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Identifying Sumatran Peat Swamp Fish Larvae Through DNA Barcoding, Evidence of Complete Life History Pattern

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Abstract

The Eastern Sumatran peat swamp ecosystem is one of the most threatened and most poorly understood biotypes. Until recently, there is no scientific record concerning ichtyoplankton composition within this system and all fish's biodiversity research at this ecosystem relied on morphological diagnosis for adult stages. Two new fish records in this system, *Rasbora pauciperforata* and *Ompok eugeneiatus* were detected. Finally the authors concluded that, eleven fish species complete their life history in Eastern Sumatran peat swamp. This investigation enlarges the COI barcode database for the molecular identification of eastern Sumatran peat swamp fishes.

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014

Keywords: Eastern Sumatran; DNA barcoding; larvae identification; peat swamp

Nomenclature

COI	cytochrome oxidase I	**min**	minute (1 min = 60 s)
ha	hectare	**K2P**	kimura 2 parameter
DNA	deoxyribonucleic acid	**Bp**	base pairs
SL	standard length	**dNTP**	deoxynucleotide
PCR	polymerase chain reaction	**OTU**	operational taxonomic unit
sRNA	large subunit ribosomal RNA	**mM**	micromolar
RNA	ribonucleic acid	**µL**	microliter

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$MgCl_2$	magnesium chloride	**yr**	year (1 yr = 12 mo)
BLAST	basic local alignment search tool	**m**	million = 10^9
MUSCLE	multiple sequence comparison by log expectation		
GBLOCK	program to select blocks of conserved sites		
MEGA	molecular evolutionary genetics analysis		

1. Introduction

There is an urgent need to fully document the biodiversity of the world within 25 yr[1], this is either because rapid loss of biodiversity and only a small fraction of the existing biodiversity is presently described[2,3]. One of the most important natural ecosystems in the world is peat-land. It comprises a unique and a complex ecosystem, which has a global important role in biodiversity conservation at genetic, species and ecosystem levels and contains many species found only or mainly in peat-lands. These species are adapted to the special acidic, nutrient poor and water-logged conditions. They cover over 400 m ha in about 180 countries[4]. Most of the world's tropical (about 62 %) are found in the Indo–Malayan region (80 % in Indonesia, 11 % in Malaysia, 6 % in Papua New Guinea, with small pockets and remnants in Brunei, Vietnam, the Philippines and Thailand[5].

Peat swamp ecosystems are considered as one of the most threatened, neglected, most poorly understood biotopes and their importance is underappreciated[4,6-8]. It is estimated a maximum of only 36 % of the historical peat swamp forest area remains[9]. Forest loss in the lowlands of Sumatra and Kalimantan, the two Indonesian provinces containing the largest areas of peat swamp forest, accounted for more than 70 % of forest clearing in the country from 1990 to 2005, resulting in a staggering 41 % loss in total area in just 15 yr[10]. The race to catalogue biodiversity before it disappears is particularly intense in the peat swamps[8]. Among the faunal groups, fish exhibit the highest endemicity to peat swamps. There are at least 219 fish species identified recorded from peat swamps, among those 80 species are restricted to this ecosystem. Many of these fishes were discovered only in the last 20 yr and many more await formal description[9].

Most of fish's biodiversity research at peat swamp system relies on morphological diagnosis focused on their adult stages or relatively large size where fish can be morphologically distinguished[7,11-14]. Morphological approach has significant limitation pertaining to morphologically cryptic taxa[15]. This is profoundly becoming the case, since[16] suggested that the conditions in peat swamps have favored the evolution of these specialized fish species and peat swamp forests in Southeast Asia are collectively ancient[9].

A DNA-based identification system, founded on the mitochondrial gene, cytochrome *c* oxidase sub unit 1 (COI), can aid the resolution of this diversity[17]. They are powerful tools with an unprecedented accuracy due to their inherently highest possible resolution, which can reach even the level of single base changes[18]. Even, this approach can be applied in diverse developmental stages, such as larvae of fishes[1,18-21] and juveniles to discover diversity, for instance through DNA barcoding of stomatopod larvae, Reference[1] found that stomatopod diversity being much higher than previously believed. This study use established DNA barcoding methodology to investigate known adult species within the peat swamp ecosystem and if previously unknown fish species are found only in larvae stages within this unique ecosystem. Finally to discuss what information could be fuelled aside from primary objective of species identification.

2. Materials and methods

2.1. Samples collection

Diverse developmental stages of peat swamp fishes were collected from eastern lowland Sumatera (02°00' N; 104°02' E; Fig.1), special emphasis was placed on the planktonic larvae. Some of adults and juveniles fishes were taken via electro fishing, nets (leading and wing fyke) to serve as references as DNA sequences of the species reported in peat swamp were not exist in GenBank database. Planktonic larvae were captured using modified hand

scope nets of 1 mm mesh without a closing system, hold to the solid concentration of suspended material. Scope frequently was generally 15 times, nevertheless frequently was occasionally reduced to keep away the net.

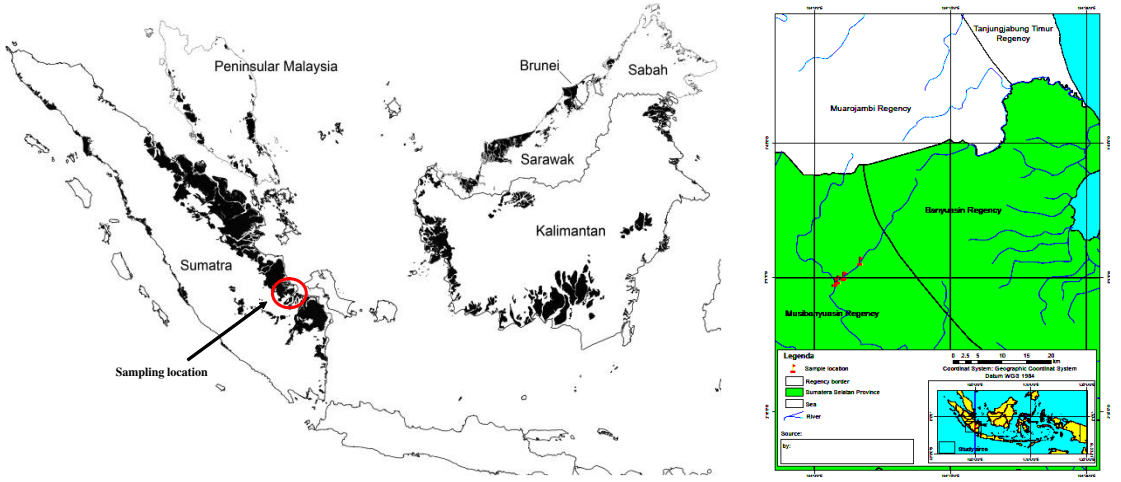

Fig.1. Location of ichtyofauna sampling sites in eastern lowland Sumatera peat swamp, modification after[9].

Diverse developmental stages of peat swamp fishes were collected from eastern lowland Sumatera peat land. All larvae sampled were filtered and transferred to bucket with 50 % ethanol and processed in the laboratory. The stipulation of larval fish encompasses developmental stages from pre-flexion to post-flexion inclusive those juvenile. Afterward, larvae were sorted employing a binocular dissecting microscope, their body length (SL) sized, specimen photo collected and preserved in 95 % ethanol. All adult specimens were stored directly in 95 % ethanol from their time of caught until analysis.

2.2. DNA extraction, PCR amplification and automated sequencing

Sixty-eight ethanol preserved individuals were utilized in the DNA analysis consisting of 14 species of morphologically identified peat swamp fish adults and 54 of unidentified larvae. DNA was extracted from muscle tissue of each specimen using the the extraction kit procedure 'DNeasy Blood & Tissue' (Geneaid and Qiagen) following to the manufacturer's instructions. The partial fragment of mitochondrial Cytochrome C Oxidase Subunit-1gene (CO1) was then amplified employing universal primers Fish-COI-F and COI-Fish-R described by Natalia et al[22]. The 16 sRNA was amplified from unidentified larvae for a second form of identification using the primers 16 sar and 16 sbr[23].

Polymerase chain reaction (PCR) amplifications for both COI and 16sRNA genes were made in a 25 µL of reaction volume consisted 16.75 µL ultrapure water, 2.5 µL of 10× PCR buffer, 2.5 µL $MgCl_2$ (15 mM), 1.0 µL of dNTP (40 mM), 0.5 µL of each primer (1 mM), 0.25 µL of tag polymerase and 1 µL DNA template. PCR cycling parameters included an initial denaturing phase of 10 min at at 95 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 48 °C and 1.5 min at 72 °C and ended with a final extension of at 7 min at 72 °C. PCR products were visualized in 1 % agarose gel, the most robust products were purified using ExoSap PCR clean-up kit and sequenced. A sequencing reaction employing the reverse primer (COI-Fish-R and 16sbr) then performed on some samples to either verify the variation or build clear pattern in the DNA sequence first utilizing the forward primer. Chromatograms were controlled[22] and checked manually.

2.3. Data analysis

Barcoding method[17] is adopted as standard methods for DNA identification. Additional sequences were acquired using BLAST searches of the Genbank database, and they were used to identify specimens. Multi-sequence

alignments for two genes undertook separately using MUSCLE[25]. GBLOCK alligment curation were used[26] to find conserved region within sequences.

Phylogeny tree of Neighbour-joining K2P model with 100 times iteration employing MEGA 5.0[27] was constructed to make a graphic ilustration and a phylogram which unidentified larvae were grouped with sequences of identified taxonomy identity. Specimen was recognized to taxonomy unit only if they formed monophyletic group with maximum 3 % (K2P) sequences divergence followed[17]. Very distinct phylogroups (e.g. clades in excess of 5 % sequence divergence) that may reflect cryptic species[28]. The COI sequences from every sample of the distinct species of adult, supplement those of the unknown larvae morphotype, were submitted separately to the GenBank database and the accession numbers were then acquired (GenBank KM213038-KM213068)

3. Results and discussion

A total of 72 conserved sequences of mitochondrial COI (~376 bp) were analysed from 35 larvae, 13 known adult samples and 24 NCBI Genbank and BOLD database for species identification. The second marker in specimen identification is the conserved large subunit ribosomal RNA (16sRNA) fragments of (~ 323 bp), the marker was able to successfully sequencing seven different samples of larval fish morphotypes. Sequencing failure was found in certain samples employing the COI (for one adult and 19 larvae), although repeated tries under vary PCR condition chemical concentrations. The reference adults represent more than 50 % of known species of this ecosystem. The neighbour–joining Kimura 2 Parameter (K2P) tree sequences in the midst of the references samples exhibited a minimum 18 putative larval OTUs and identified that seems reflect different species based on profound of genetic divergence (Tab.1).

An escalation in genetic different was notified with increases through species to genus extent and hereinafter. Maximum intraspecific variations occurred of 2.8 % a K2P divergence, whilst variations round genera spread from 6.9 % to 25.7 % based on K2P distance (Fig.2). A total of 10 of 18 OTUs larval (55 %) clustered with the reference adult species in monophyletic clades (Fig.2). Those comprised 23 larvae, a 66 % on the whole evaluation of larval placement to species.

Table 1. K2P distances within and among clades using CO1 sequences for peat swamp adults and larvae. OTUs conform to different larvae cluster identified.

OTUs and groups	Maximum divergence within groups (%)	Minimum divergence among groups (%)	Closest sister group
1	0.003	0.069	OTUs 1
Pectenocypris korthusae	0.014	0.069	*Pectenocypris korthusae*
Rasbora. pauciperforata	0.003	0.149	OTUs 1
Rasbora. cephalotaenia	0.000	0.137	OTUs 1
2	n.a	0.152	*Rasbora gracilis*
Rasbora dorsiocellata	0.028	0.122	*Rasbora sumatrana*
3	0.003	0.222	*Rasbora gracilis*
4	n.a	0.149	*Ompok eugeneitus*
Helostoma temminkii	0.000	0.257	*Chitala lopis*
Trichogaster trichopterus	0.000	0.114	*Trichogaster pectoralis*
Trichogaster pectoralis	0.000	0.114	*Trichogaster trichopterus*
Trichopsis vittata	0.022	0.211	*Trichogaster trichopterus*
Anabas testudinae	0.000	0.233	*Channa micropeltis*
5	n.a	0.257	OTUs 6
6	n.a	0.257	OTUs 5
7	n.a	0.166	*Betta fusca*
Hemibagrus nemurus	0.000	0.195	*Bagrichthys macropterus*
8	n.a	0.130	*Mystus bleekeri*

Most of the unidentified larvae in OTU represented by a single individual formed single lineage that potentially cryptic species. Excluding these, OTUs 1 and OTUs 3 were comprised to more individual. The maximum K2P divergence within OTUs 1 was 0.3 % and its proximate sister group was *Pectenocypris korthusae* with a minimum divergence of 6.9 % (Tab.1 and Fig.2). While maximum K2P divergence within OTUs 3 was 0.3 %, with a minimum difference of 22.2 % to *Rasbora gracilis* (Tab.1 and Fig.2). In addition, a bootstrap analysis within Neighbour–joining phylogeny tree (1000 bootstrap) disclosed similarly high degree of support and a total of 184 variable sites were recognized in the midst of the *Rasbora spp (*Fig.3). The number of noted species and cryptic species recently discovered in Rasbora based on[29] were three and 40 (a total of 43 species), hence Cryptic Index-value of Rasbora come to 14.33 (43/3). A second form of identification from 16sRNA confirmed 3 larval into the clade of reference species in NJ (Fig.4). However, within this marker the two unknown larval (OTUs 3 and OTUs 6) were not fitted well with the clades of reference species.

Two new fish records were found in this system, *Rasbora pauciperforata* and *Ompok eugeneiatus* and a minimum 11 fish species complete their life history in eastern Sumatran peat swamp. They are: *Rasbora pauciperforata; Rasbora dorsiocellata; Helostma temminkii; Trichogaster trichopterus; Rasbora. Cephalotaenia; Trichogaster pectoralis; Trichopsis vittata; Anabas testudinae; Pectenocypris korthusae, Parosphromenus deissneri* and *Hemibagrus nemurus.*

The present investigation deputizes the pioneer molecular study of eastern Sumatran peat swamp freshwater ichtyofauna. The DNA barcoding technique used in this survey was powerful in recognizing larvae from just 10 of 18 OTUs larval (55 % or 66 % on the whole evaluation of larval placement to species) to the species extent. This tone generated not from a default of the barcoding approach, yet even either because of lacking reference sequences for described peat swamp ichtyofauna or from diversity revealed by means of using DNA barcoding being much superior. The DNA barcoding methodology used in Indo–Pacific coral reef stomatopods study was able in placement larvae from only 36 % gonodactylid OTUs to species-level. Although owning reference sequences for 91 % of identified Indo–West Pacific Gonodactylid stomatopods[1]. Even when almost all recorded adult references were collected instead there were still encountered unidentified larvae[20].

The molecular identifications of larval fishes yet have some shortage and bottlenecks. The most serious matter is the COI database being patchy, particularly for those non-economic[29]. Similar argument occur to the case of tropical peat swamp ichtyofauna, whereas the inaccessibility, the belief of supporting lower diversity and not welcoming place imply that they received relatively little consideration from scientists[4,6,8]. Thus, it can be understand that barcode database for molecular recognition of larval fish in the peat swamp region still far more than complete. Reference[29], found three more families, six more genera and 13 more species corresponds for larval identification solely because the barcoding database was becoming more equipped and reliable for species identification.

However, the question concerning the possibility existence of species captured as larvae but unwitting in adult peat swamp communities still available since no ancillary species confronted to the adult communities were took in larvae at that area. Hence, outcomes of this investigation denote that either biodiversity in this region is much higher or biodiversity in eastern Sumatran peat swamp has still to be discovered and portrayed. Using DNA barcoding for larvae identification, Paul and Sarah[1] provides a mechanism to measure undiscovered biodiversity of well-studied fauna within The Indo-West Pacific and noted that the biodiversity in this region underestimated by at least half.

The extent of genetic variation monitored for the COI gene sequence was superiorly congruent with the taxonomic degree. Hence, a 3 % of stand shows mostly suffice to differentiate species of peat swamp freshwater ichtyofauna. A 3% distinction in COI has been accepted for utilize in species recognition[17]. The distribution of pairwise differences in midst of DNA barcoding of references expressed little to no overlap in the distribution of discrepancy under and between species.

The potential for cryptic species were identified based on existence polyphyletic lineage of three clusters within genus Rasbora with genetic divergences larger than 5 % and relatively substantial quantity of sites, were revealed (see Fig.2). This argument can apply referring[28] where most distinct phylogroups (e.g. clades in the excess of 5 % sequences divergence) that might mirror cryptic species. Indeed, without morphological examination, identifying and classifying specific DNA fragments under that a species stipulation is problematic. It has been proposed that this problem be overtook thru reverse taxonomy in which invention of novel OTUs leading the collection and identification of the new sequences[30]. At the time when elaborate morphological comparison is established in the

new cryptic species, little but obvious distinction might be frequently discovered. Afterward, the novel species shall be formally identified[33].

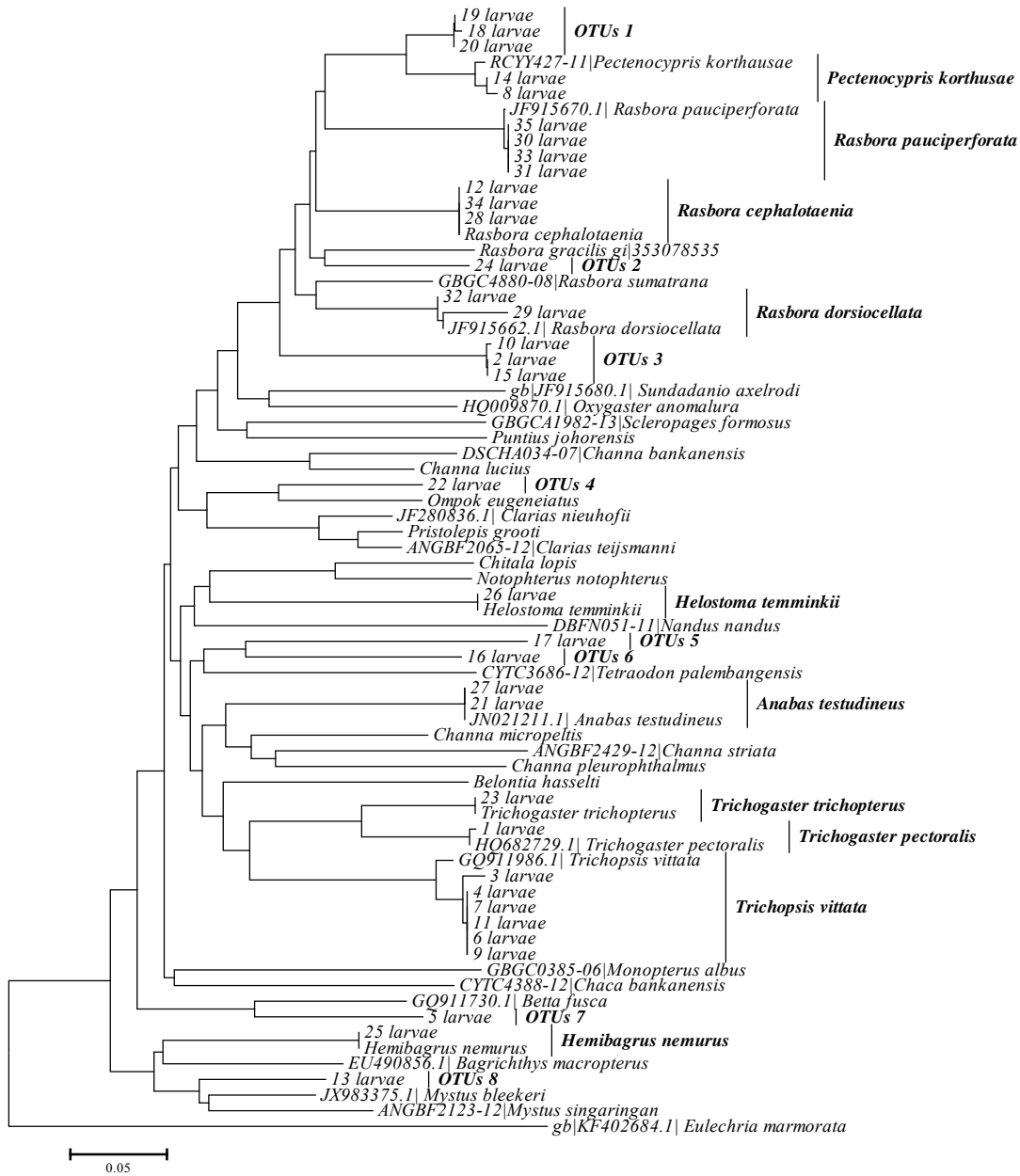

Fig. 2. Neighbour-joining phylogeny tree of CO1 sequences was displaying the assignment of larval haplotype. Branch length scale represents K2P distance.

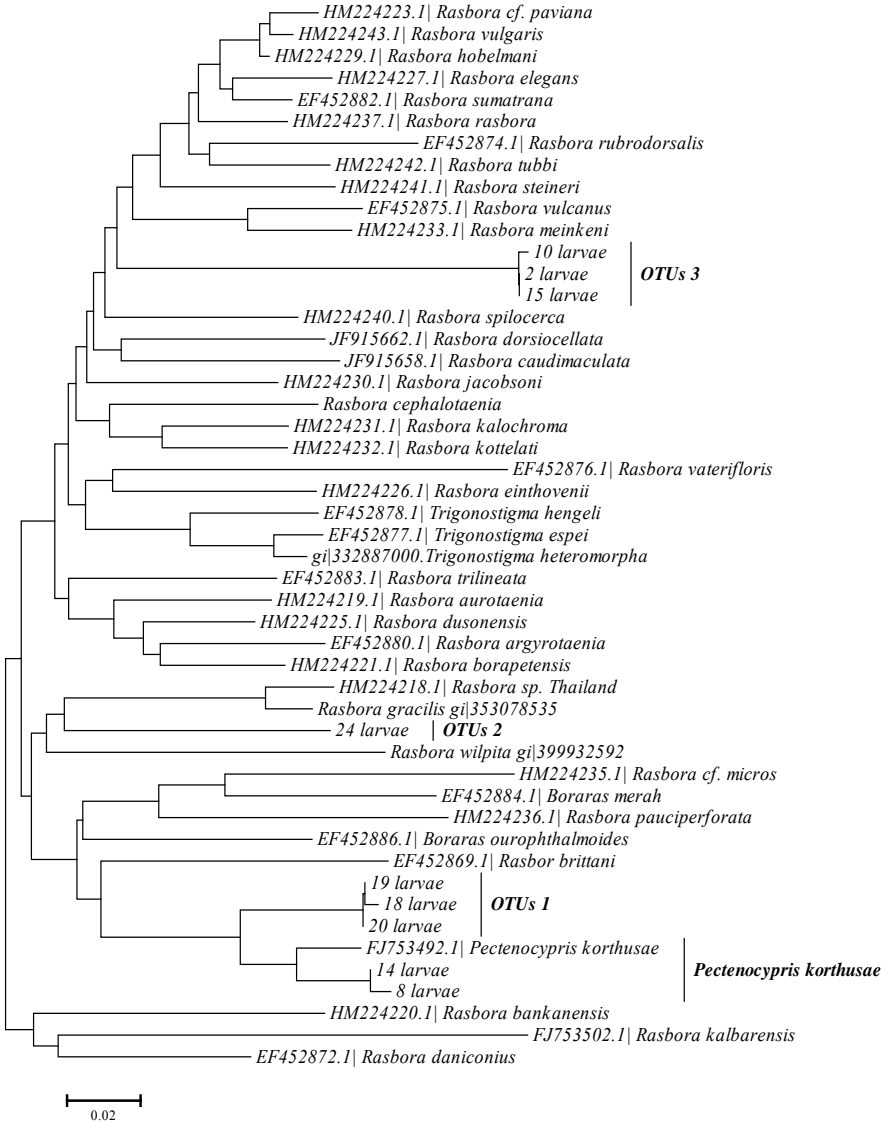

Fig.3. Phylogeny tree of Neighbour–joining of CO1 sequences was displaying the assignment of larval OTUs 1 and OTUs 3 from all known genus Rasbora (Teleostei: Cyprinidae).

In this study, barcodes able to uncover new records of fishes, however it is also likely they were misidentified in barcode library, except that their catalogue specimens were re–examined under qualified fish taxonomists. Defining a species name to a sequence perhaps not constantly is feasible, but knowing that this biodiversity exists and having the DNA sequence is yet worth[1]. Moreover, understanding, the dimensions and distribution of biodiversity is crucial in the perspective of investigating regional patterns of biodiversity gradients[31].

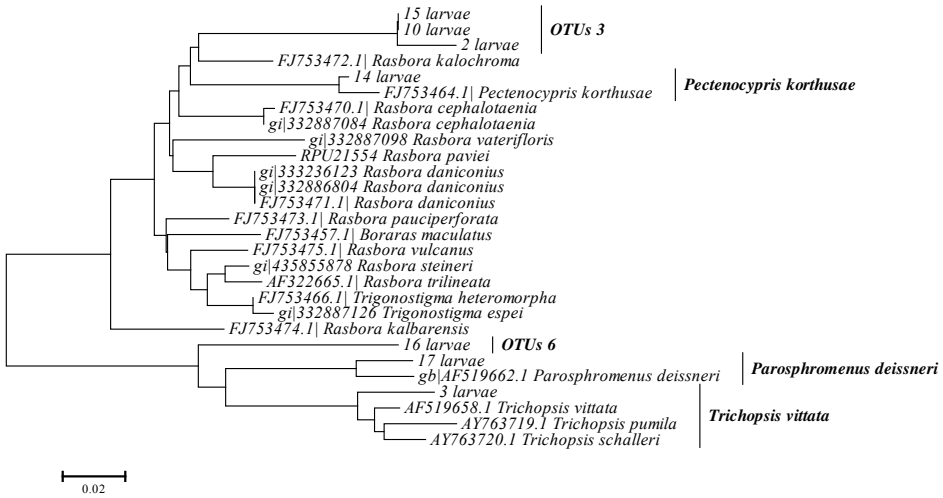

Fig.4. Phylogeny tree of Neighbour–joining of 16sRNA sequences was displaying the assignment of larval OTUs 1, OTUs 3 and unknown larvae.

Despite planktonic larvae appear reasonably prevalent in tropical peat swamp system, yet scientific document concerning comprehensive larvae identification for this system extremely limited. The limited availability of identification keys means it is almost impossible to identify larval specimens solely by their external appearance[20]. In addition, in the early life the morphology of the same species can change quickly and significantly during its development from pre-flexion larvae to post-flexion to the pre-juvenile stage. Thus, the same species at different developmental stages may be identified as a different species when using morphological characters[29]. This present investigation establishes barcode database for larval fish molecular identification of eastern Sumatran peat swamp and verifies the validity barcoding approach for peat swamp larvae identification. Another interesting finding is the confirmations a minimum eleven species that are complete all life histories in this ecosystem. The finding explains the importance of peat swamp ecosystem regarding biodiversity and its particular role in maintaining the existence at least those eleven species. In addition, increasing taxonomic resolution to species level for larvae identification will contribute to our knowledge of larval strategies, timing and dispersal that are undisputedly fundamental factor in fisheries management.

4. Conclusion

Eight larval COI sequences were not known for any published or own barcode sequences that demonstrate the poor sampling of fishes from the Sumatran peat land swamps. Two new fish records in this system, *Rasbora pauciperforata* and *Ompok eugeneiatus* were detected and eleven fish species were confirmed complete their life history in eastern Sumatran peat swamp. They are: *Rasbora pauciperforata; Rasbora dorsiocellata; Helostma temminkii; Trichogaster trichopterus; Rasbora. Cephalotaenia; Trichogaster pectoralis; Trichopsis vittata; Anabas testudinae; Pectenocypris korthusae, Parosphromenus deissneri* and *Hemibagrus nemurus.* This investigation enlarges the COI barcode database for the molecular identification of eastern Sumatran peat swamp fishes. This study contributes to the knowledge of larval strategies, timing and dispersal, which are indisputably fundamental factors in fisheries management.

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