DNA barcoding analysis of larval fishes in Peninsular Malaysia

I.A. Azmir¹, Y.B. Esa^{2,3*}, S.M.N. Amin^{2,4}, M.Y.I. Salwany² and M.Y.F. Zuraina⁵

¹School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Negeri Sembilan, Kampus Kuala Pilah, Pekan Parit Tinggi, 72000 Kuala Pilah, Negeri Sembilan, Malaysia

²Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor Malaysia

³International Institute of Aquaculture and Aquatic Sciences (I-AQUAS), Universiti Putra Malaysia, 71050 port Dickson, Negeri Sembilan, Malaysia

⁴FAO World Fisheries University, Pukyong National University, Busan, 48547, South Korea

⁵Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, Selangor, Malaysia

*Corresponding Author Email : yuzine@upm.edu.my

Paper received: 15.12.2020

Revised received: 17.03.2020

Accepted: 14.08.2020

Abstract

Aim: To identify fish larvae to species level by DNA barcoding method using the mitochondrial Cytochrome c Oxidase I (COI) gene. The lack of species identification work on fish larvae in Peninsular Malaysia has warrant this study to be conducted.

Methodology: Identification of fish larvae species from mangrove areas of Pendas Johor, Matang Perak, Pekan Pahang and Setiu Terengganu was conducted. Samples were collected from April 2015 to September 2015 using a bongo net, towed at a depth of about 0.5 m from the surface for 5 min against the tidal flow. From the total of 354 fish larvae collected, a representative of 177 fish larvae was selected and sequenced using COI gene.

Results: Results from BLAST and BOLD SYSTEM search showed all sequences had high percentage identity index and similarity (90% to 100%). Fish larvae were identified through phylogenetics analysis showing monophyletic status between query sequences with reference sequences obtained from own collection and GenBank. The *Sillago vittata* and *Sillago sihama* sequences was found to be in separate clusters despite their similar genus. A few strong match of specimens from different genus was found with high bootstrap value ($n > 90\%$) through Neighbour-Joining (NJ) and Maximum-likelihood (ML) analysis e.g the *Paramugil parmatus* with *Liza melinoptera* (NJ = 100%, ML = 99%) and *Pseudogobius oligactis* with *Eugnathogobius oligactis* (NJ = 92%, ML 94%).

Interpretation: Identification of fish larvae were best conducted with the aid of molecular method, DNA barcoding in particular rather than comparative taxonomical studies alone that able to identify fish larvae sample to genus level at best.

Key words: Cytochrome c Oxidase I (COI) gene, DNA barcoding, Fish larvae, Mangroves, Peninsular Malaysia, Phylogenetic

How to cite : Azmir, I.A., Y.B. Esa, S.M.N. Amin, M.Y.I. Salwany and M.Y.F. Zuraina: DNA barcoding analysis of larval fishes in Peninsular Malaysia. *J. Environ. Biol.*, **41**, 1295-1308 (2020).

Introduction

Malaysia supports a sizeable area of mangrove forest, which covers 505,382 ha, and has been automatically ranked as the second largest area covered with mangrove forest among Asian countries (Sandilyan and Kathiresan, 2014). About 99,180 ha of mangrove area is distributed in Peninsular Malaysia (Tan, 2007), and the rest of them are in Sabah and Sarawak. Mangrove is a productive area used by aquatic animals, especially the newly hatched fish eggs and fish larvae. The mangrove ecosystem provides high food sources, low predation rate and stable ocean conditions for growth of its occupants (Frietas and Muelbert, 2004). The fish larvae generally reside in shallow waters as larger predators usually avoid such environments; the mangrove (Ara et al., 2011a). However, the vast majority of marine teleost fish have a pelagic larval phase that differs greatly in morphology from the adult. The pelagic stage may last as briefly as 1 week in some damselfish (Pomacentridae) or more than 64 weeks in some porcupine fish (Diodontidae) (Leis and McCormick, 2002). Given this, an established amount of taxonomic key specifically tailored for morphological characteristic identification of fish larvae is required. Otherwise, the identification process will be very challenging considering the rapid development of fish larvae to juvenile stage (short planktonic period) in some fish species, for instance the Damselfish (Wellington and Victor, 1989). As to mediate the process, considerable skills and taxonomic expertise is compulsory (Azmir et al., 2017).

Taxonomic ambiguities have been successfully resolved with the technique called DNA barcoding, which utilizes a short, standardized gene region to identify species (Azmir et al., 2017; Chu et al., 2019). Application of DNA barcoding utilizes the mitochondrial gene instead of nuclear gene as they possess the advantages of having the lack of introns, haploid mode of inheritance, ample selection of primers (Herbert et al., 2003) and a larger number of copies of mitochondrial DNA (mtDNA) present in each cell significantly raises the sensitivity of analysis (Branicki et al., 2003). Thus, the cytochrome c oxidase I (COI) gene was selected for the role of 'universal barcode' considering their relatively high rate of molecular evolution and low in number of insertion or deletion mutations if compared to the ribosomal sequences of 16S (Hebert et al., 2003). According to Ward et al. (2005), COI gene has also proven as a sensitive tool to help discriminate animal identities.

The utilization of mitochondrial DNA, especially cytochrome c oxidase I (COI) gene as a marker in the identification process is fitting as they are present in almost all eukaryotes and have higher evolution rates than nuclear genes, hence confirms it to be the best to discriminate between closely related taxa (Hebert et al., 2003). Through this method, scientists are able to uncover the identity of not only a fully formed organism but also organisms with phenotypically varied or at any stage of development, including egg. In addition, only small amount of

DNA sample retrieved from scale, fin clip, muscle and etc. is enough for amplification and analysis using polymerase chain reaction (PCR) technology (Avisé, 1994).

DNA barcoding has made such headway in solving ambiguities of species identification at relatively low cost (Lewis et al., 2016), thus enabling the historically difficult task of linking marine-larval fish to adult becomes more feasible. In addition, DNA barcoding method also enables the identification of microscopic fish larvae down to species level (Shao et al., 2002). Therefore, the aim of this study was to utilize the DNA barcoding method to genetically identify fish larvae samples from selected mangrove areas in Peninsular Malaysia.

Materials and Methods

Data collection : Fish samples both adults and larvae were collected from mangrove areas in Peninsular Malaysia with seine nets for adults. Fish samples were collected from April 2015 to September 2015. Four mangrove areas were surveyed, namely, Pendas in Johor, Pekan in Pahang, Matang in Perak, and Setiu in Terengganu (Fig. 1). Both Pendas and Matang are located on the west side of Peninsular Malaysia; Pekan and Setiu on the east of Peninsular Malaysia. Sampling was conducted during full moon as moon phases influences the distribution of larval fish species by changing their abundance and composition in the mangrove creeks (Lima et al., 2015). Sampling work was focused during the highest tide by referring to tide tables (National Hydrographic Malaysia, 2015).

Fig. 1 : Sampling stations of fish larvae from four selected mangrove areas in Peninsular Malaysia (Foon et al., 2015).

Sampling of larval fish was done with bongo net (500 µm mesh size) towed at 0.5 m depth from the surface with flow meter attached at the net opening for the rate assessment of water flow. One cycle of towing took 5 min at the speed of 2 knots. The sampling area took place at the selected mangrove area (50 m² area surrounding). Sample caught were preserved in 95% ethanol before it was stored in laboratory. The collection of adult samples was done at the same time and preserved in ice before they were identified to species level by referring to appropriate literature (Atan *et al.*, 2010; Mansor *et al.*, 1998). Details of collection localities were available in Barcode of Life Data System (BOLD) (Ratnasingham and Hebert, 2007) under the project file of 'DNA Barcoding of Fish Larvae from Mangrove areas in Peninsular Malaysia (DNAFL)' and 'DNA Barcoding of Fish from Mangrove areas in Peninsular Malaysia (DBAFM)'.

DNA extraction and purity : DNA extraction of fish larvae and adult fish specimens was done using different kit according to the amount of tissue available. Hence, the extraction method of adult sample was categorized as type A, while fish larvae was categorized as type B. Due to ample amount of adult fish tissue sample available, isolation of 20 mg muscle tissue in 1.5 ml microcentrifuge tube was possible. Type A DNA extraction was done using ReliaPrep™ gDNA Tissue Miniprep System (Promega, USA). The fish larvae were microscopic in size and some of them weighed less than 3mg. Type B of DNA extraction was done with MyTaq™ Extract-PCR Kit (Bioline, USA). The concentration and purity of DNA were subsequently tested using NanoDrop 1000 V3.8 (Thermo Scientific, USA).

PCR amplification and agarose gel electrophoresis : A fragment of 621bp to 636bp of mitochondrial gene was amplified by polymerase chain reaction (PCR), employing primer sets of FishF1 and FishF2 (Ward *et al.*, 2005) at the 5' end of COI gene:

FishF1 (forward): 5'-TCAACCAACCACAAAGACATTGGCAC-3'

FishR1 (reverse): 5'-TAGACTTCT GGGTGGCCAAAGAATCA-3'

FishF2 (forward): 5'- TCGACTAATCATAAAGATATCGGCAC-3'

FishR2 (reverse): 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'

Amplification reaction was performed in a total volume of 25 µl, with Promega (Madison, WI) containing 5X Green Fo Taq® Reaction buffer, 25 mM MgCl₂ solution, deoxynucleotide (dNTP) 40 mM, Forward primer 0.1M, Reverse primer 0.1M, Go Taq® DNA polymerase (5 µl µl⁻¹), DNA template (20 ng) and 13.5 µl of nuclease-free water

The PCR condition was as follows: initial denaturing at 94°C for 2 min, 35 cycles of denaturing at 94°C for 30 sec, annealing at 54°C for 50 sec, extension at 72°C for 40 sec and final extension at 72°C for 5 min. PCR mixture was prepared under sterile conditions to ensure contaminant free.

All PCR products were visualized on 1% agarose gel (Sigma-Aldrich, USA) to check the presence of amplicons of desired size (650bp). Ready to load DNA ladder of 100bp (Promega, USA) was applied in the well of agarose gel stained with 1 µl of GelRed stain (Thermo Fisher Scientific, USA). Electrophoresis condition was set at 80V, 400A, 60 min in 1X TBE buffer. Gel products were viewed and documented via Alphamager HP (Biotechne, USA). Sequencing was done using conventional Sanger sequencing conducted by a private company (First Base Laboratory Sdn. Bhd. Malaysia).

Data analysis : Firstly, the sequences obtained were submitted to BOLD system (<http://www.boldsystems.org/>) and to BLAST of GenBank (<http://www.ncbi.nlm.nih.gov/>) for species identification. Then, the sequencing result of both adult and fish larvae specimens were viewed in Chormas Lite (V 2.1.1) software for trace file viewing and Sequence Scanner (V 2.0) was applied to check on nucleotide base quality. CLUSTAL X (V 2.1) (Larkin *et al.*, 2007) was applied for aligning multiple sequences then the trace files were trimmed manually in BioEdit software (V 7.2.5) (Hall, 1999). Haplotype of the sequences were assessed in DsaSP (V 5.10) and then submitted to Barcode of Life Data System (BOLD) (Ratnasingham and Hebert, 2007) for gaining their own barcode status and GenBank to obtain the accession number.

Phylogenetic assessment : The query data set in this study, which was the fish larvae samples, were assessed for their genetic distances among specimens caught. The pairwise genetic distance among intraspecies, intragenus and intrafamily was calculated in BOLD by 'Distance Summary' command. The calculation was based on the Kimura 2-paramater (K2P) (Kimura, 1980) evolutionary model, while the sequences were aligned using MUSCLE algorithm in MEGA 7 software to ensure no gaps nor were stop codons present in the alignment through amino acid translation. A distance analysis using neighbour-joining (NJ) (Saitou and Nei, 1987) analysis was done with a close-neighbour-interchange (CNI) option implemented in MEGA 7 applying the K2P evolutionary model. Phylogenetic confidence was estimated by bootstrapping replicates of 1000.

Phylogenetic relationship was inferred using three different methods of Bayesian analysis, maximum likelihood (ML) and Neighbour-joining (NJ) (Saitou and Nei, 1987). The bootstrap confidence of 1000 replicates were applied on NJ and ML analyses to evaluate highest likelihood of a tree. Bayesian phylogenetic analysis was done in MrBayes V 3.2.2 (Huelsenbeck and Ronquist, 2001). Markov Chain Monte Carlo (MCMC) process was used to approximate the posterior probabilities of trees which tend to be higher than bootstrap support values calculated in NJ and ML tree. The process was set to 4 X 10⁶ generations with tree being sampled every 100 generations. Saturation test was done on all codons using DAMBE V 6.4.29 (Xia, 2013). The trees were rooted using

sequences of *Megaptera novaeangliae* (Balaenopteridae) and *Hemibagrus nemurus* (Bagridae), obtained from GenBank.

Results and Discussion

DNA barcodes fish larvae species assignments : The discrimination of 38 out of 41 species (93%) of fish larvae was successfully conducted using DNA barcoding method and further supported with the genetic distance calculation and sequence divergence approach. The results demonstrated the existence of a barcode gap for the species analyzed, thus confirming its effectiveness as a powerful measure for species identification. The presence of barcode gap between lowest interspecific and the highest intraspecific genetic distance indicated correct species identification between samples (Shen *et al.*, 2016). Out of 153 fish larvae sequenced, they corresponded to be the representative of 18 families, 33 genera and 41 species of fish larvae. The number of specimens found per species varied from 1 specimen to 7 specimens per species. Single specimen (singleton) was found in many species including *Ambassis* sp., *Chelonodon patoca*, *Dendrophysa russelii*, *Eugnathogobius variegates*, *Exyrias puntang*, *Hippichthys cyanospilos*, *Hippichthys penicillus*, *Leiognathus ruconius*, *Monacanthus chinensis*, *Mugilogobius chulae*, *Ostorhinchus fasciatus*, *Paramonacanthus sulcatus*, *Paramugil parmatus*, *Photopectoralis bindus*, *Sillago sihama*, *Sillago vittata* and *Upeneus margarethae* (Table 1).

The correct discrimination of 95% of analyzed species (38/41) was done based on the unique haplotypes obtained from 117 barcoded sequences. Out of 117 sequences, 98 haplotypes were assigned with GenBank accession and BOLDSYSTEM index number (Table 2). All amplified fragments of fish larvae specimens inhabiting the mangrove areas of Peninsular Malaysia contained 621 bp and sequences showed good quality, with no evidence of insertions, deletions or stop codon. There were 285 (45.9%) conserved sites, 336 (54.1%) variable sites and 288 (46.4%) parsimony informative sites found in the partial sequences of successfully amplified mitochondrial *COI* gene. No pseudogene in DNA structure was confirmed through translation of 98 haplotypes for conserved 621 bp fragment containing 206 amino acids.

The within species average K2P distance was 1.00% compared to 20.19% for species within genera (Table 3). Thus, it was approximately 20 times more divergent than conspecific individuals. This value was an appropriate threshold value according to Hebert *et al.* (2003) for flagging genetically divergent specimen as definite species. DNA barcoding distinguished species based on nucleotide sequences whenever interspecific variation was greater than the intraspecific (Ajmal Khan *et al.*, 2014).

The mean K2P distance values were found incongruous for conspecific and congeneric comparisons (1.00% and 20.19%)

with the ranged recorded in the study of Neotropical freshwater fish where the value was from zero to 8.5% and from zero to 24.8% (Pereira *et al.*, 2013); from zero to 3.3% and from 1.9% to 28.2% Yucatan fish (Valdez-Moreno *et al.*, 2010). High number of mean values observed in congeneric species reflected low number of closely related species analyzed. The low number of species analyzed might be a contributing factor to these mean values. Other than that, it was also caused by a few genera represented by only one or more species (Pereira *et al.*, 2013). Thus, an increase in number of species per genus can help in decreasing the mean value of congeneric.

Assignment of fish larvae species were from the public reference records in BOLD Indo-Pacific libraries, GenBank and also based on the adult dataset collected in this study. About 17% (7 species) of the query matched the own collection of adult datasets, while the remaining query were matched with the reference barcodes libraries (BOLD and GenBank). Successful species assignments were achieved in large number of cases with 98% and above similarity (78%, 32/41) with divergent value below 2%. Value of <2% for species divergent indicated an extreme distant fish populations among the studied samples (Ward *et al.*, 2008), which at the same time indicated that the populations were part of single species (Hebert *et al.*, 2003) and genetic variability did not increase when the species originated from similar region (Shen *et al.*, 2016).

About 22% (9/41) were found matched to 90-98% similarity with the reference barcode libraries with >2% divergence between species. The exception of having >2% divergence occurred in *Opisthonema libertate* (2.63%), *Sardinella melanura* (2.79%), *Stolephorus indicus* (2.79%), *Ophiocara porocephala* (3.14%), *Periophthalmus* sp. (4.51%), *Sicyopterus* sp. (6.8%), *Hemigobius hoevenii* 2.62%), *Thryssa hamiltonii* (3.46%) and *Gerres filamentosus* (2.79%). Nevertheless, all species with >2% conspecific divergence were unambiguously identified using *COI* barcoding proven by K2P distance to the nearest neighbours of min 16.01% (Table 4).

Possibility of species mis-assignment may occur to a morphologically close sister species, especially when there is shortfall in DNA barcode data of species within a given group (Landi *et al.*, 2014). Nevertheless, the K2P distanced to their nearest neighbors were in the range of 16.01% until 21.5% where Hebert *et al.* (2003) have set a 10-fold sequence difference between the average interspecific and average intraspecific as a threshold for animal species identification.

Discrepancies in species assignment : A fraction of small dubious species assignments was detected on 7% of the specimen whereby they were assigned up to genus level only (<90% match) (Table 5). A few studies have also found ambiguities and mismatches through DNA barcode data (Diaz *et al.*, 2016; De Carvalho *et al.*, 2011; Mabragaña *et al.*, 2011). However, the

Table 1 : Species assignment of 153 barcoded fish larvae from four mangrove areas surveyed in Peninsular Malaysia

Family	Species	%		Occurrence			
		Genbank Identity	BOLD Similarity	Pendas	Pekan	Matang	Setiu
Ambassidae	<i>Ambassis dussumieri</i>	95	100				1
	<i>Ambassis marianus</i>	99		4	5		
	<i>Ambassis nalua</i>	99		1	1		
	<i>Ambassis</i> sp.	82					1
Apogonidae	<i>Ostorhinchus fasciatus</i>	99		1			
Clupeidae	<i>Hilsa kelee</i>	99		2			
	<i>Opisthonema libertate</i>	99		2			
	<i>Sardinella melanura</i>	100		2			
Eleotridae	<i>Ophiocara porocephala</i>	-	85			2	
Engraulidae	<i>Thryssa kammalensis</i>	99			13		
	<i>Thryssa hamiltonii</i>	99			6		
	<i>Stolephorus indicus</i>	99		1		1	
Gerreidae	<i>Gerres filamentosus</i>	93		2			
Gobiidae	<i>Boleophthalmus boddarti</i>	100		2	1	2	
	<i>Brachygobius kabiliensis</i>	100				1	
	<i>Exyrias puntang</i>	99		1			
	<i>Eugnathogobius variegatus</i>	99			1		
	<i>Hemigobius hoevenii</i>	99				20	
	<i>Glossogobius aureus</i>	99		8			
	<i>Gobiopterus lacustris</i>	99				10	
	<i>Mugilogobius chulae</i>	97			1		
	<i>Periophthalmus</i> sp.	*			1	1	
	<i>Pseudogobiopsis oligactis</i>	97			2		
	<i>Pseudogobius javanicus</i>	99		1	2		
	<i>Pseudogobius masago</i>	98			10	1	
	<i>Pseudogobius olorum</i>	85				10	
	<i>Sicyopterus</i> sp.	99			2	1	
Leiognathidae	<i>Photopectoralis bindus</i>	99		1			
	<i>Leiognathus ruconius</i>	99		1			
Monacanthidae	<i>Monacanthus chinensis</i>	99		1			
	<i>Paramonacanthus sulcatus</i>	99		1			
Mugilidae	<i>Paramugil parmatius</i>	99			1		
Mullidae	<i>Upeneus margarethae</i>	99					1
Scatophagidae	<i>Scatophagus argus</i>	100		2	1		
Sciaenidae	<i>Dendrophysa russelii</i>	99				1	
Sillaginidae	<i>Sillago sihama</i>	99			1		
	<i>Sillago vittata</i>	*		1			
Sparidae	<i>Sparus aurata</i>	99		15			
Syngnathidae	<i>Hippichthys cyanospilos</i>	95		1			
	<i>Hippichthys penicillus</i>	94		1			
Tetraodontidae	<i>Chelonodon patoca</i>	98		1			
	Undertermined					3	

availability of high-quality reference sequences in public sequence libraries (BOLD and GenBank) are important to avoid any discrepancies in confirmation of species. Lack of sufficient and adequate data will lead the blast searches to an ambiguous result. This situation might occur from misidentification of voucher specimen, contamination during sample processing, insufficient taxonomic identification or synonym and syntax problems (Ward, 2012; Radulovici et al., 2010; Tautz et al., 2003).

In this study, three congeneric species of *Pseudogobius*; *Pseudogobius masago*, *Pseudogobius olorum* and *Pseudogobius javanicus* exhibited high average congeneric distance (mean distance 19.47%, minimum distance 18.03%), with two former species clustered as sister taxa, while *Pseudogobius javanicus* formed a separate branch in NJ (Fig. 2) and Bayesian (Fig. 4). In ML tree, all three species formed separate branch with unique set of COI haplotypes (Fig. 3). The

Table 2 : Summary of 98 observed mitochondrial DNA haplotypes in larval fish specimens from mangrove areas of Peninsular Malaysia, Malaysia

Species	BOLDSYSTEM index Number (BIN)	GenBank accession	Sampling area	Sample ID
<i>Ambassis dussumieri</i>	BOLD:ADB1883	KX223884	Setiu Terengganu	SA14A1
<i>Ambassis marianus</i>	BOLD:ACQ5401	KX223888	Pendas Johor	PR3A5_1
<i>Ambassis marianus</i>	BOLD:ACQ5401	KX223889	Pendas Johor	PC3A1
<i>Ambassis marianus</i>	BOLD:ACQ5401	KX223887	Pekan Pahang	CA1A1
<i>Ambassis marianus</i>	BOLD:ACQ5401	KX223886	Pendas Johor	PA1A4
<i>Ambassis marianus</i>	BOLD:ACQ5401	KX223885	Pekan Pahang	CA2A2
<i>Ambassis marianus</i>	BOLD:ACQ5401	KX144849	Pendas Johor	PP1A2
<i>Ambassis nalua</i>	BOLD:ADA4639	KX223932	Pendas Johor	PC2A6
<i>Ambassis nalua</i>	BOLD:ADA4639	KX144850	Pekan Pahang	CA3A3
<i>Ambassis sp.</i>	BOLD:ACE0269	KX223890	Setiu Terengganu	Sp1A2
<i>Boleophthalmus boddarti</i>	BOLD:AAW7430	KY849515	Matang Perak	BBM2
<i>Boleophthalmus boddarti</i>	BOLD:AAW7431	KY849544	Matang Perak	BBM1
<i>Boleophthalmus boddarti</i>	BOLD:ADC7579	KY849516	Pekan Pahang	BBC2
<i>Boleophthalmus boddarti</i>	BOLD:AAW7430	KY849511	Pendas Johor	BBP1
<i>Boleophthalmus boddarti</i>	BOLD:AAW7431	KY849537	Pendas Johor	BBP8
<i>Brachygobius kabiliensis</i>	BOLD:ADB2718	KX223927	Matang Perak	MG92G12
<i>Chelonodon patoca</i>	BOLD:AAF2666	KX223894	Pendas Johor	PB1U1
<i>Sillago vittata</i>	BOLD:ADB2362	KX223895	Pendas Johor	PI111
<i>Dendrophysa russelii</i>	BOLD:AAD4938	KX223896	Matang Perak	Mp1Q1
<i>Eugnathogobius variegatus</i>	BOLD:AAW8350	KX223930	Pekan Pahang	CG36G19
<i>Exyrias puntang</i>	BOLD:ACT3463	KX223899	Pendas Johor	PG11G8
<i>Gerres filamentosus</i>	BOLD:AAM2324	KX223900	Pendas Johor	PH1H4
<i>Gerres filamentosus</i>	BOLD:ADB1938	KX281922	Pendas Johor	PC4H1
<i>Glossogobius aureus</i>	BOLD:ADB2538	KX223901	Pendas Johor	PG1G4
<i>Glossogobius aureus</i>	BOLD:ADB2538	KX281923	Pendas Johor	PG23G11
<i>Glossogobius aureus</i>	BOLD:ADB2538	KX223898	Pendas Johor	PG22G10
<i>Gobiopterus lacustris</i>	BOLD:ADB1051	KX223905	Matang Perak	MG5G5
<i>Gobiopterus lacustris</i>	BOLD:ADB1051	KX223902	Matang Perak	MG4G4
<i>Gobiopterus lacustris</i>	BOLD:ADB1051	KX281924	Matang Perak	MG87G11
<i>Gobiopterus lacustris</i>	BOLD:ADB1051	KX281925	Matang Perak	MA3G32
<i>Gobiopterus lacustris</i>	BOLD:ADB1051	KX223903	Matang Perak	MG101G13
<i>Hemigobius hoevenii</i>	BOLD:ADD2192	KX281927	Matang Perak	MG120G33
<i>Hemigobius hoevenii</i>	BOLD:AAE5276	KX223907	Matang Perak	MA2G30
<i>Hemigobius hoevenii</i>	BOLD:AAE5276	KX223906	Matang Perak	MG13G35
<i>Hemigobius hoevenii</i>	BOLD:AAE5276	KX281926	Matang Perak	MG171G19
<i>Hemigobius hoevenii</i>	BOLD:AAE5276	KX281928	Matang Perak	MG160G18
<i>Hemigobius hoevenii</i>	BOLD:AAE5276	KX223908	Matang Perak	MG80G9
<i>Hemigobius hoevenii</i>	BOLD:AAE5276	KX223909	Matang Perak	MG64G38
<i>Hilsa kelee</i>	BOLD:AAC0856	KX223912	Pendas Johor	PE2K1
<i>Hilsa kelee</i>	BOLD:AAC0856	KX223913	Pendas Johor	PK4K3
<i>Hippichthys cyanospilos</i>	BOLD:ADB2646	KX223915	Pendas Johor	PW1Y1
<i>Hippichthys penicillus</i>	BOLD:ADB2645	KX223914	Pendas Johor	PW2Y2
<i>Leiognathus ruconius</i>	BOLD:ADB1713	KX223916	Pendas Johor	PS4L2
<i>Monacanthus chinensis</i>	BOLD:AAB6368	KX223920	Pendas Johor	PT1M1
<i>Mugilogobius chulae</i>	BOLD:ADB2817	KX223921	Pekan Pahang	CG18G3
<i>Ophiocara porocephala</i>	BOLD:ADB1822	KX223922	Matang Perak	MG54e2
<i>Ophiocara porocephala</i>	BOLD:ADB1822	KX281930	Matang Perak	MG47e1
<i>Opisthonema libertate</i>	BOLD:AAF2460	KX223911	Pendas Johor	PK1K2
<i>Opisthonema libertate</i>	BOLD:AAF2460	KX223910	Pendas Johor	PK12K6
<i>Ostorhinchus fasciatus</i>	BOLD:AAC1242	KX223924	Pendas Johor	PD1J
<i>Paramonacanthus sulcatus</i>	BOLD:AAC5161	KX223925	Pendas Johor	PT2M2
<i>Paramugil parmatus</i>	BOLD:ABU7210	KX223926	Pekan Pahang	CN1m1
<i>Periophthalmus sp.</i>	BOLD:ADB2719	KX223929	Pekan Pahang	CG26G10
<i>Periophthalmus sp.</i>	BOLD:ADB2243	KX223928	Matang Perak	MG99G23
<i>Photopectoralis bindus</i>	BOLD:AAC5681	KX223931	Pendas Johor	PC1L1

Cont....

<i>Pseudogobiopsis oligactis</i>	BOLD:ADB1314	KX281931	Pekan Pahang	CG17G2
<i>Pseudogobiopsis oligactis</i>	BOLD:ADB1314	KX223933	Pekan Pahang	CG35G16
<i>Pseudogobius javanicus</i>	BOLD:AAD9462	KX223936	Pendas Johor	PG2G5
<i>Pseudogobius javanicus</i>	BOLD:AAD9462	KX223937	Pekan Pahang	CG32G17
<i>Pseudogobius javanicus</i>	BOLD:AAD9462	KX223935	Pekan Pahang	CG41G14
<i>Pseudogobius masago</i>	BOLD:ACC0583	KX144852	Pekan Pahang	CG19G4
<i>Pseudogobius masago</i>	BOLD:ACC0583	KX144851	Pekan Pahang	CG33G13
<i>Pseudogobius masago</i>	BOLD:ACC0583	KX223938	Matang Perak	MG118G16
<i>Pseudogobius masago</i>	BOLD:ACC0583	KX223939	Pekan Pahang	CG21G6
<i>Pseudogobius masago</i>	BOLD:ACC0583	KX223940	Pekan Pahang	CG24G9
<i>Pseudogobius masago</i>	BOLD:ACC0583	KX223941	Pekan Pahang	CG27G11
<i>Pseudogobius olorum</i>	BOLD:ADB2106	KX223943	Matang Perak	MG159G17
<i>Pseudogobius olorum</i>	BOLD:ADB2106	KX223944	Matang Perak	MG15G36
<i>Pseudogobius olorum</i>	BOLD:ADB2106	KX281936	Matang Perak	MG2G2
<i>Pseudogobius olorum</i>	BOLD:ADB2106	KX281934	Matang Perak	MG12G34
<i>Pseudogobius olorum</i>	BOLD:ADB2106	KX223942	Matang Perak	MG3G3
<i>Sicyopterus</i> sp.	BOLD:AAE7961	KX223934	Matang Perak	MA1G31
<i>Sardinella melanura</i>	BOLD:AAB7198	KX223945	Pendas Johor	PK6K4
<i>Sardinella melanura</i>	BOLD:ADB1709	KX281937	Pendas Johor	PK7K5
<i>Scatophagus argus</i>	BOLD:AAB3531	KX223948	Pendas Johor	PS1S4
<i>Scatophagus argus</i>	BOLD:AAB3531	KX223947	Pekan Pahang	CS1S1
<i>Scatophagus argus</i>	BOLD:AAB3531	KX223946	Pendas Johor	PS3S3
<i>Sicyopterus</i> sp.	BOLD:ADB1338	KX281932	Pekan Pahang	CG34G18
<i>Sicyopterus</i> sp.	BOLD:ADB1338	KX223893	Pekan Pahang	CG23G8
<i>Sillago sihama</i>	BOLD:ABZ1631	KX223949	Pekan Pahang	CG14Z1
<i>Sparus aurata</i>	BOLD:ADB2220	KX223953	Pendas Johor	P13V1
<i>Sparus aurata</i>	BOLD:ADB2220	KX223952	Pendas Johor	PI5V2_1
<i>Sparus aurata</i>	BOLD:ADB2220	KX223951	Pendas Johor	PI7V4
<i>Sparus aurata</i>	BOLD:ADB2220	KX223950	Pendas Johor	PI6V3
<i>Sparus aurata</i>	BOLD:ADB2220	KX223954	Pendas Johor	PG9V7_1
<i>Stolephorus indicus</i>	BOLD:ADB1383	KX281939	Matang Perak	MK15E1
<i>Stolephorus indicus</i>	BOLD:AAB7978	KX223955	Pendas Johor	PK15E1
<i>Thryssa hamiltonii</i>	BOLD:AAB6419	KX223956	Pekan Pahang	CE11E16
<i>Thryssa hamiltonii</i>	BOLD:AAB6419	KX281941	Pekan Pahang	CE12E17
<i>Thryssa hamiltonii</i>	BOLD:AAB6419	KX281942	Pekan Pahang	CE3E9
<i>Thryssa hamiltonii</i>	BOLD:AAB6419	KX281940	Pekan Pahang	CE7E13
<i>Thryssa hamiltonii</i>	BOLD:AAB6419	KX223957	Pekan Pahang	CE1E7
<i>Thryssa hamiltonii</i>	BOLD:AAB6419	KX223958	Pekan Pahang	CE18E20
<i>Thryssa kammalensis</i>	BOLD:ADB1427	KX223962	Pekan Pahang	CG5E2
<i>Thryssa kammalensis</i>	BOLD:ADB1427	KX223959	Pekan Pahang	CG6E3
<i>Thryssa kammalensis</i>	BOLD:ADB1427	KX223960	Pekan Pahang	CG4E1
<i>Thryssa kammalensis</i>	BOLD:ADB1427	KX223961	Pekan Pahang	CG7E4
<i>Upeneus margarethae</i>	BOLD:AAB9714	KX223963	Setiu Terengganu	SR1N1

Table 3 : Genetic divergences of Kimura 2-parameters (K2P) at different taxonomic level for fish larvae specimens from mangrove areas of Peninsular Malaysia, Malaysia

Comparison within			Distance			
	Taxa	Comparisons	Min (%)	Mean (%)	Max (%)	SE (%)
Species	22	138	0	1	10.29	0.01
Genus	5	118	13.29	20.19	25.85	0.02
Family	5	924	16.01	23.31	30.96	0

discordance formed might signal the presence of cryptic species among studied samples.

In the case of *Sillago vittata* from GenBank and larva of

Sillago vittata, each of the two nominal species, formed cohesive units in NJ and ML trees but were differently clustered in the Bayesian tree (Fig. 4) with genetic divergence value of 5.52%, indicating the samples could be considered as two different

Table 4 : DNA barcode gap between fish larvae in selected mangrove areas of Peninsular Malaysia, Malaysia

Species	Mean intra-sp	Max intra-sp	Nearest species	Distance to NN
<i>Dendrophysa russelii</i>	N/A	N/A	<i>Ostorhinchus fasciatus</i>	26.01
<i>Hilsa kelee</i>	0.49	0.49	<i>Opisthonema libertate</i>	19.5
<i>Opisthonema libertate</i>	2.63	2.63	<i>Hilsa kelee</i>	19.5
<i>Sardinella melanura</i>	2.79	2.79	<i>Hilsa kelee</i>	19.54
<i>Stolephorus indicus</i>	2.79	2.79	<i>Hilsa kelee</i>	20.86
<i>Thryssa hamiltonii</i>	1.46	3.46	<i>Thryssa kammalensis</i>	20.3
<i>Thryssa kammalensis</i>	0.27	0.49	<i>Thryssa hamiltonii</i>	20.3
<i>Ambassis dussumieri</i>	N/A	N/A	<i>Ambassis marianus</i>	13.29
<i>Ambassis marianus</i>	0.49	0.97	<i>Ambassis dussumieri</i>	13.29
<i>Ambassis nalua</i>	0	0	<i>Ambassis sp.</i>	21.76
<i>Ambassis sp.</i>	N/A	N/A	<i>Ambassis nalua</i>	21.76
<i>Ophiocara porocephala</i>	3.14	3.14	<i>Monacanthus chinensis</i>	20.09
<i>Boleophthalmus boddarti</i>	0.89	1.92	<i>Sicyopterus sp.</i>	20.13
<i>Brachygobius kabiliensis</i>	N/A	N/A	<i>Monacanthus chinensis</i>	19.75
<i>Eugnathogobius variegatus</i>	N/A	N/A	<i>Pseudogobius olorum</i>	19.14
<i>Exyrias puntang</i>	N/A	N/A	<i>Scatophagus argus</i>	20.52
<i>Glossogobius aureus</i>	0.43	0.65	<i>Exyrias puntang</i>	20.9
<i>Gobiopterus lacustris</i>	0.49	0.81	<i>Sicyopterus sp.</i>	20.21
<i>Hemigobius hoevenii</i>	1.09	2.62	<i>Pseudogobius olorum</i>	18.95
<i>Mugilogobius chulae</i>	N/A	N/A	<i>Sicyopterus sp.</i>	16.01
<i>Periophthalmus sp.</i>	4.51	4.51	<i>Mugilogobius chulae</i>	19.85
<i>Pseudogobiopsis oligactis</i>	1.14	1.14	<i>Sillago sp.</i>	20.93
<i>Pseudogobius javanicus</i>	0.43	0.65	<i>Mugilogobius chulae</i>	16.9
<i>Pseudogobius masago</i>	0.4	0.65	<i>Pseudogobius olorum</i>	18.03
<i>Pseudogobius olorum</i>	1.07	1.63	<i>Sicyopterus sp.</i>	17.5
<i>Sicyopterus sp.</i>	6.8	10.29	<i>Mugilogobius chulae</i>	16.01
<i>Ostorhinchus fasciatus</i>	N/A	N/A	<i>Ambassis marianus</i>	19.58
<i>Paramugil parmatus</i>	N/A	N/A	<i>Monacanthus chinensis</i>	19.61
<i>Gerres filamentosus</i>	2.79	2.79	<i>Ambassis dussumieri</i>	21.5
<i>Leiognathus ruconius</i>	N/A	N/A	<i>Photopectoralis bindus</i>	22.07
<i>Photopectoralis bindus</i>	N/A	N/A	<i>Sillago sp.</i>	21.77
<i>Upeneus margarethae</i>	N/A	N/A	<i>Monacanthus chinensis</i>	20.52
<i>Scatophagus argus</i>	0.32	0.48	<i>Ambassis marianus</i>	19.29
<i>Sillago sihama</i>	N/A	N/A	<i>Hilsa kelee</i>	20.4
<i>Sillago sp.</i>	N/A	N/A	<i>Pseudogobiopsis oligactis</i>	20.93
<i>Sparus aurata</i>	0.68	1.47	<i>Sillago sp.</i>	21.99
<i>Hippichthys cyanospilos</i>	N/A	N/A	<i>Hippichthys penicillus</i>	19.09
<i>Hippichthys penicillus</i>	N/A	N/A	<i>Hippichthys cyanospilos</i>	19.09
<i>Monacanthus chinensis</i>	N/A	N/A	<i>Paramonacanthus sulcatus</i>	16.24
<i>Paramonacanthus sulcatus</i>	N/A	N/A	<i>Monacanthus chinensis</i>	16.24
<i>Chelonodon patoca</i>	N/A	N/A	<i>Paramugil parmatus</i>	22.99

Note: N/A is not available.

Table 5 : Nearest match in identification of fish larvae specimens from mangrove areas of Peninsular Malaysia, Malaysia

Query Species	GenBank		BOLD	
	Species assignment	Identity (%)	Species assignment	Similarity (%)
<i>Ambassis sp.</i>	<i>Ambassis marianus</i>	82	<i>Ambassis nalua</i>	85
<i>Sillago vittata</i>	<i>Cirripectes stigmatus</i>	84	<i>Sillago sp.</i>	Unresolved
<i>Periophthalmus sp.</i>	<i>Periophthalmus modestus</i>	84	<i>Periophthalmus kalolo</i>	82
<i>Ophiocara porocephala</i>	<i>Lutjanus sp.</i>	83	<i>Ophiocara porocephala</i>	85
<i>Pseudogobius olorum</i>	<i>Pseudogobius olorum</i>	85	<i>Pervagor aspricaudus</i>	85

Fig. 2 : Neighbour-Joining (NJ) tree showing relationships among 98 haplotypes of fish larvae sampled from selected mangrove areas in Peninsular Malaysia. Each node represented with bootstrap value (%) after 1000 pseudoreplications of NJ analysis.

species. A similar case of deep genetic divergence between the two species also occurred between the Brazilian cusk eel, *Genypterus brasiliensis* and the Pink cusk-eel, *G. blacodes*. The finding was further supported by both morphological and molecular approaches proving that *Genypterus blacodes* and *G. brasiliensis* should be pronounced as a separate species (Mabragaña et al., 2011). Hebert et al., (2003) demonstrated that collection of multiple samples within each species would help to avoid paraphyly or polyphyly assignment of a species, as in case of *sillago vittata*. Simultaneously, the lack of large and robust database with reliable reference sequences might be the reason for this shortcoming similar to the situation faced in the study of

Indo-Pacific larvae by Leis (2015) and fish larvae study in Eastern Atlantic Ocean by Ardura et al. (2016).

Vague species assignment also occurred in few specimens where the issue stems from recent taxonomic changes in species nomenclature, for example in the cases of *Paramugil parmatius/Liza melinoptera* and *Pseudogobius oligactis/Eugnathogobius oligactis*. Both cases were found to be a synonym name of assigned species whereby the former assigned name was most updated naming used (Fishbase.org, 2017). According to Pusey (2001), synonymy simply occurs when naming of the species is done in different manner by two or

Fig. 3 : Maximum likelihood (ML) tree showing relationships among 98 haplotypes of fish larvae sampled from selected mangrove areas in Peninsular Malaysia. Each node represented with bootstrap value (%) after 1000 of pseudoreplications.

more authors. This situation will require revision to make sure that the nomenclature remains correct, relevant and accurately reflects evolutionary history.

High values of intraspecific genetic divergence (>2%) : There were several species displaying >2% intraspecific divergence where 9 species (21.95%) were further subdivided into two or more sublusters. Each cluster showed a strong bootstrap value of

100%, except in *Thryssa hamiltonii* and *Hemigobius hoevernii*. Deep intraspecific divergence has also been reported in few other barcoding studies involving bird (Milá *et al.*, 2012), scorpion (Coelho *et al.*, 2014), mollusk (Ye *et al.*, 2015) and cichlid fish (Colatrelli *et al.*, 2012) which suggested underestimation of species diversity.

Sardinella melanura (2.79%), *Stolephorus indicus*

Fig. 4 : Bayesian inference (BI) showing relationships among 98 haplotypes of fish larvae sampled from selected mangrove areas in Peninsular Malaysia.

(2.79%) and *Opisthonema libertate* (2.63%) belonged to the Clupeiformes order with a worldwide distribution of 402 described species in Indo-West Pacific Ocean inhabiting brackish and marine waters and also freshwater (Li and Orti, 2007). However, in the study of Hebert *et al.* (2003), species divergence cut-off value of 3% was applied in the lepidoteran species delimitation resulting in 98% success on species recognition. Due to this, *Gerres filamentosus* (2.79%) and the rest of species with below 3% intraspecific genetic divergence value were still considered as morphospecies, thus their species assignment was not to be contested.

The status of *Thryssa hamiltonii* (mean, 1.46%, max 3.46%) species assignment was in question from biogeographical perspective, as whether they were sampled from local populations or they were transported from the oceanic

currents. *T. hamiltonii* was believed to be transported from distant spawning locations as Clupeiformes larvae have a long planktonic life of 6 weeks to 8 weeks (Smith *et al.*, 1977) and members of this family have high plasticity in its habitat use for reproduction making them a facultative diadromous fish (Mai and Vieira, 2013).

Four species reported in this study represented mangrove resident fishes, the *Ophiocara porocephala* (mean 3.14%) from Eleotridae family while *Hemigobius hoeverii* (mean 1.19%, max 2.62%), *Periophthalmus* sp. (mean 4.51%) and *Sicyopterus* sp. (mean 6.8%, max 10.29%) were gobiids. The gobiids are highly diverse family and especially rich in the Indo-Pacific region, including freshwaters of the Indo-Malay Archipelago (Larson *et al.*, 2014). The gobiids have faced a lot of

morphological ambiguities as compared to other fish families for example Terapontidae, Sillaginidae and Ambassidae (Krishna *et al.*, 2012). *Sicyopterus pugnans* and *Sicyopterus lagocephalus* has been reported in neighbouring region, the Philippines (Bayot *et al.*, 2014), hence shed a light of possible species assignment for *Sicyopterus* sp. in this study. Genetic divergence between three specimens of *Sicyopterus* sp. was found in a huge gap as they were collected from different region of Peninsular Malaysia (2 samples from East Malaysia and one from West Malaysia).

In some species sharp genetic discontinuities between two regions may occur in the absence of dispersal and gene flow, such as discontinuities in the oceanographic regimes (Aziz *et al.*, 2016, Gonzalez *et al.*, 2016) which later influence their genetic divergence and population structure (Li *et al.*, 2017). As for *Periophthalmus* sp., strong bootstrap value in ML and NJ supported the monophyletic relationship between the two specimens, however, due to insufficient records of DNA barcode reference libraries in BOLD for Indo-Pacific fishes, the broad use of libraries in automated identification became limited (Hubert *et al.*, 2012).

Since barcodes of same sequences of species invariably were clustered in the same clade, despite the differences in locality (one specimen from the West Peninsular Malaysia and the other from East Peninsular Malaysia), Krishna *et al.* (2012) concluded that across geography barcodes of same species do not contain much variations. However, high conspecific divergences occasionally may reflect actual cases of undescribed species or sub-species (Landi *et al.*, 2014). For example, members of gobiids from Oxudercinae subfamilies was claimed as not a well-known taxa (Okuyama, 1988) were considered to have ambiguity in their morphological taxonomy (Krishna *et al.*, 2012) in which Leis and Carson-Ewart (2000) claimed to have lacked in reference materials resulted in no description was able to be drawn for the group.

Thus, from this study it can be concluded that molecular methods in particular DNA barcoding is the best method for the identification of fish larvae.

Even though, previously molecular method was considered intimidating to fellow taxonomists, integration of both methods is expected to do wonders in solving the identity of phenotypically ambiguous sample. The integrative method should receive good support from future researchers as it was not undermining any field of expertise and in fact will help solve more unsolved cases, especially for fish larvae.

Acknowledgments

This paper was a part of doctoral thesis of Izzati Adilah Azmir. The first author would like to thank South east Asian

Regional Center for Graduate Study and Research in Agriculture (SEARCA) for the scholarship award for conducting the project. This project was funded by Fundamental Research Grant Scheme (FRGS) 07-02-141544FR and LRGS/1/2019/UPM/1 (vote no. 5545102) funded by the Ministry of Education, Malaysia.

References

- Agorreta, A., D.S. Mauro, U. Schliewen, J.L.V. Tassell, M. Kovačić, R. Zardoya and L. Rüber: Molecular phylogenetics of Gobioidae and phylogenetic placement of European gobies. *Molec. Phylog. Evol.*, **69**, 619-633 (2013).
- Ajmal Ali, M., G. Gyulai, N. Hidvegi, B. Kerti, F.M. Al Hemaied, A.K. Pandey and J. Lee: The changing epitome of species identification—DNA barcoding. *Saudi J. Biol. Sci.*, **21**, 204-231 (2014).
- Ara, R., A. Arshad, S.M.N. Amin, S.K. Daud and M.A. Ghaffar: Environment and diversity of ichthyoplankton in the seagrass beds of Sungai Pulai estuary, Johor Peninsular Malaysia. *J. Food, Agric. Environ.*, **9**, 733-738 (2011).
- Ardura, A., E. Morote, M. Kochzius and E.G. Vazquez: Diversity of planktonic fish larvae along a latitudinal gradient in the Eastern Atlantic Ocean estimated through DNA barcodes. *PeerJ.*, **4**, e2438 (2016).
- Atan, Y., H. Jaafar and A.R.A. Majid: Ikan Laut Malaysia glosari nama sahah spesies ikan. Dewan Bahasa dan Pustaka (2010).
- Avise, J.C.: Molecular Markers, Natural History, and Evolution. Chapman & Hall, New York, pp. 511 (1999).
- Aziz, N.M.A., Y. Esa and A. Arshad: DNA barcoding and phylogenetic analysis of Malaysian groupers (Subfamily: Epinephelinae) using mitochondrial Cytochrome c oxidase I (COI) gene. *J. Environ. Biol.*, **37**, 725-733 (2016).
- Azmir, I., Y. Esa, S.M.N. Amin, I.S. Md Yasin and F. Md Yusof: Identification of larval fish in mangrove areas of Peninsular Malaysia using morphology and DNA barcoding methods. *J. Appl. Ichthyol.*, **33**, 998-1006 (2017).
- Bayot, F.A., K.N.A. Joya and W.R. Jacinto: Phylogenetic analysis and barcoding of gobies in Maragondon river, Cavite, Philippines. *Inter. J. Curr. Sci.*, **12**, 50-6 (2014).
- Branicki, W., Kupiec, T. and Pawlowski, R.: Validation of cytochrome b sequence analysis as a method of species identification. *J. Forens. Sci.*, **48**, 1-5 (2003).
- Chu, C., K.H. Loh, C.C. Ng, A.L. Ooi, Y. Konishi, S.P. Huang and V.C. Chong: Using DNA Barcodes to Aid the Identification of Larval Fishes in Tropical Estuarine Waters (Malacca Straits, Malaysia). *Zool. Stud.*, **58**, e30 (2019).
- Coelho, P., P. Sousa, D.J. Harris and A.V. der Meijden: Deep intraspecific divergences in the medically relevant fat-tailed scorpions (Androctonus, Scorpiones). *Acta Tropica.*, **134**, 43-51 (2014).
- Colatreli, O.P., N.V. Meliciano, D. Toffoli, I.P. Farias and T. Hrbek: Deep phylogenetic divergence and lack of taxonomic concordance in species of Astronotus (Cichlidae). *Int. J. Evol. Biol.*, 915265 (2012).
- De Carvalho, D.C., D.A. Oliveira, P.S. Pompeu, C.G. Leal, C. Oliveira and R. Hanner: Deep barcode divergence in Brazilian freshwater fishes: the case of the São Francisco River basin. *Mitoch. DNA*, **22**, 80-86 (2011).
- Díaz, J., G.V. Villanova, F. Brancolini, F. del Pazo, V.M. Posner, A. Grimberg and S.E. Arranz: First DNA barcode reference library for

- the identification of South American freshwater fish from the lower Paraná river. *PLoS ONE*, **11**, e0157419 (2016).
- Fishbase. *Boleophthalmus boddarti*. Accessed on 26th May 2017. <http://www.fishbase.org/summary/Boleophthalmus-boddarti.html>
- Foon, J.K., S.K. Tan and G.R. Clements: A new species of *Kenyirius* (Pulmonata, Camaenidae) from Peninsular Malaysia. *J. Trop. Biol. Conser.*, **12**, 25–34 (2015)
- Freitas, D.M. and J.H. Muelbert: Ichthyoplankton distribution and abundance off Southeastern and Southern Brazil. *Brazil. Arch. Biol. Technol.*, **47**, 601-612 (2004).
- Gonzalez, E.B., H. Knutsen and P.E. Jorde: Habitat discontinuities separate genetically divergent populations of a rocky shore marine fish. *PLoS ONE*, **11**, e0163052 (2016).
- Hall, T. A.: BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic Acids Symposium Series, London. Information Retrieval Ltd., **41**, pp. 95-98 (1999).
- Hebert, P.D., A. Cywinska, S.L. Ball and J.R. DeWaard: Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B*, **270**, 313-21 (2003).
- Hebert, P.D., E.H. Penton, J.M. Burns, D.H. Janzen and W. Hallwachs: Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of the United States of America., **101**, 14812-14817 (2004a).
- Hebert, P.D., M.Y. Stoeckle, T.S. Zemlak and C.M. Francis: Identification of birds through DNA barcodes. *PLoS Biol.*, **2**, e312 (2004b).
- Hubert, N., C.P. Meyer, H.J. Bruggemann, F. Guérin, R.J.L. Komono, B. Espiau, R. Causse, J.T. Williams and S. Planes: Cryptic diversity in Indo Pacific coral-reef fishes revealed by DNA-barcoding provides new support to the centre-of-overlap hypothesis. *PLoS ONE*, **7**, e28987 (2012).
- Huelsenbeck, J.P. and F. Ronquist: MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754-755 (2001).
- Kimura, M.: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mole. Evol.*, **16**, 111-120 (1980).
- Krishna, P.V., K.M. Rao and D.S. Rao: Identification of selected estuarine fishes by DNA barcoding from river Krishna region, Andhra Pradesh, India. *Inter. J. Res. Pharmace. Biomed. Sci.*, **3**, 1044-1049 (2012).
- Landi, M., M. Dimech, M. Arculeo, G. Biondo, R. Martins, M. Carneiro, G.R. Carvalho, S.L. Brutto and F.O. Costa: DNA barcoding for species assignment: The case of Mediterranean marine fishes. *PLoS One*, **9**, e106135 (2014).
- Larkin, M. A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez and J.D. Thompson: Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947-2948 (2007).
- Larson, G., D.R. Piperno, R.G. Allaby, M.D. Purugganan, L. Andersson, M.A. Kalin, L. Barton, C.C. Vigueira, T. Denham, K. Dobney, A.N. Doust, P. Gepts, M.T. Gilbert, K.J. Gremillion, L. Lucas, L. Lukens, F.B. Marshall, K.M. Olsen, J.C. Pires, P.J. Richerson, R.R. de Casas, O.I. Sanjur, M.G. Thomas and D.Q. Fuller: Current perspectives and the future of domestication studies. *Proc. Natl. Acad. Sci.*, **111**, 6139-46 (2014).
- Leis, J.M. and B.M.C. Ewart: The larvae of Indo-Pacific Coastal Fishes: An identification guide to marine fish larvae. Brill Publisher, Leiden, The Netherlands. p. 850 (2000).
- Leis, J. M., and McCormic, M. I.: The biology, behaviour and ecology of the pelagic, larval stage of coral reef fishes. In: Coral Reef Fishes. (Ed.: P.J. Sale). Elsevier Science, USA. pp. 171-199 (2002).
- Leis, J.M.: Taxonomy and systematics of larval Indo-Pacific fishes: a review of progress since 1981. *Ichthyol. Res.*, **62**, 9-28 (2015).
- Lewis, L. A., Richardson, D. E., Zakharov, E. V. and Hanner, R.: Integrating DNA barcoding of fish eggs into ichthyoplankton monitoring programs. *Fishery Bulletin*, **114**, 153-166 (2016).
- Li, C. and G. Orti: Molecular phylogeny of Clupeiformes (Actinopterygii) inferred from nuclear and mitochondrial DNA sequences. *Molec. Phyloge. Evolut.*, **44**, 386-398 (2007).
- Li, Y., A. Ludwig and Z. Peng: Geographical differentiation of the *Euchiloglanis* fish complex (Teleostei: Siluriformes) in the Hengduan Mountain Region, China: Phylogeographic evidence of altered drainage patterns. *Ecol. Evol.*, **7**, 928-940 (2017).
- Lima, A.R.A., M. Barletta, M.F. Costa, J.A.A. Ramos, D.V. Dantas, P.A.M.C. Melo, A.K.S. Justino and G.V.B. Ferreira: Changes in the composition of ichthyoplankton assemblage and plastic debris in mangrove creeks relative to moon phases. *J. Fish Biol.*, **89**, 619-640 (2015).
- Mabragaña, E., J.M.D. de Astarloa, R. Hanner, J. Zhang and M.G. Castro: DNA barcoding identifies Argentine fishes from marine and brackish waters. *PLoS ONE*, **6**, e28655 (2011).
- Mansor, M.I., H. Kohno, H. Ida, H.T. Nakamura, Z. Aznan and S. Abdullah: Field Guide to Important Commercial Marine Fishes of the South China Sea. Malaysia: *Marine Fishery Resources Development and Management SEAFDEC.*, MFRDMD/SP/2. (1998).
- Mat Jaafar, T.N., M.I. Taylor, S.A. Mohd Nor, M. de Bruyn and G.R. Carvalho: DNA barcoding reveals cryptic diversity within commercially exploited Indo-Malay Carangidae (Teleostei: Perciformes). *PLoS ONE*, **7**, e49623 (2012).
- Mai, A.C.G. and J.P. Vieira: Review and consideration on habitat use, distribution and life history of *Lycengraulis grossidens* (Agassiz, 1829) (Actinopterygii, Clupeiformes, Engraulididae). *Biota Neotro.*, **13**, 121-130 (2013).
- Milá, B., E.S. Tavares, A.M. Saldaña, J. Karubian, T.B. Smith and A.J. Baker: A trans-Amazonian screening of mtDNA reveals deep intraspecific divergence in forest birds and suggests a vast underestimation of species diversity. *PLoS ONE*, **7**, e40541 (2012).
- National Hydrographic Malaysia.: Tide Tables Malaysia 2015. Volume 1. Malaysia. (2015)
- Okiyama, M.: An atlas of the early stage fishes in Japan. Tokai University Press, Tokyo, Japan. p. 1154 (1988).
- Pereira, L.H., R. Hanner, F. Foresti and C. Oliveira: Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna. *BMC Genetics*, **14**, 20 (2013).
- Pusey, B.: The new and the known: describing freshwater fish species. *Using Rainforest Research.*, p. 2 (2001). Retrieved from http://www.wet Tropics.gov.au/site/user-assets/docs/new_Fishes.pdf
- Radulovici, A.E., P. Archambault and F. Dufresne: DNA barcodes for marine biodiversity: moving fast forward. *Diversity*, **2**, 450-472 (2010).
- Ratnasingham, S. and P.D.N. Hebert: Barcoding Bold: The barcode of life data system (www.barcodinglife.org). *Mole. Ecol. Not.*, **7**, 355–364 (2007).
- Russell, F.S.: The eggs and planktonic stages of British marine fish.

- Academic Press, London, New York, San Francisco. p. 524 (1976).
- Saitou, N. and M. Nei: The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mole. Biol. Evol.*, **4**, 406-425 (1987).
- Sandilyan, S. and Kathiresan, K.: Decline of mangroves – A threat of heavy metal poisoning. *Ocean & Coastal Management*, **102**, 161-168 (2014).
- Shao, K.T., K.C. Chen and J.H. Wu: Identification of marine fish eggs in Taiwan using light microscopy, scanning electric microscopy and mtDNA sequencing. *Mar. Fresh. Res.*, **53**, 355-365 (2002).
- Shen, Y., L. Guan, D. Wang and X. Gan: DNA barcoding and evaluation of genetic diversity in Cyprinidae fish in the midstream of the Yangtze River. *Ecol. Evol.*, **6**, 2702-2713 (2016).
- Smith, R.L. and J.A. Cameron: Acute Effects-Pacific herring roe in the Gulf of Alaska. Environmental assessment of the alaskan continental shelf. Annual Reports, **12**, 596-635 (1977).
- Tan, K.H.: Protection and management of mangroves in Malaysia: Challenges and strategies. In: Proceedings of national conference on coastal and marine biodiversity, Kuala Lumpur (2007).
- Tautz, D., P. Arctander, A. Minelli, R.H. Thomas and A.P. Vogler: A plea for DNA taxonomy. *Tren. Ecol. Evolut.*, **18**, 70-74 (2003).
- Trivedi, S., A.A. Aloufi, A.A. Ansari and S.K. Ghosh: Role of DNA barcoding in marine biodiversity assessment and conservation: an update. *Saudi J. Biol. Sci.*, **23**, 161-171 (2016).
- Valdez-Moreno, M., L. Va'squez-Yeomans, M. Eli'as-Gutierrez, N.V. Ivanova and P.D.N. Hebert: Using DNA barcodes to connect adults and early life stages of marine fishes from the Yucatan Peninsula, Mexico: potential in fisheries management. *Mar. Freshwater Res.*, **61**, 665-671 (2010).
- Ward, R.D., T.S. Zemlak, B.H. Innes, P.R. Last and P.D. Hebert: DNA barcoding Australia's fish species. *Phil. Trans. R. Soc. B*, **360**, 1847-1857 (2005).
- Ward, R.D., F.O. Costa, B.H. Holmes and D. Steinke: DNA barcoding of shared fish species from the North Atlantic and Australasia: minimal divergence for most taxa, but *Zeus faber* and *Lepidopus caudatus* each probably constitute two species. *Aqua. Biol.*, **3**, 71-78 (2008).
- Ward, R.D.: FISH-BOL, A case study for DNA barcodes. In: DNA Barcodes: Methods and Protocols, Methods in Molecular Biology (Eds: W.J. Kress and D.L. Erickson). Springer Science+Business Media, LLC, New York. **858**, 423-439 (2012).
- Wellington, G.M. and B. Victor: Planktonic larval duration of one hundred species of Pacific and Atlantic damsel fishes (Pomacentridae). *Mar. Biology*, **101**, 557-567 (1989).
- Winterbottom, R.: Search for the gobi old sister group (Actinopterygii: Percomorpha). *Bull. Mar. Sci.*, **52**, 395-414 (1993).
- Xia, X.: DAMBE5: A comprehensive software package for data analysis in molecular biology and evolution. *Molec. Biol. Evol.*, **30**, 1720-1728 (2013).
- Ye, Y.Y., C.W. Wu and J.J. Li: Genetic population structure of *Macridiscus multifarius* (Mollusca: Bivalvia) on the basis of mitochondrial markers: strong population structure in a species with a short planktonic larval stage. *PLoS ONE*, **10**, e0146260.