) at point of sampling. The water parameters were measured using the following equipment: Secchi disk and tape were used to measure water depth (WD), and turbidity (TURB), SCT Meter YSI Model 33 (YSI Inc., USA) was used to measure water temperature (TEMP) and salinity (SAL), while YSI 550A (YSI Inc., USA) was used to measure water pH and dissolved oxygen (DO). No ecological analysis was intended as this was a one-off sampling measurement.

DNA Barcoding referencing of fish species

Specimens of twenty-two adult fish species without available molecular sequences of 12S rRNA gene in the public databases were obtained from local wet markets for analysis. Samples were identified based on the FAO species identification guide book (Carpenter and Niem 2001). Ikan Laut Malaysia (Atan et al. 2010) and Fishes of Malaysia (Ambak et al. 2012). Each specimen was photographed, and whole specimens were permanently stored in 70% ethanol at the Molecular Ecology Research Laboratory, USM. The pectoral fin clips of each species (one to three specimens) were preserved in 96% ethanol for molecular identification. The combined report from Mansor et al. (2012a), Mansor et al. (2012b), and Mansor et al. (2012c) had recorded a total of 120 morphologically identified adult fish species in the Merbok River, of which 68 species (Mansor et al. 2012b) had been classified according to their habitat category; estuarine (E), marine (M), marine-estuarine dependent (MED), freshwater-estuarine dependent (FED) and freshwater (F),

while the remaining 52 species had not been previously classified. The genomic DNA of adult specimens (22 species without 12S rRNA reference sequences) was extracted using the modified hexadecyltrimethylammonium bromide (CTAB) protocol (Grewe et al. 1993) from approximately 1.0 mm of the preserved fin clip. A segment of the 12S rRNA gene was amplified from the extracted DNA using the primer pairs: MiFish-U-F 5'-GTC GGT AAA ACT CGT GCC AGC-3' and MiFish-U-R 5'-CAT AGT GGG GTA TCT AAT CCC AGT TTG-3' (Miya et al. 2015). The 25 µL PCR reaction mix contained 2.5 µL of 10X $MgCl_2$ free PCR buffer, 2.0 µL of 50mM $MgCl_2$ 1.0 µL of 10mM dNTP, 0.5 µL of each 5µM forward and 5µM reverse primers, 0.25 µL of 5U/µL *Taq* polymerase (iNtRON, Gyeonggido, Korea), 1.0 µL of DNA template and 16.75 µL of double-distilled water. The thermal conditions were; a pre-denaturation step of 2 minutes at 95°C, followed by 35 cycles of 20 seconds at 94°C, 15 seconds at 47.9°C, 15 seconds at 72°C, followed by final extension of 5 minutes at 72°C and then stored at 4°C. Sequencing of the PCR products was done at the First Base Laboratories Sdn. Bhd. (Selangor, Malaysia) on an ABI3730XL capillary sequencer (Applied Biosystems, USA). To aid molecular confirmation of each species, samples from the same specimens were also analysed with the COI gene based on the following primer pair: FishF1 5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3' and FishR1 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' (Ward et al. 2005). The thermal conditions were; a pre-denaturation step of 4 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 50 seconds at 47.9°C, 1 minute at 72°C, followed by final extension of 7 minutes at 72°C and then stored at 4°C. The sequencing protocol was the same as for the 12S rRNA gene.

Forward and reverse sequences were trimmed and aligned using MEGA7 software (Kumar et al. 2016). The COI sequences were then compared to the Barcoding of Life Database (BOLD) System. Its comprehensive features including morphological information (photographic record) and other supporting data for species identification permit effective cross referencing to the 12S rRNA gene sequence for the same sample (and species). The newly generated 12S rRNA gene sequence of each species has been submitted to NCBI (GenBank) (https://www.ncbi.nlm.nih.gov) with accession numbers: KY379960-KY379968, KY778751-KY778754, MG729393, MG729396, MG729397, MG748713, MG748714, MK330865-MK330867.

DNA metabarcoding

Genomic DNA extraction and amplification

The genomic DNA extraction of the larval specimens was conducted following the protocol of the adult specimens with some modifications: 1) the larval specimens that were preserved in five

replicate tubes for each location containing 70% ethanol were first cut and minced; 2) then, the minced samples were pooled into six separate labelled 1.5 mL microcentrifuge tubes based on sampling stations (St1, St2, St3.1, St3.2, St4.1, St4.2). The number of individuals varied among sites, but as earlier mentioned, the volume has been standardised for all sites by maintaining a uniform number of scoops (5X). The extracted DNA was purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA) following the manufacturer's instruction to remove excess inhibitor that could potentially inhibit the amplification of mitochondrial DNA (mtDNA). The purity and quantity of the extracted and purified DNA were measured using UV spectrophotometer Q3000 (Quawell, USA) before and after purification. The mitochondrial genome amplification and enrichment step were then conducted on the purified DNA of each pooled sample extract using REPLI-g Mitochondrial DNA kit (Qiagen, USA) following the provided protocol. The amplification of the whole mitochondrial genome was aimed to get complete mitogenomes of almost all fish species in one shot. This is to reduce the cost for sequencing and analysis when compared to individual mitogenome. After the amplification steps, samples St3.1 and St3.2 were pooled and was labelled as sample St3. At the same time, samples St4.1 and St4.2 were also pooled and labelled as sample St4 for the library preparation step in the Illumina MiSeq NGS platform (refer to Results for pooling clarification). Successfully amplified samples were sent for pre-processing and next-generation sequencing at the Shanghai Majorbio Pharmaceutical Technology Co., Ltd. (Shanghai, China).

Library preparation and sequencing

The NGS shotgun sequencing was conducted on an Illumina MiSeq (Illumina, San Diego, USA) with paired-end 250 bp insert size. The library preparation was done to add adapter sequences onto the ends of the DNA fragments. The steps involved in library preparation were; 1) fragmentation, 2) end-repair, 3) A-tailing, 4) ligation and 5) paired-end sequencing. Firstly, DNA samples were sheared into approximately 400 to 500 bp fragments using an ultrasonicator, Covaris M220 (https://covaris.com/products/afa-ultrasonication/m-series/). Then, the sheared DNA fragments were purified using QIAquick PCR Purification Kit (Qiagen, Germany). The fragmented DNA was then end-repaired, and the 5'-end were phosphorylated. Next, the blunt 3'-ends were A-tailed by adding an adenine (A) base to form an overhang. During the A-tailing, the overhang A-tail allows adapters containing thymine (T) base to pair with the DNA fragments. The A-tailing of the 3'-ends is important to facilitate ligation of DNA template to the sequencing adapters. The ligase enzyme covalently links the adapter and DNA fragments during adapter-fragment ligation. The ligated DNA products were then PCR amplified using TruSeq™ DNA Sample Prep Kit (Illumina,

California, USA) to enrich the DNA ligation products. Finally, the genomic DNA library was assessed by electrophoresis, nanodrop and qubit as a part of the library assurance (QA) and quality control (QC) procedures. The genomic library with satisfactory QA and QC was continued with the cluster generation and sequencing. NGS data pre-processing steps were conducted on each raw sequence read which involved quality control procedures to filter sequence reads with low-quality and remove of the adapter sequences prior to analysis of sequence reads of each sample. All the above procedures starting from library preparation until sequencing (1–5) and NGS data pre-processing, were conducted at the Shanghai Majorbio Pharmaceutical Technology Co., Ltd. (Shanghai, China).

Bioinformatics procedure

The data generated from the shotgun sequencing was then analysed using several bioinformatics software and run in the Linux platform. Quality analysis of the MiSeq reads was done using FastQC available from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Adapters and low-quality reads were filtered and trimmed using Trimmomatic (Bolger et al. 2014) using the following parameters: ILLUMINCLIP (to perform adapter removal): TruSeq2-PE.fa:2:30:10; LEADING (to cut bases at the start of the read):3; TRAILING (to cut bases at the end of the read):3; SLIDINGWINDOW (to perform sliding window trimming):4:28; MINLEN (the minimum length specified to cut the reads):100. The clean paired-end reads obtained after quality trimming with an average length of 100 to 250 bp and average GC content of 44% to 45% proceeded to *de novo* assembly for scaffold formation. Following the default parameter settings, the *de novo* assembly was done using MEGAHIT (v.1.0.2) assembler software (Li et al. 2015). The parameters used were: i) the min-count: 2; ii) k-min: 21; iii) k-max: 99; iv) k-step: 20; and v) min-contig-len: 200 (supplementary materials: Table S1).

The assembled scaffolds were divided into taxonomic classified reads and taxonomic unclassified reads using Kraken 2 software (Wood et al. 2019). The reads with taxonomic classification were further blast on a mitochondrial genome reference database of *COI* and 12S rRNA genes (RefSeq) of 35,655 current fish sequences downloaded from NCBI (GenBank) in FASTA file format for BLAST analysis with scaffolds of each sample. The BLAST analysis on COI and 12S rRNA gene reference databases was performed by using ‘megablast’ using the following criterion (blast identity: ≥ 97% (Mariac et al. 2018, Fujii et al. 2019), word size: 28, e-value: 0.0001) for species-level assignment and diversity analysis. The scaffolds were realigned with the sequences of the identified species and reference sequence of 120 fish species from

Merbok River to confirm the annotation and taxonomic classification. Only scaffolds with ≥ 97% similarity with the reference sequences were assigned to species.

Metabarcoding results verification

Species-specific primer design

To verify the metabarcoding results of fish larvae identification, species-specific primer pairs were developed for 15 fish species randomly selected based on the DNA metabarcoding results (supplementary materials: Table S3). These primer pairs targeted the *COI* gene region because of its well-developed reference database in both the NCBI and BOLD systems compared to other genes. The sequences of these 15 species were downloaded from the two databases, and primer development was conducted through an online tool, Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser et al. 2007). All primer pairs were designed following the standard criteria for primer design, such as the primer length (18 to 22 bp), product size (200 to 630 bp), GC content (45% to 65%), and melting temperature (Tm: 50°C to 65°C).

PCR amplification of larval samples using newly designed primer pairs

PCR amplification was conducted on the pooled genomic DNA of the four sampling stations (St1, St2, St3, and St4) using the 15 newly designed species-specific primers. The 25 μL PCR reaction contained 16.75 μL of double-distilled water, 2.5 μL of 10×PCR buffer, 2.0 μL of $MgCl_2$, 1.0 μL of dNTP, 0.5 μL of each forward and reverse primer and 0.25 μL of *Taq* polymerase (iNtRON, Gyeonggido, Korea) and 1.0 μL DNA template of pooled samples. The same PCR conditions were applied for each primer pair: pre-denaturation step of 2 minutes at 95°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 45°C to 55°C and 50 seconds at 72°C, final extension step of 10 minutes at 72°C and stored at 4°C. Successfully amplified PCR products were sent to First BASE Laboratories Sdn. Bhd. for Sanger-sequencing on the ABI3730XL sequencer (Applied Biosystem, USA).

Diversity analysis

The read abundance of fish larvae were used to analyse the diversity within the four stations (alpha diversity). The data was tabulated with the size bins combined across the samples, square

root-transformed by measuring diversity indices (*i.e.*, Shannon, Margalef, Menhinick, Evenness, and Equitability). For larval fish diversity among stations, Bray-Curtis similarity was conducted on the relative abundance to assess and visualise the Merbok River's beta diversity, displayed through a two-dimensional nonmetric-multidimensional scaling (NMDS) ordination based on their similarity (%). The alpha and beta diversity analyses were conducted using PRIMER7 and PERMANOVA+ (version 7; Primer-E, Ivybridge, UK).

RESULTS

General water condition of the Merbok River

As only a single measurement was taken, the water quality assessment was only a snapshot of the general water conditions and for classifying the stations into habitat types (freshwater, estuarine, marine or combinations of these). Based on salinity, the stations were classified as mesohaline (salinity range 5.0–17.9 ppt) at the upper zone (St1 and St2) and polyhaline (salinity range: 18.0–29.0) at the middle and lowest zones (St3.1, St3.2, and St4.1, St4.2). St3.1 and St3.2 were combined and renamed St3, and similarly St4.1, St4.2 were also combined and renamed St4. The pooling was done with the potential for capturing higher diversity and considering of the relatively short distance within the combined sets and their similar water quality characteristics. Water parameters were recorded for each sampling site (Table 1); water depth, turbidity, temperature, salinity, pH, and dissolved oxygen.

Table 1. The environmental parameters of Merbok River including the water depth, turbidity, salinity, pH, temperature, and dissolved oxygen during the time of sampling

Parameters	Locations					
	Lalang River (St1) 5°42'00.1"N 100°30'17.2"E	Semeling River (St2) 5°41'14.0"N 100°28'41.6"E	Keluang River (St3) 5°39'17.8"N 100°26'45.0"E	Teluk Wang (St4) 5°38'00.9"N 100°25'56.6"E	Gelam River (St5) 5°38'38.4"N 100°25'00.0"E	Terus River (St6) 5°38'11.2"N 100°23'52.4"E
Water depth (cm)	38.5	65.3	124.0	125.3	98.6	113.5
Turbidity (cm)	38.5	65.3	124.0	124.3	88.2	92.5
Salinity (ppt)	10	10.3	19	22	23	24
pH	5.8	5.8	5.9	6.3	6.3	6.4
Temperature (°C)	27.3	28.8	30.6	31.3	31.2	31.1
Dissolved oxygen (mg/L)	4.40	4.98	6.70	6.50	7.15	7.77

Fish larvae assignment and diversity based on the metabarcoding method

The Illumina MiSeq platform sequencer generated 3,123,982, 2,668,052, 2,388,913 and 2,566,647 paired-end raw reads from each of the four samples; St1, St2, St3 and St4, respectively.

After sequence quality trimming, the final paired-end reads were 1,400,112, 1,581,822, 1,422,667 and 1,642,143 for sample St1, St2, St3 and St4, respectively. These high-quality and cleaned reads were assembled into a total of 1,939 scaffolds, 3,486 scaffolds, 1,900 scaffolds and 1,932 scaffolds for samples St1, St2, St3, and St4, respectively. The *de novo* assembly analysis revealed a minimum scaffold length of 200 bp, maximum scaffold lengths of 6,419 bp to 6,753 bp and average scaffold length of 610 bp to 758 bp (Supplementary material Table S1).

The BLAST analysis annotated a total of 1,658 (18%) and 1,367 (15%) scaffolds to the *COI* and 12S rRNA genes, respectively. Scaffolds annotated to COI and 12S rRNA were further proceeded with BLAST analysis for taxonomic assignment of the fish larvae with an acceptable limit of blast identity at ≥ 97%. The combined results of BLAST analysis of 2,014 scaffolds annotated to *COI* (1,071 scaffolds), and 12S rRNA (943 scaffolds) genes (Table S2) revealed a total of 89 species, 65 genera, and 41 families in the Merbok River. Among these species, 88 species were identified by the *COI* gene, while the 12S rRNA gene identified 78 species. Although this study had standardized the sampling replicates for each site and standardized pooling of the DNA samples for amplification and NGS, low annotation rate still occurred after the assembly. The total amount of mitochondrial DNA in the samples is unknown and uneven for pooled taxa, together with the presence of nuclear DNA from the larvae samples and non-target DNA (contaminants) that may be present in the samples such as from the gut of the larvae. This could affect the proportion of mtDNA in the total DNA extracts and the annotation (Tang et al. 2014).

Species detection through metabarcoding of larval fish (89 species) was lower than previously recorded morphologically identified adult species (120 species). The number of species detected by the metabarcoding approach were; 12 (St1), 26 (St2), 46 (St3), and 76 (St4). Six species were detected at all stations; *Oryzias javanicus*, *Oryzias dancena*, *Oreochromis niloticus*, *Oreochromis aureus*, *Lutjanus malabaricus*, and *Siganus fuscescens*. In terms of habitat category, the first four of these common species are freshwater-estuarine (FE), while *Lutjanus malabaricus* and *Siganus fuscescens* are marine-estuarine (ME) species.

Another six species were recorded at three of the four locations. Among these, one species was detected in St1, St2 and St3: *Oryzias melastigma* (FE). In comparison, the other five species were detected in St2, St3, and St4: *Netuma thalassina* (MFE), *Alepes djedaba* (marine habitat, M), *Lutjanus argentimaculatus* (MFE), *Pennahia pawak* (M) and *Terapon jarbua* (MFE). A more significant number, 40 species, were detected at two of the four sampling stations. *Osphronemus goramy* (F) was detected at St1 and St2 only. Four species were detected in St2 and St3: *Ambassis gymnocephalus* (MFE), *Elops hawaiencsis* (MFE), *Clarias batrachus* (F), and *Mastacembelus erythrotaenia* (F), while six species were detected in St2 and St4: *Mystus cavasius* (FE), *Mystus vittatus* (FE), *Gerres oyena* (ME), *Hyporhamphus quoyi* (MFE), *Lutjanus johnii* (ME), and *Johnius*

carouna (MFE). The rest (33) of the two-site species were detected in St3 and St4 only, these two sites being nearest the coast.

Thirty-seven species were site specific, detected in only a single sampling station. Four species were only detected at St1: *Brachygobius xanthomelas* (F), *Traypauchen vagina* (ME), *Trichogaster pectoralis* (F), and *Monopterus albus* (FE). Two species were site-specific to St2; *Macrognathus aculeatus* (FE) and *Liza planiceps* (MFE). One species was detected only at St3; *Pennahia argentata* (M) habitat species. The remaining 30 species were detected only at St4. The larvae occurrence generally parallel the expected habitat with related freshwater species at the upper stations and marine related ones at the lower stations. However, many species were also common in several stations which is not unexpected as a considerable number of the recorded species are multi-habitat tolerant according to the FishBase classification. Details of the occurrence of species at the sampling stations and habitat category, as detected by *COI*/12S rRNA gene, are shown in table 2. The relative abundance of fish larvae among sampling sites is shown in figure 2.

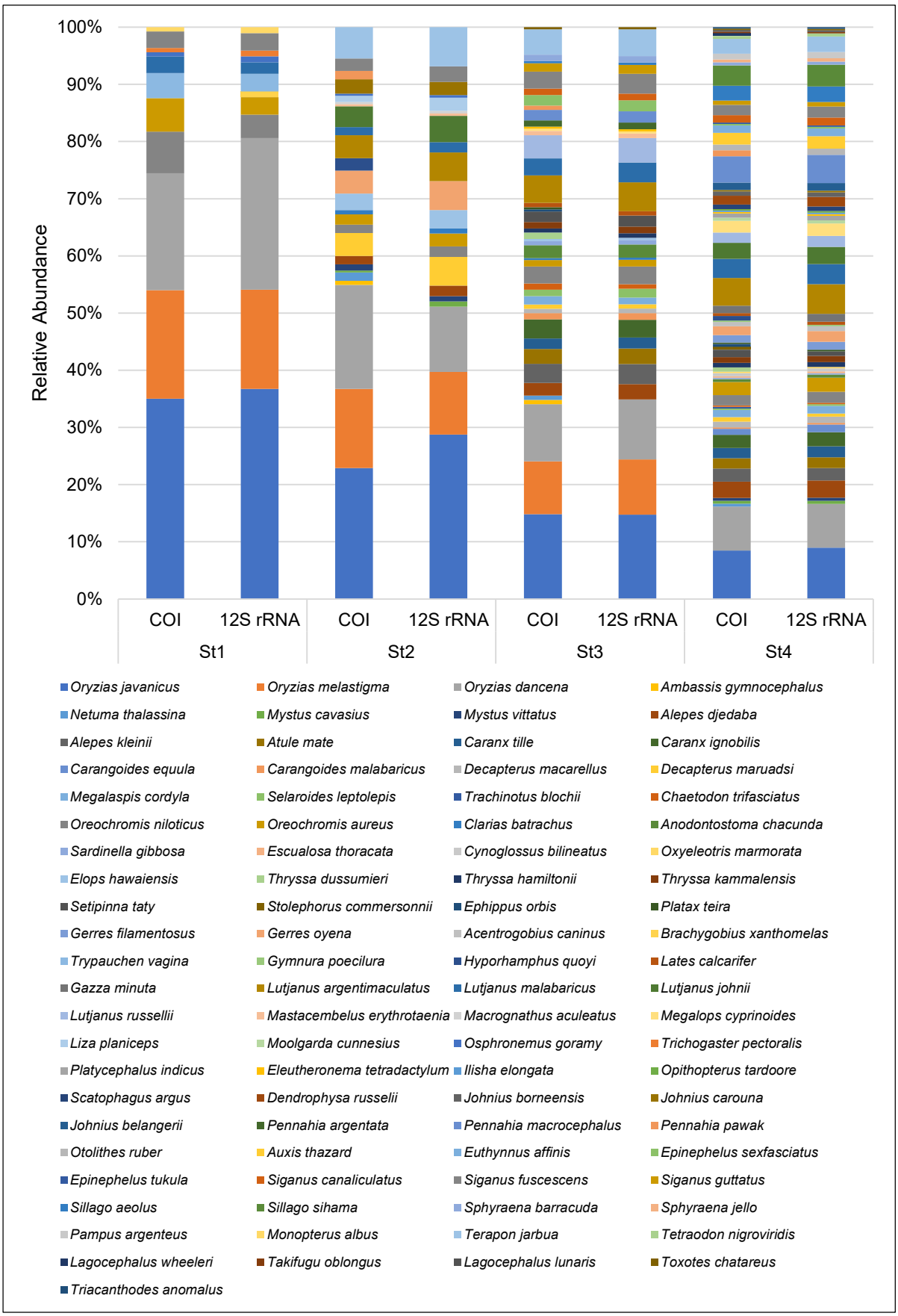

Fig. 2. The relative abundance of fish larvae species detected among sampling sites (St1, St2, St3 and ST4) based on the number of scaffolds reads of the COI and 12S rRNA genes.

Table 2. Presence/absence of larval fish species along the Merbok River based on metabarcoding analysis of COI (▲) and 12S rRNA (◊) genes and habitat category of each species, where F: freshwater; FE: freshwater estuarine; MFE: marine, freshwater estuarine; M: marine; and ME: marine estuarine

No.	Family	Species	Habitat category	St1	St2	St3	St4
1.	Adrianichthyidae	*Oryzias javanicus*	FE	▲ ◊	▲ ◊	▲ ◊	▲ ◊
2.	Adrianichthyidae	*Oryzias melastigma*	FE	▲ ◊	▲ ◊	▲ ◊	
3.	Adrianichthyidae	*Oryzias dancena*	FE	▲ ◊	▲ ◊	▲ ◊	▲ ◊
4.	Ambassidae	*Ambassis gymnocephalus*	FE		▲	▲	
5.	Ariidae	*Netuma thalassina*	MFE		▲	▲	▲
6.	Bagridae	*Mystus cavasius*	FE		▲ ◊		▲ ◊
7.	Bagridae	*Mystus vittatus*	FE		▲ ◊		▲ ◊
8.	Carangidae	*Alepes djedaba*	M		▲ ◊	▲ ◊	▲ ◊
9.	Carangidae	*Alepes kleinii*	M			▲ ◊	▲ ◊
10.	Carangidae	*Atule mate*	ME			▲ ◊	▲ ◊
11.	Carangidae	*Caranx tille*	ME			▲ ◊	▲ ◊
12.	Carangidae	*Caranx ignobilis*	ME			▲ ◊	▲ ◊
13.	Carangidae	*Carangoides equula*	M				▲ ◊
14.	Carangidae	*Carangoides malabaricus*	M			▲ ◊	▲ ◊
15.	Carangidae	*Decapterus macarellus*	M			▲ ◊	▲ ◊
16.	Carangidae	*Decapterus maruadsi*	M			▲ ◊	▲ ◊
17.	Carangidae	*Megalaspis cordyla*	ME			▲ ◊	▲ ◊
18.	Carangidae	*Selaroides leptolepis*	ME			▲ ◊	▲ ◊
19.	Carangidae	*Trachinotus blochii*	ME				▲
20.	Chaetodontidae	*Chaetodon trifasciatus*	M			▲ ◊	▲ ◊
21.	Cichlidae	*Oreochromis niloticus*	F	▲ ◊	▲ ◊	▲ ◊	▲ ◊
22.	Cichlidae	*Oreochromis aureus*	F	▲ ◊	▲ ◊	▲ ◊	▲ ◊
23.	Clariidae	*Clarias batrachus*	F		▲ ◊	▲ ◊	
24.	Clupeidae	*Anodontostoma chacunda*	MFE			▲ ◊	▲ ◊
25.	Clupeidae	*Sardinella gibbosa*	M			▲ ◊	▲ ◊
26.	Clupeidae	*Escualosa thoracata*	MFE				▲ ◊
27.	Cynoglossidae	*Cynoglossus bilineatus*	ME				▲ ◊
28.	Eleotridae	*Oxyeleotris marmorata*	FE				▲ ◊
29.	Elopidae	*Elops hawaiensis*	MFE		▲ ◊	▲ ◊	
30.	Engraulidae	*Thryssa dussumieri*	ME			▲	▲
31.	Engraulidae	*Thryssa hamiltonii*	MFE			▲ ◊	▲ ◊
32.	Engraulidae	*Thryssa kammalensis*	ME			▲ ◊	▲ ◊
33.	Engraulidae	*Setipinna taty*	ME			▲ ◊	▲ ◊
34.	Engraulidae	*Stolephorus commersonnii*	ME				▲
35.	Ephippidae	*Ephippus orbis*	M			▲	▲
36.	Ephippidae	*Platax teira*	M			▲	▲ ◊
37.	Gerreidae	*Gerres filamentosus*	MFE				▲ ◊
38.	Gerreidae	*Gerres oyena*	ME		▲ ◊		▲ ◊
39.	Gobiidae	*Acentrogobius caninus*	MFE				▲ ◊
40.	Gobiidae	*Brachygobius xanthomelas*	F	◊			
41.	Gobiidae	*Trypauchen vagina*	ME	▲ ◊			
42.	Gymnuridae	*Gymnura poecilura*	M				▲ ◊
43.	Hemiramphidae	*Hyporhamphus quoyi*	MFE		▲		▲
44.	Latidae	*Lates calcarifer*	MFE			▲ ◊	▲ ◊
45.	Leiognathidae	*Gazza minuta*	ME				▲ ◊
46.	Lutjanidae	*Lutjanus argentimaculatus*	MFE		▲ ◊	▲ ◊	▲ ◊
47.	Lutjanidae	*Lutjanus malabaricus*	ME	▲ ◊	▲ ◊	▲ ◊	▲ ◊
48.	Lutjanidae	*Lutjanus johnii*	ME		▲ ◊		▲ ◊
49.	Lutjanidae	*Lutjanus russellii*	ME			▲ ◊	▲ ◊
50.	Mastacembelidae	*Mastacembelus erythrotaenia*	F		▲ ◊	▲ ◊	
51.	Mastacembelidae	*Macrognathus aculeatus*	FE		▲ ◊		
52.	Megalopidae	*Megalops cyprinoides*	MFE			▲ ◊	▲ ◊
53.	Mugilidae	*Liza planiceps*	MFE		▲ ◊		
54.	Mugilidae	*Moolgarda cunnesius*	MFE				▲ ◊
55.	Osphronemidae	*Osphronemus goramy*	F	▲ ◊	▲ ◊		
56.	Osphronemidae	*Trichogaster pectoralis*	F	▲ ◊			

57.	Platycephalidae	*Platycephalus indicus*	ME				▲ ◊
58.	Polynemidae	*Eleutheronema tetradactylum*	MFE			▲ ◊	▲ ◊
59.	Pristigasteridae	*Ilisha elongata*	ME				▲ ◊
60.	Pristigasteridae	*Opithopterus tardoore*	ME				▲ ◊
61.	Scatophagidae	*Scatophagus argus*	MFE				▲ ◊
62.	Sciaenidae	*Dendrophysa russelii*	MFE				▲ ◊
63.	Sciaenidae	*Johnius borneensis*	MFE				▲ ◊
64.	Sciaenidae	*Johnius carouna*	MFE		▲ ◊		▲ ◊
65.	Sciaenidae	*Johnius belangerii*	ME				▲ ◊
66.	Sciaenidae	*Pennahia argentata*	M			▲ ◊	
67.	Sciaenidae	*Pennahia macrocephalus*	M			▲ ◊	▲ ◊
68.	Sciaenidae	*Pennahia pawak*	M		▲	▲	▲
69.	Sciaenidae	*Otolithes ruber*	ME				▲ ◊
70.	Scombridae	*Auxis thazard*	M				▲ ◊
71.	Scombridae	*Euthynnus affinis*	M				▲ ◊
72.	Serranidae	*Epinephelus sexfasciatus*	M			▲ ◊	▲ ◊
73.	Serranidae	*Epinephelus tukula*	M				▲ ◊
74.	Siganidae	*Siganus canaliculatus*	ME			▲ ◊	▲ ◊
75.	Siganidae	*Siganus fuscescens*	ME	▲ ◊	▲ ◊	▲ ◊	▲ ◊
76.	Siganidae	*Siganus guttatus*	ME			▲ ◊	▲ ◊
77.	Sillaginidae	*Sillago aeolus*	M			▲ ◊	▲ ◊
78.	Sillaginidae	*Sillago sihama*	ME				▲ ◊
79.	Sphyraenidae	*Sphyraena barracuda*	ME			▲ ◊	▲ ◊
80.	Sphyraenidae	*Sphyraena jello*	ME				▲ ◊
81.	Stromatidae	*Pampus argenteus*	M				▲ ◊
82.	Synbranchidae	*Monopterus albus*	FE	▲			
83.	Terapontidae	*Terapon jarbua*	MFE		▲ ◊	▲ ◊	▲ ◊
84.	Tetraodontidae	*Tetraodon nigroviridis*	FE				▲ ◊
85.	Tetraodontidae	*Lagocephalus wheeleri*	M				▲
86.	Tetraodontidae	*Takifugu oblongus*	ME				▲ ◊
87.	Tetraodontidae	*Lagocephalus lunaris*	ME				▲ ◊
88.	Toxotidae	*Toxotes chatareus*	FE			▲ ◊	▲ ◊
89.	Triacanthodidae	*Triacanthodes anomalus*	M				▲ ◊

Detection of non-target species

This study detected non-target species from the remaining 7,243 scaffolds reads that were not taxonomically classified as fish species (Fig. 3). Most of the reads are classified as bacteria (5,021 reads) known as fish associated bacteria (from phylum Proteobacteria, Actinobacteria, Firmicutes and Bacteriodetes), reads that taxonomically remained unassigned (1642 reads), other eukaryote (507 reads) (*e.g.*, shrimps and molluscs), and archaea (73 reads).

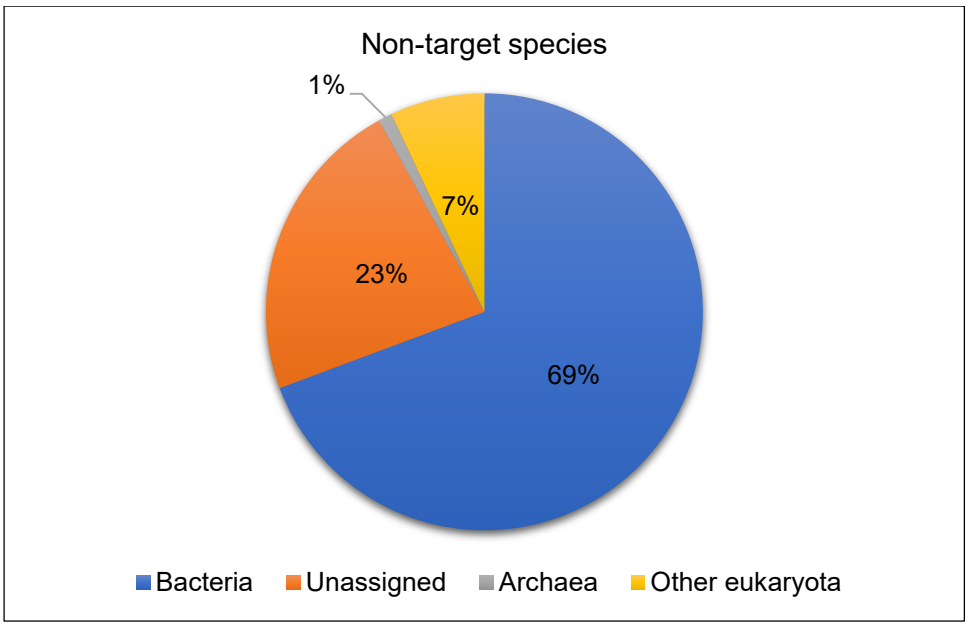

Fig. 3. Reads abundance of non-target DNA.

Comparison of larval fish diversity among four stations along Merbok River

The beta diversity of the fish larvae among different sampling sites and different genes was compared using Bray-Curtis similarity plotted in the two-dimensional non-metric multidimensional scaling (NMDS) (Fig. 4). As expected, the *COI* and 12S rRNA genes were clustered together according to each station. Based on the NMDS, two major clusters with 36% similarity were formed; St1 and St2 were grouped in a cluster with 52% similarity, while St3 and St4 were grouped in a cluster with 62% similarity. In the St1 and St2 clusters, two clusters formed show species diversity identified using COI and 12S rRNA genes with 89% similarity between both genes in St1 and 90% similarity between both genes from St2. In the St3 and St4 clusters, the clustering is similar to the St1 and St2. The COI and 12S rRNA genes were grouped in a cluster with 94.9% similarity, while the COI and 12S rRNA genes in St4 were clustered with 95% similarity (Fig. 4).

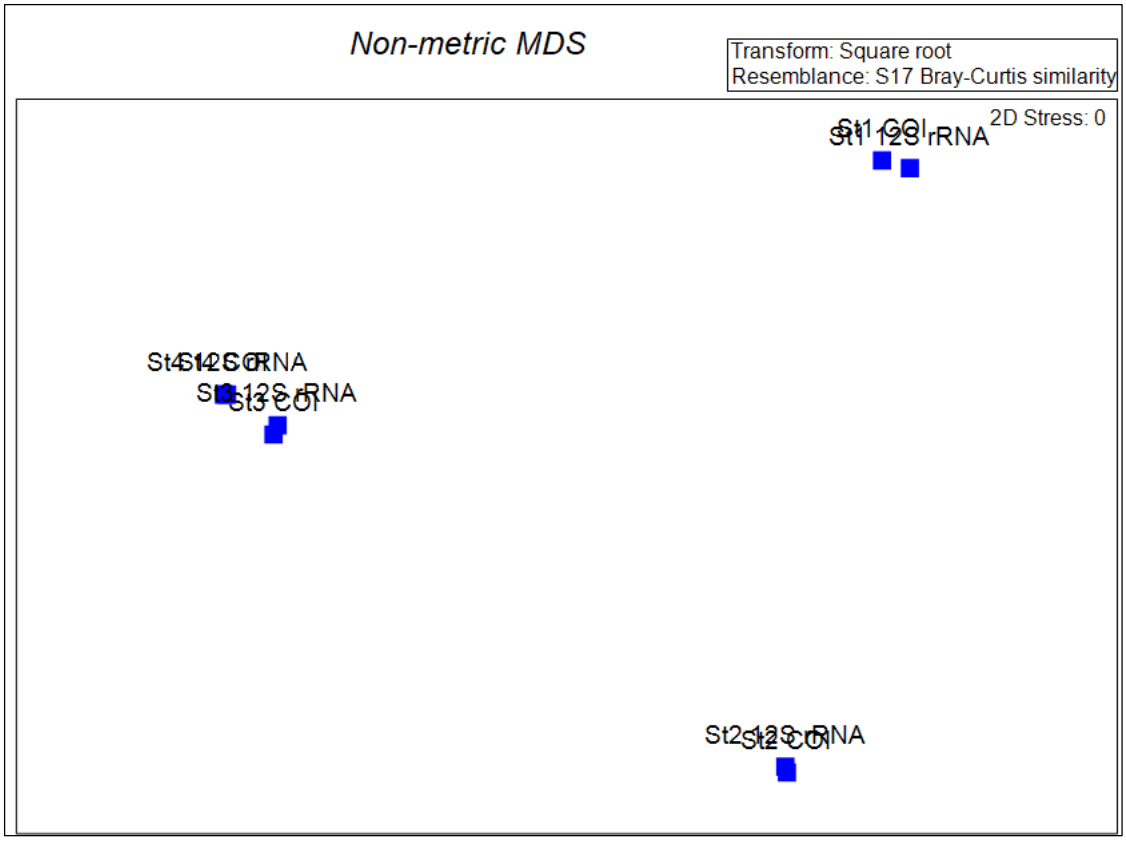

Fig. 4. Bray-Curtis similarity plotted by two-dimensional non-metric multidimensional scaling (NMDS) showing the similarity of larval fish species diversity among all four stations of Merbok River identified using the *COI* and 12S rRNA genes.

Validation of larval fish species

Only nine of the 15 newly designed *COI* primers were successfully amplified. These primers detected five species and, unexpectedly, also a shrimp species. Of these, only three of the five species in this validation step, had been detected in the DNA metabarcoding analysis. Surprisingly, none of the primers were specifically designed for these three species. The species detected were *Oryzias javanicus* (99%), *Decapterus maruadsi* (98%), and *Pennahia macrocephalus* (96%). The other two species, *Ambassis marianus* (99%) and an unknown species with the closest match to *Carangoides chrysophrys* at 83% were not detected in the DNA metabarcoding analysis. More unexpectedly, a shrimp species, *Acetes sibogae* of family Sergestidae, (98%) was also amplified.

DISCUSSION

Accuracy of diversity estimates using metabarcoding

This study reports the utilization of the DNA metabarcoding approach to assess the larval fish distribution and diversity in a biodiverse mangrove river system. It is a pioneering application of this technique in a Malaysian aquatic system and further supports its reliability for biodiversity assessment and potential future applications. In general, the larval distribution in the Merbok River was according to the expected zonation but not exclusively, with freshwater-estuarine species predominating the upper stations (St1 and St2), marine-estuarine and marine species equally distributed in the middle zone (St3), and marine-estuarine species dominating the lower zone (St4), although fully marine species was also abundant. The species diversity detected in this study was lower than the morphologically identified adult specimens reported in previous studies (references). A total of 91 species (metabarcoding and Sanger sequencing of designed primer) was detected in the current study compared to 120 morphologically identified adults in earlier studies (Mansor et al. 2012a b c). The study also elucidated the alpha and beta diversities of fish larvae in the river. Furthermore, a comparison between the two approaches showed an overlap of only 47 species (28.7%). More than half of the species morphologically documented in previous studies were not detected in the current study most probably due to different sampling seasons.

The discordance between the current and previous studies may be explained by variation in seasonal abundance of the species. Larvae samples were collected during the rainy season, presumed to be the spawning peak for most fishes with higher plankton availability as food for fish larvae (Ikejima et al. 2003; Chew and Chong 2011; Ooi 2012). On the other hand, the earlier studies applied a whole-year sampling strategy, mainly from fishermen catches which increased the probability of higher collection. For instance, the abundance of engraulids (*T. dussumieri*, *T. hamiltoni*, *T. kammalensis*, *S. taty*, and *S. commersonnii*) during the larvae sampling period in August strongly signified that this was the spawning season for members of family Engraulidae which paralleled the findings by Ooi and Chong (2011). The catfish, *Arius argyropleuron* was not detected by the metabarcoding approach, although it was the most abundant fish species recorded in earlier studies (Mansor et al. 2012a b c). This species has a major spawning peak in April and a minor in July, not coinciding with our sampling period. Valdez-Moreno et al. (2010) compared adult and larval data. They found only 34 matches between the two groups, while another 75 records of species from larval data were not matched to the adult species. This they attributed to the seasonal diversity of larvae, influenced by species-specific spawning time. On the other hand, we recorded 44 species that had not been assigned in previous studies of adult populations. The detection of these species by metabarcoding highlights the usefulness of DNA metabarcoding to uncover species undetected by morphological assessments (Emilson et al. 2017). The inclusion of these metabarcoded fish species has generated a more comprehensive list of species present in Merbok River with complementary data from fish larvae. Our data has also provided insights into

the spawning time and habitats of the identified species. Species misidentification of previous studies may have also contributed to these discrepancies, although this is not expected to be a major reason since adult specimens would have well-defined characteristics. Thus, we believe other factors also played a major role in the differences of findings, including technical and bioinformatics issues.

Issues related to sample handling and bioinformatic analysis

One of the factors that could affect the accuracy of taxonomic assignment and biodiversity estimates is the selection of markers. Most metabarcoding studies on fish larvae have utilised the typical DNA barcoding marker of the *COI* gene (Maggia et al. 2017, Mariac et al. 2018, Nobile et al. 2019). The 12S rRNA gene utilised in this study has a proven record of delivering species-level identification of fish in metabarcoding investigations of eDNA and larval fish (Thomsen et al. 2012, Miya et al. 2015, Sato et al. 2017, Ratcliffe et al. 2020, Kim et al. 2021). However, the fish database of the12S rRNA gene is still incomplete, which could have led to missed species detection. Thus, a comprehensive and precise reference database of the DNA marker is important prerequisite to obtain an accurate diversity assessment (Taberlet et al. 2012, Clarke et al. 2014, Bucklin et al. 2016, Weigand et al. 2019) and to avoid false positive and false negative species identification due to poor reference database (Bucklin et al. 2016). Although we rectified this by generating reference sequences of the 12S rRNA for species that had been morphologically cataloged in the area but with no available voucher sequences in the databases, the discrepancies were significant. This is likely due to several other factors that may be affecting the larval supply during sampling, which are larval distribution in small and isolated areas that are difficult to be reached, the survival of the larvae before and after arrival at the nursery grounds, and predation that occur during the larval settlement in the nursery grounds (Pineda et al. 2010).

The high number of reads annotated to non-target taxa *i.e.*, non fish species, a likely consequence of contamination, were observed. Bacteria had the highest composition, followed by archaea and other eukaryotes such as shrimps and molluscs. While the detection of these non-target organisms shows the versatility of metabarcoding to detect non-target organisms, this generality could affect the accuracy of metabarcoding for biodiversity estimates. In many cases, contaminants could occur naturally from the host or in the environment where the samples are collected (McKnight et al. 2019). Ficetola et al. (2015) and Liu et al. (2020) attributed contamination as one of the factors that could affect the accuracy of metabarcoding and influence false-positive and false-negative detection. The presence of these contaminants during sampling and DNA extraction and inadequate bioinformatic analyses could mask the presence of target organisms or species,

especially when DNA from the contaminants is more abundant than the target organisms. We adhered to a strict protocol throughout the process; clean equipment and closed containers were used to prevent cross-contamination among sites, but breakthrough contamination could still occur. This was revealed by the unexpected detection of the native shrimp species, *Acetes sibogae.* Although great precaution was taken, the samples may have been contaminated with *A. sibogae* tissues during sampling or laboratory. The abundance and higher affinity of DNA templates of this shrimp species to P12 and P13 primers could also explain the detection of *A. sibogae* in St3 and St4. An eDNA study conducted by Thomsen et al. (2012), targeting fish species at The Sound of Elsinore, Denmark also unexpectedly detected four species of birds that occasionally cross the sampling area during migration.

The potential risk of missed target species due to the presence of contaminants can be overcome by increasing the number of technical replicates (Ficetola et al. 2015). However, the high number of replicates leads increased sequencing costs. Our study pooled five sampling replicates to increase the chances of identifying more species. However, such a strategy is also associated with several disadvantages. It may dilute the DNA of rare species or low abundant species present in the bulk samples, resulting in non-detection of these species and further loss of these rare fish lineage information (Kelly et al. 2014; Shaw et al. 2016; Sato et al. 2017). Sato et al. (2017) stated that the pooling of samples in eDNA metabarcoding is unsuitable if the objective of the study is to assess species richness and alpha diversity of species. To improve the species detection and to avoid false positive and false negative due to pooling of samples, it is advised to sort and extract the specimens individually according to their size before pooling the extracted DNA together or to cut the specimens in similar size for pool DNA extraction (Ji et al. 2013; Elbrecht et al. 2017) which must be considered in future investigations.

Another plausible reason for failure to detect targeted species is low concentration or low affinity of the target species DNA templates to the tested primer pairs, which could be outcompeted by higher affinity DNA templates (Lobo et al. 2017). This was evident by amplifying the abundant *Oryzias javanicus* although not a target species of the newly designed primers. Lower DNA concentration of target species than non-target species may cause false negative of DNA metabarcoding data, as Smith (2017) noted. Furthermore, mismatches between primers and target templates can prevent certain species from being amplified by PCR and lead to failure in species detection (Bru et al. 2008; Deagle et al. 2014; Elbrecht and Leese 2015; Piñol et al. 2015). Lobo et al. (2017) suggested that newly designed primers need to be tested and optimized on individual specimens or using assembled mixtures prior to large-scale analysis of bulk samples. This should be done to prevent mismatches between primers and target templates in bulk samples of metabarcoding. These factors must be taken into account in future investigations.

The large metabarcoding dataset is one of the challenges and difficulties in the bioinformatic analysis for precise taxonomic assignment. One of the major steps in bioinformatic analysis is the trimming of raw sequence reads. The step involves the removal of sequences containing excessive ambiguous or low-confidence base calls (Bokulich et al. 2013; Edgar et al. 2015) to improve the accuracy of the reads. During the trimming process, the parameters must be carefully considered to remove sequencing errors and reads effectively, which can affect downstream diversity and abundance analysis, and loss of reads of low-abundance taxa (Piper et al. 2019). The best-hit classification using alignment-based tools such as BLAST is the most widely used method for taxonomic assignment compared to other methods such as the sequence composition method and phylogenetic method (Piper et al. 2019). However, this simple classification method is prone to over-classifying the query sequence resulting in incorrect species-level taxonomy, especially when the reference data is mislabelled, absent or incomplete, and consequently false-positive and false-negative results (Koski et al. 2001). To overcome this issue that came from the lack of reference data, the sequence may still be robustly assigned to higher taxonomic rank, for example, family level (Porter et al. 2018).

Suitability and cost-efficiency of metabarcoding for biomonitoring of fish larvae

Studies on fish larvae generate important insights on spawning locations and seasons, reproductive biology, nursery grounds, migratory routes of fishes as well as for biomonitoring of habitats (Kidwai and Amjad 2001; Frantine-Silva et al. 2015; Maggia et al. 2017; Mariac et al. 2018; Nobile et al. 2019; Ratcliffe et al. 2021). DNA-metabarcoding coupled with a comprehensive reference database of fish species is now recognised as an efficient cost-effective technique for large-scale studies involving environmental and bulk samples, when laboratory facilities are available (Maggia et al. 2017; Nobile et al. 2019). Besides, it does not require high-level taxonomic expertise for individual identification (Kacev et al. 2018). A recent morphological checklist of the adult fish survey by Zainal Abidin et al. (2021) from the Merbok River and nearby landing sites recorded an additional 75 species (with an overlap of 12 species of larvae) from those of Mansor et al. (2012a b c). We believe more species are yet to be documented through a more rigorous sampling procedure coupled with DNA metabarcoding.

Knowledge of species and population distribution patterns is critical in strategizing biodiversity conservation effort (Thomsen and Willerslev 2015). The current metabarcoding data have furnished helpful information on the species identity and distribution along with an anthropogenically important river system and the alpha- and beta-diversity estimates. The utilisation of eDNA studies in conservation strategies have been highlighted in various organisms; Hajibabaei

et al. (2011) on freshwater benthic macroinvertebrate, Calvignac-Spencer et al. (2013) on carrion flies (blow and flesh flies) to monitor mammal diversity and Ji et al. (2013) on the diversity of insects and birds. Our DNA metabarcoding analysis has provided strong evidence that the Merbok River is still healthy for supporting fish diversity, a piece of welcome news for the local community. However, a more holistic survey on larval and adult fish, including non-commercial species in Merbok River, should be conducted, including temporal and ecological studies. These morphological and molecular databases would be beneficial for strategizing the management and conservation of fisheries in this area. DNA metabarcoding has proven a rapid and cost-effective identification tool for the Merbok River, which could be a model for similar research in other aquatic ecosystems in Malaysia.

CONCLUSIONS

We detected 89 species of fish larvae through metabarcoding with two additional species identified in the validation using newly designed primers, making a total of 91 identified species with >97% species identity with existing databases. Although lower in species richness compared to the morphologically identified species of adult specimens in previous studies, we opine that the probability of low species richness is due to the lower sampling effort, which only focused on a single season (rainy season), and possible technical issues in the sampling, laboratory and bioinformatics analyses that should be addressed in future projects. Nevertheless, the current findings further support the suitability of DNA metabarcoding as a cost-effective approach for investigating species distribution and diversity in this region. In addition, it contributed novel data not recorded in previous studies. We suggest a more holistic fish larvae survey at Merbok River by considering seasonal changes and increased sampling sites. This would enable more comprehensive data to understand the patterns in fish larvae ecology and distribution within the estuarine mangrove habitats and thus their conservation.

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NFMAHA and AA analysed the data. NFMAHA drafted the manuscript. All authors contributed and participated in revising and approving the manuscript.

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Supplementary Materials

Table S1. Summary of assembled scaffolds statistics using MEGAHIT (v1.0.2). (download)

Table S2. Number of scaffolds annotated to *COI* and 12S rRNA genes that were assigned to fish larvae species with blast identity at ≥ 90%. (download)

Table S3. List of newly designed species-specific primer pairs with description of its primer length, product size, GC content (%) and melting temperature (°C). The successfully amplified primer pairs are marked as '√'. (download)