Original Research

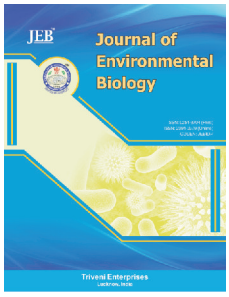
Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

DOI : http://doi.org/10.22438/jeb/39/5(SI)/26

JEB™

p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP



Phylogeography and population structure of *Tenualosa toli* inferred from Cytochrome b mitochondrial DNA fragment

Authors Info

P. Puvanasundram[1]*, Y. B. Esa[1], K.A.A. Rahim[2] and S.M. Nurul Amin[1]

[1]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

[2]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia

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

Key words

Clades
Cyt b sequences
Haplotypes
Phylogenetic structure
Tenualosa toli

Publication Info

Paper received : 16.07.2017
Revised received : 19.09.2017
Re-revised received : 25.11.2017
Accepted : 28.12.2017

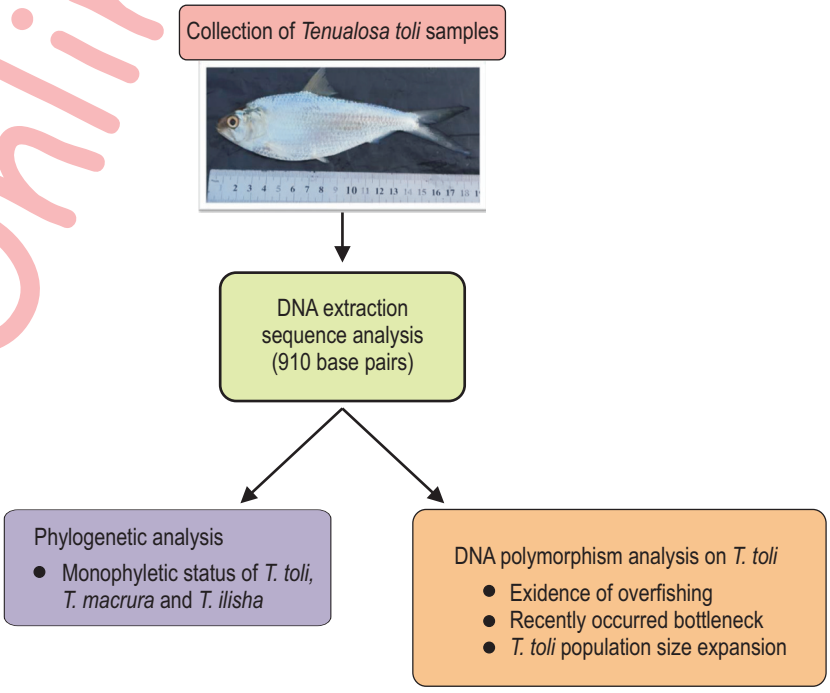
Abstract

Aim : Shad fish of genus *Tenualosa* subfamily Alosinae (shads) family Clupeidae, are commercially and culturally important estuarine fish in many Asian countries, especially in Sarawak. In this study, the phylogenetics of three species from the genus Tenualosa (*Tenualosa toli, Tenualosa macrura* and *Tenualosa ilisha*) was determined.

Methodolgy : Sequence analysis of 910 base pairs of Cytochrome b gene were conducted on the samples of indigenous *T.toli* (N=111) and *T. macrura* (N=24), which were collected from Sarawak including the samples of non-native *T. ilisha* (N=4) obtained from Bangladesh.

Results : A total of 28 haplotypes were found with *T. toli* producing 15 haplotypes, where 13 haplotypes were unique haplotypes while 2 shared haplotypes among the 6 populations.

Interpretation : Phylogenetic analysis supported the monophyletic status between the three shad species. The highest intraspecific genetic divergences were recorded between imported samples and samples from other localities. There is evidence of overfishing and recently occurred bottleneck events which led to a population size expansion of *T. toli*, especially in Sebuyau, Daro and Mukah.



Introduction

Shad fish of genus *Tenualosa* subfamily Alosinae (shads) family Clupeidae are commercially and culturally important estuarine fish in many Asian countries, especially in Sarawak. This genus consists of 5 different species worldwide, mainly *Tenualosa ilisha*, T*enualosa macrura*, *Tenualosa revesii*, *Tenualosa thibaudeaui* and *Tenualosa toli* (Blaber *et al*., 2005). However, only two are found in Malaysia, which are locally known as *terubok*; *T. macrura* (sea *Terubok*) and *T. toli* (river *Terubok*), where the distribution of both species are confined along the coastal waters of Sarawak. *T. macrura* closely resembles *T. toli* but has a smaller head and a longer tail compared with the latter (Blaber *et al*., 1999). *T. toli* resembles *T. ilisha*, which has a longer head (28 to 32% standard length), shorter caudal fin (25 to 31% standard length), more gillrakers and spots along the flank (Whitehead, 1985). *T. ilisha*, also known as Hilsa, is a large anadromous fish that can be found in the coastal area, brackish, estuarine and also fresh water rivers of Bangladesh, India and Myanmar (Whitehead, 1985; Salini *et al*., 2004). A recent study by Arai and Amalina (2014) established the first record of *T. ilisha* in Malaysia waters, specifically in Perak River in Peninsula Malaysia. These results suggest that the *T. ilisha* found in Malaysia belongs to a different population from the one found in Bangladesh, India and other countries.

There are 3 core terubok areas in Sarawak mainly Lassa, Saribas and Lupar River as the population of Sarawak river shad (*T. toli*) can only be found within these three water bodies. *T. toli* spawns in both the Batang Lupar and Batang Lassa over an extended period, but each female spawns only once (Blaber *et al*., 1996). This species has long been a prized target by local fishermen for the high price commanded by the roe and the delicious taste of its flesh. The total catch landing for this species has been reported to be depleted to a very low level, due to over-exploitation including other subsidiary factors such as environmental degradations and water pollution (Blaber *et al*., 2005). The current status of *T. toli* within Batang Lupar, Batang Lassa and Batang Saribas, which are known as 'core terubok area' were recorded by Khairul Adha *et al.* (2014). The smaller terubok (*T. toli*) also known as 'empirit' are found locally and abundantly in the area, fishery of *T. macrura* occurs at a large scale and local fishermen tend to use smaller mesh size of net (50 mm to 100 mm) for *T. macrura* fishery leading to the increase in 'empirit' fishery as well.

Morphological based identification is a common method applied on adult fishes but this is difficult to apply on juvenile fish. In order to further support morphological identification, the use of different methods for identification is required. The application of molecular markers, mainly DNA markers, are reliable in stock management as well as juvenile fish identification. The genetic diversity data is useful in research related to evolution, conservation and management of natural resources as well as genetic improvement programs (Tanya and Kumar, 2010).

Phylogenetic analysis using DNA characters is one of the best molecular approaches to determine and confirm the systematic and taxonomy status among organisms (Avise, 2000; Esa *et al.,* 2008, 2012; Jeffrine and Esa, 2006). The mitochondrial DNA (mtDNA) has many properties that make it useful for reconstructing phylogenetic history and tracing maternal geologies (Avise, 1994; Stepien and Kocher, 1997). This includes rapid rate of sequence divergence (at least in vertebrates), which allows discrimination of recently diverged lineages, maternal inheritance and absence of recombination. They have also contributed valuable information on population genetic structures, phylogeography and barcoding studies of various fishes (Kamarudin and Esa 2009; Cook *et al.,* 2006; Esa *et al.,* 2000; Esa and Khairul Adha, 2013).

Mitochondrial DNA Cytochrome b (Cyt b) is an available and universal primer gene, used in many fields to identify species (Hsieh *et al.,* 2001; Irwin *et al.,* 1991; Pääbo and Wilson, 1988). Phylogenetic studies on fish were also conducted using this gene such as the phylogenetic analysis of fishes of the subfamily Schozothoracinae using Cyt b gene (Barat *et al.,* 2012). Cyt b gene is proven in clarifying the status of particular species. For example, Esa *et al.* (2012) successfully constructed the phylogeny of Malaysian freshwater fishes in the family Cyprinidae inferred from the Cyt b gene.

The taxonomy of clupeiformes has extensively been studied (Whitehead, 1985), but their phylogenetic relationships are still poorly understood. The lack of phylogenetic relationship studies among tropical shads of the genus *Tenualosa* means that their taxonomic status under the species concept criterion that utilize genetic characters such as the genetic species concept (Baker and Bradley, 2006) have never been tested. Study conducted by Abdul Aziz *et al.* (2015) on the mitochondrial DNA diversity of Terubok (*Tenualosa toli*) from Daro and Mukah, Sarawak inferred by partial (Cyt b) concluded that the haplotype and nucleotide diversity was relatively low for both of these populations. In this study, a single haplotype was shared among 76 individuals implying that genetic deprivation occurred due to overfishing.

This study aims to construct phylogenetic tree of genus *Tenualosa* inferred from sequencing of Cyt b. mitochondrial DNA to examine the systematic and evolutionary relationship among the member of genus *Tenualosa.* In addition, this study also aims to resolve the population structure among selected population of *T. toli.*

Materials and Methods

Sample collection : Samples of *T. toli* used in this study were collected from Sebuyau (N= 25), Sadong Jaya (N=21), Satok market (N=9), Batang Lupar (N=20), Daro (N=12) and Mukah (N=25). Samples of *T. macrura* were collected from Sadong Jaya (N= 7), Kota Samarahan (N=11), Daro (N=1) and Sibu (N=5).

Sampling location are shown in Fig. 1. On the other hand, samples of *T. ilisha* (N=4) were collected from Bangladesh. All samples were identified based on morphological features using key identification of clupeoid species by Whitehead (1985). Tissue and fin samples were collected and preserved in 95% ethanol and subsequently stored at -20°C. *Sardinella maderensis* sequences, which were used as outgroup taxa, were obtained from Genbank (Accession number: AF472583.1, DQ 19799.1).

DNA extraction, polymerase chain reaction (PCR) and purification of PCR product : Total DNA was extracted using Wizard® Genomic DNA Purification system by Promega according to the manufacturer's protocol. The DNA quality and approximate yield were determined by electrophoresis in 1 to 2% agarose gel 75V for 60 min.

A 1140 base pair (bp) segment of Cyt b. gene was amplified with the oligonucleotide primers (5'-CGAAGCTTGATATGAAAAACCATCGTTG- 3', forward) and (5'-AACTGCAGTCATCTCCGGTTTACAAGAC- 3', reverse) (Pääbo, 1990). Approximately, 50-100 ng of template DNA was amplified in a 25 µl reaction mixture which contains 5X buffer, 25 mM $MgCl_2$, 40 mm of dNTP, 0.1M of each primer and 0.5 unit of Taq DNA polymerase. The cycle parameters consisted of 35 cycles of denaturation (95°C at 30s), annealing (47.3°C at 30s) and extension (72°C at 60s). The annealing temperature was determined through optimization. The amplified products were visualized on 1% agarose gels, run for approximately 75V for 60 min.

The PCR products were then purified using Wizard ® SV Gel and PCR Clean-Up system by Promega according to the manufacturer's protocol and then sequenced bi-directionally by First Base Laboratories Sdn Bhd, Malaysia using forward primers, which were used earlier for PCR amplifications. Sequencing was done using BigDye® Terminator v3.0 Cycle sequencing kit (ACGT) on a ABI 377 automated sequencer (PE Applied Biosystem).

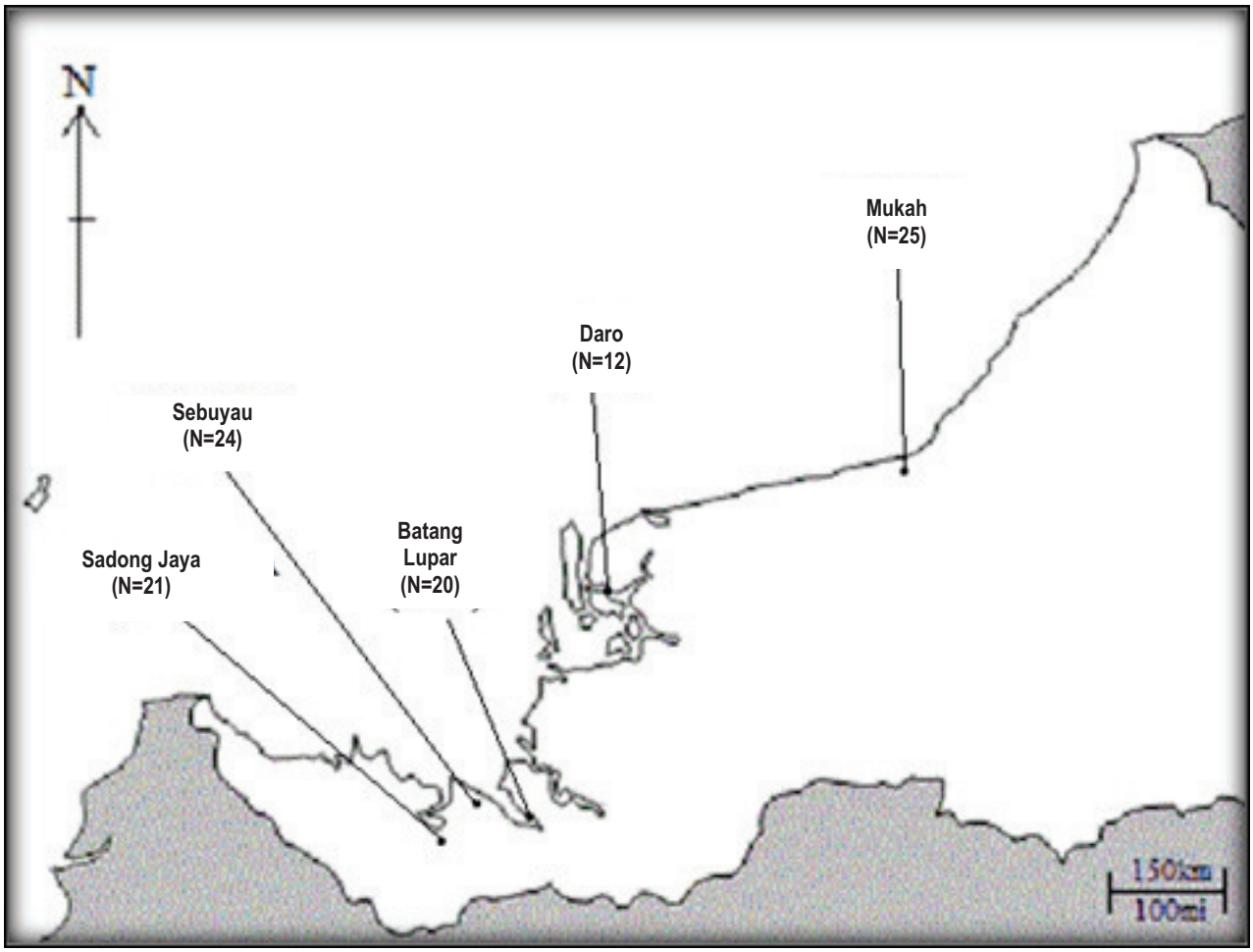


Fig. 1 : Map indicating sampling location of *Tenualosa toli* in specific locations in Sarawak, Malaysia

Data analysis : DNA sequencing results were viewed in the Chromas Lite (version 2.1.1 copyright© 1998-2013) software (Technelysium Pty Ltd), http://www.technelysium.com.au. The multiple sequence alignment were done using CLUSTALX version 2.1 (Larkin *et al*. 2007). Haplotypes were detected using FaBox (1.41) an online fasta sequence toolbox and the sequences were then deposited into Genbank to obtain the accession number. The accession number of all the haplotypes deployed in this study is shown in Table 1.

Analyzation of the Cyt b sequences were conducted using MEGA version 7.0.18 (Tamura *et al*. 2013). The pairwise genetic distance between each haplotype were calculated using Kimura two-parameter evolution model (Kimura, 1980) implemented in MEGA version 7.0.18.

Phylogenetic relationships were inferred using three different methods of analysis: neighbour-joining (NJ) (Saitou and Nei, 1987), maximum parsimony (MP) and maximum likelihood (ML). A distance analysis using the NJ method was done using a close neighbor-interchange (CNI) option implemented in MEGA version 7.0.18. (Tamura *et al.,* 2013). The NJ clustering was performed using the Kimura two-parameter evolutionary model (Kimura, 1980). Phylogenetic confidence were estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets. The model with the best maximum likelihood (ML) score using Tamura Nei (TN93+I) were used to construct ML tree (Tamura and Nei, 1993). Bootstrap tree were computed using 1000 replicate data sets. All phylogenetic tree were rooted with an outgroup from the genus *Sardinella* which was *Sardinella maderensis*.

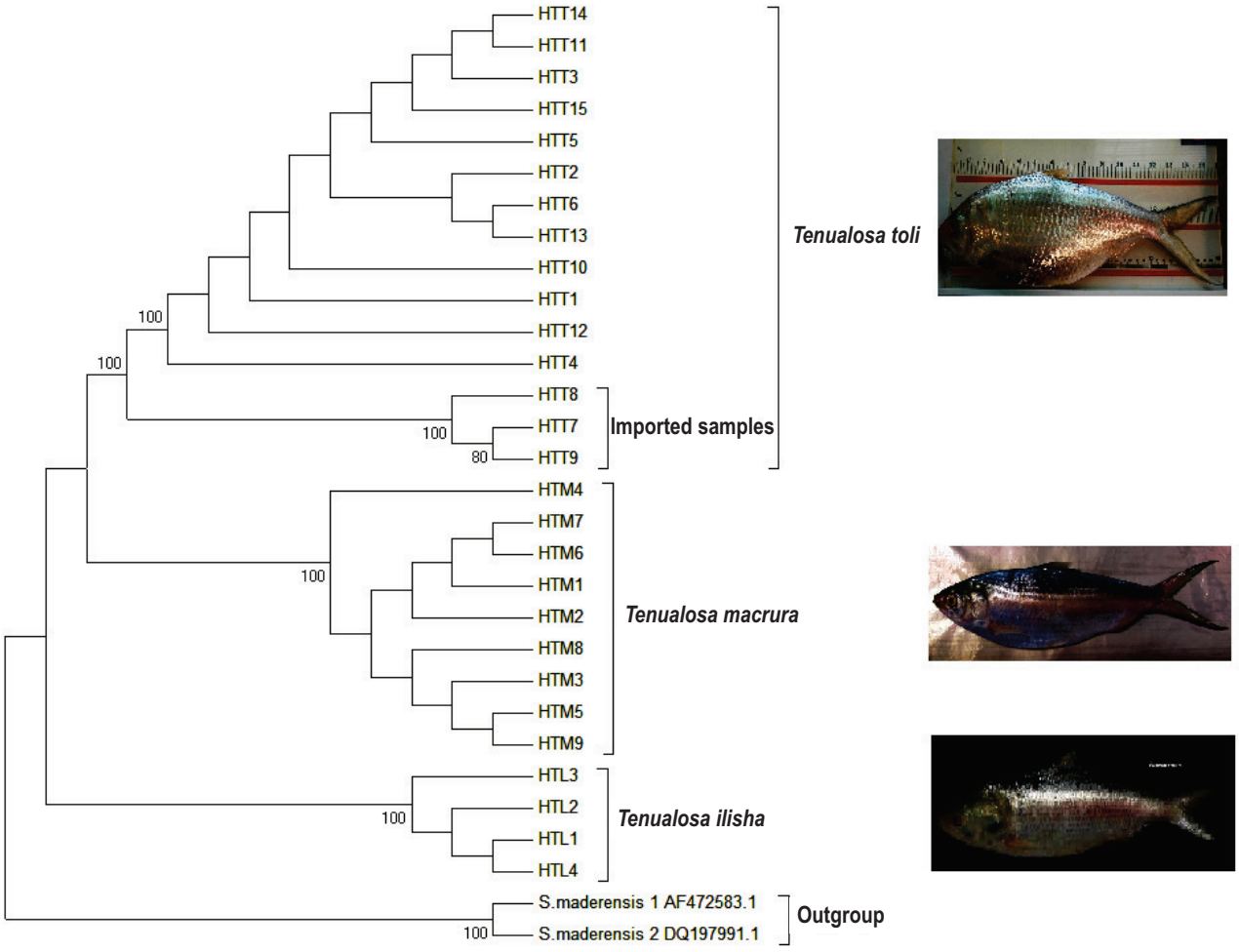


Fig. 2 : Phylogenetic relationships among *Tenualosa toli*, *Tenualosa macrura*, *Tenualosa ilisha* and outgroup (*Sardinella maderensis*) haplotypes utilized in the present study. The bootstrap percentage values presented at each nodes corresponds to the Neighbour-Joining/Maximum Likelihood (NJ/ML) analyses

Table 1 : List of haplotypes with corresponding species name and Genbank Accession number (Abbreviation: TT= *Tenualosa toli*, TM= *Tenualosa macrura*, TL= *Tenualosa ilisha)*

Haplotype	Species name	Collected from	Number of samples	Genbank Number	Accession
HTT1	*T. toli*	Sebuyau	12	Kt630278	
		Sadong Jaya	12		
		Batang Lupar	11		
		Daro	7		
		Mukah	13		
HTT2	*T. toli*	Sebuyau	2	Kr261091	
HTT3	*T. toli*	Sebuyau	8	KR261092	
		Sadong Jaya	8		
		Batang Lupar	7		
		Daro	4		
		Mukah	9		
HTT4	*T. toli*	Sebuyau	1	Kx859096	
HTT5	*T. toli*	Sebuyau	1	Kr261093	
HTT6	*T. toli*	Sebuyau	1	Kx859097	
HTT7	*T. toli*	Satok market	7	Kr827625	
HTT8	*T. toli*	Satok market	1	Kr827626	
HTT9	*T. toli*	Satok market	1	Kx859098	
HTT10	*T. toli*	Daro	1	Ku888655	
HTT11	*T. toli*	Mukah	1	Kx859099	
HTT12	*T. toli*	Mukah	1	Kx859100	
HTT13	*T. toli*	Mukah	1	Kx859101	
HTT14	*T. toli*	Batang Lupar	1	Kx859102	
HTT15	*T. toli*	Batang Lupar	1	Kx859103	
HTM1	*T. macrura*	Sadong Jaya	1	Kr261094	
HTM2	*T. macrura*	Sadong Jaya	4	KT630282	
		Samarahan	8		
		Daro	1		
		Sibu	3		
HTM3	*T. macrura*	Sadong Jaya	1	Kr261095	
HTM4	*T. macrura*	Sadong Jaya	1	Kx084540	
HTM5	*T. macrura*	Samarahan	1	Kx859104	
HTM6	*T. macrura*	Samarahan	1	Kx859105	
HTM7	*T. macrura*	Samarahan	1	Kt630283	
HTM8	*T. macrura*	Sibu	1	Kx859106	
HTM9	*T. macrura*	Sibu	1	Kx859107	
HTL1	*T. ilisha*	Bangladesh	1	Kx859108	
HTL2	*T. ilisha*	Bangladesh	1	Ku888657	
HTL3	*T. ilisha*	Bangladesh	1	Ku888658	
HTL4	*T. ilisha*	Bangladesh	1	KX859109	

In order to obtain the genetic structure, hierarchical analysis of molecular variance (AMOVA) as well as pairwise Fst values of different population of *T. toli*, Arlequin version 3.5.5 (Excoffier and Lischer, 2010) was utilized in this study. Demographic history were estimated based on two different approaches mainly Tajima's D (Tajima, 1989) and Fu's FS (Fu, 1997 where the values were obtained through neutrality test conducted in Arlequin version 3.5.5. This value signifies population expansion. Mismatch distribution which is mainly frequency distribution of pairwise differences between sequences were also conducted through this software. This analysis produces three values, mainly θ0 (before population growth), θ1 (after population growth) and Ƭ (time since expansion time expressed in units of mutational time) (Rogers and Harpending, 1992). Associated graph for this analysis were obtained through Dnasp version 5.0.1 (Librado and Rozas, 2009). This software was also utilized for DNA polymorphism analysis in order to obtain nucleotide and haplotype diversity. PopArt version 1.7 (Bandelt *et al.,* 1999) was used to obtain minimum spanning network.

Results and Discussion

A total of 139 samples, each comprising of a Cyt b sequence length of 910 bp, were used in the present study and a

Table 2 : Numbers of *Tenualosa toli* haplotypes with corresponding locality and relative frequency

Haplotype	Sebuyau (SB) (N=24)	Sadong Jaya (SJ) (N=21)	Daro (D) (N=12)	Mukah (M) (N=25)	Batang Lupar (BL) (N=20)	Imported samples (IS) (N=9)
HTT1	0.500	0.571	0.583	0.520	0.550	
HTT2	0.083					
HTT3	0.333	0.381	0.333	0.360	0.350	
HTT4	0.042					
HTT5	0.042					
HTT6		0.048				
HTT7						0.778
HTT8						0.111
HTT9						0.111
HTT10			0.083			
HTT11				0.040		
HTT12				0.040		
HTT13				0.040		
HTT14					0.050	
HTT15					0.050	
Nucleotide diversity (PiJC)	0.001	0.001	0.001	0.001	0.001	0.000
Number of haplotypes	5	3	3	5	4	3
Haplotype diversity (Hd)	0.656	0.552	0.591	0.620	0.600	0.417
Number of polymorphic sites	4	2	2	7	3	2

TT= *Tenualosa toli*

total of 28 different haplotypes were identified. The extracted samples yielded 15 *T. toli* haplotypes (HTT1-HTT15), 9 *T. macrura* haplotypes (HTM1-HTM9) and 4 *T. ilisha* haplotypes (HTL1-HTL4). The lists of haplotypes used in this study are presented in Table 1. Overall, 709 bp (77.9%) were conserved sites, 201 bp (22.1%) were variable sites and 184 bp (20.2%) were parsimony informative sites from the total of 910bp of the Cyt b gene fragment. All haplotypes were deposited in Genbank with accession number as listed in Table 1.The percentage of the average total nucleotide composition from the sequenced samples were A=24.0%, T=28.3%, C=30.7%, G=16.9%.

For phylogenetic analyses, Neighbour Joining (NJ) and Maximum Likelihood (ML) phylogram produced similar tree topologies. However, the positioning of *T. ilisha* was different for the MP phylogram as in Fig. 2, which divides samples into two major clusters. The first cluster grouped all the *T. toli* samples and *T. macrura* samples with a high bootstrap supports value while another cluster was composed of *T. ilisha* sequences. This is because data handling in all analysis are different. In NJ and ML, data handling is done in an easy manner as the information of multiple alignment of sequences is reduced to a more simple form. In this process, however, some of the information will be lost, especially regarding identities of ancestral and derived nucleotides at each position when multiple alignments is conducted (Brown, 2002). MP on the other hand assumes that evolution follows the shortest possible route and that the correct tree is the one that requires fewer nucleotide changes to produce observed differences between sequences (Brown, 2002). MP and ML map the history of gene sequences compared to NJ which uses distance based methods (Holder and Lewis, 2003).

All sequences were grouped together according to their taxonomic identification based on their morphological characteristics. In the NJ and ML phylogram tree, *T. toli* samples were grouped into two clusters. The first cluster consisted of all haplotype except for HTT7, HTT8 and HTT9, where these haplotypes were separated from the main clade and or could be genetically divided. This indicates that these haplotypes are comprised of distinctive individuals belonging to the same species. The first clade consists of sequences obtained from Sebuyau, Sadong Jaya, Batang Lupar, Daro and Mukah, which showed high genetic similarities to each other. HTT7, HTT8 and HTT9 are unique haplotypes that consist of samples obtained from Satok market.

Among the 15 *T. toli* haplotypes, which represents 6 different populations, 2 had shared haplotypes whereas 13 were unique haplotypes (Table 2). The 111 partial Cyt b sequences of *T. toli* consisted of 39 (4.2%) variable sites, 871 (95.7%) conserved sites and 28 (3.1%) parsimony informative sites out of 910 bp. The protein translation of 910 bp fragment of all sequences produced 27 haplotypes based on 303 amino acid residues and these residues showed 52.8% variable sites. HTT1 and HTT3 are common haplotype, which were found in all the selected populations in Sarawak. *T. toli* samples from Sebuyau and Mukah produced 3 unique haplotypes, whereas Batang Lupar produced 2 unique haplotypes. Sadong Jaya and Daro each produced 2 unique haplotypes. Both Sebuyau and Mukah produced 5 haplotypes, which was the highest number of haplotypes produced in one particular population.

The utilization of mtDNA in this study managed to provide insight into the genetic makeup of *T. toli* collected from various localities in Sarawak. Phylogenetic analysis of partial Cyt b fragment supported the reciprocally monophyletic relationship between the three *Tenualosa* species. The high genetic divergences found between species (>13%K2P) further showed their genetic distinctiveness. Genetic distance values greater than 11% indicate specific species recognition (Baker and Bradley, 2001). The present mtDNA data suggests that *T. toli* and *T. macrura* differed genetically as high genetic divergence between the indigenous *T. toli* and *T. macrura* (13.9%-15.3%) found in this study supported their taxonomic status as distinct species. It is difficult to morphologically distinguish these species, especially during their juvenile stage (Blaber, 2009), which further supports the advantage of molecular markers over morphological characterization for species identification.

In this study, DNA polymorphism analysis of *T. toli* across 6 different populations mainly Sebuyau, Sadong Jaya, Batang Lupar, Daro, Mukah and imported samples were conducted. The nucleotide diversity in all population was low (0.001), whereas haplotype diversity ranged from 0.417 (Imported samples) to 0.656 (Sebuyau) (Table 2). The highest number of polymorphic site (7 sites) was found in haplotypes from Mukah. On the other hand, highest haplotye diversity were found in haplotypes from Sebuyau as it contained the highest number of haplotypes,

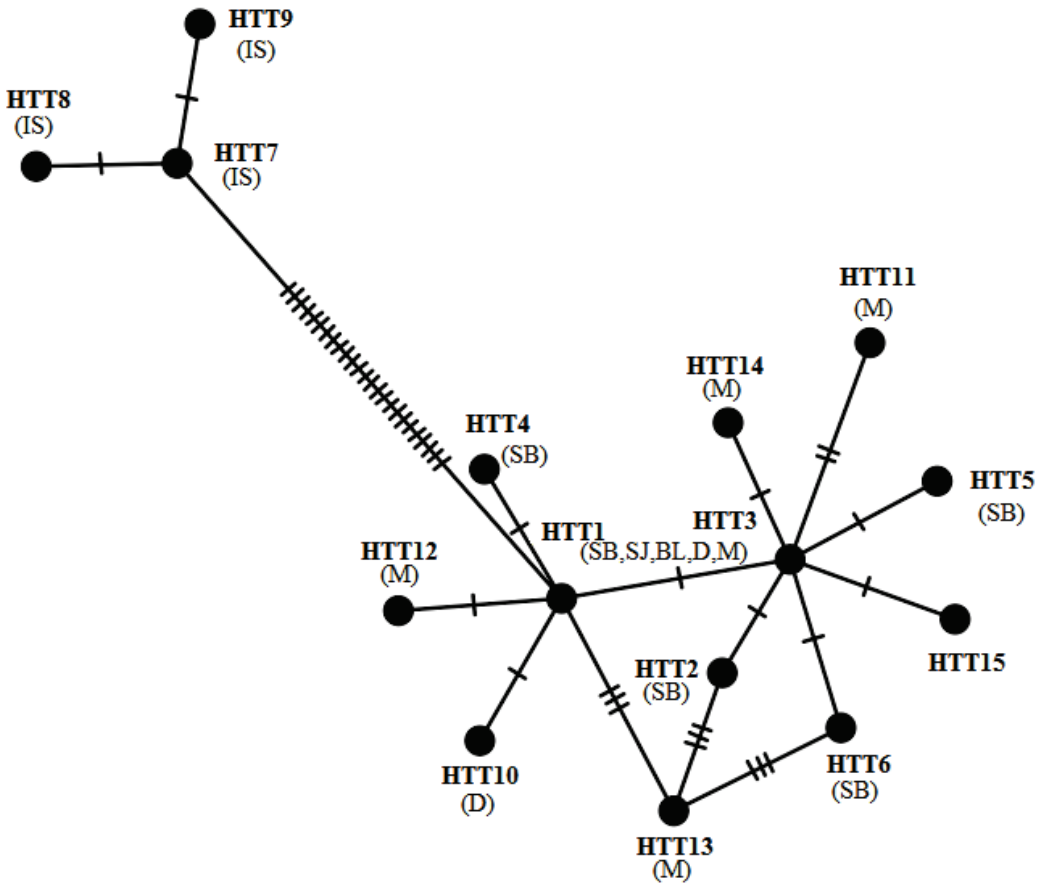


Fig. 3 : Minimum spanning network showing relationships among 15 mitochondrial DNA Cyt b haplotypes of *Tenualosa toli* with their respective population. The hatch marks among the haplotypes show single mutational steps

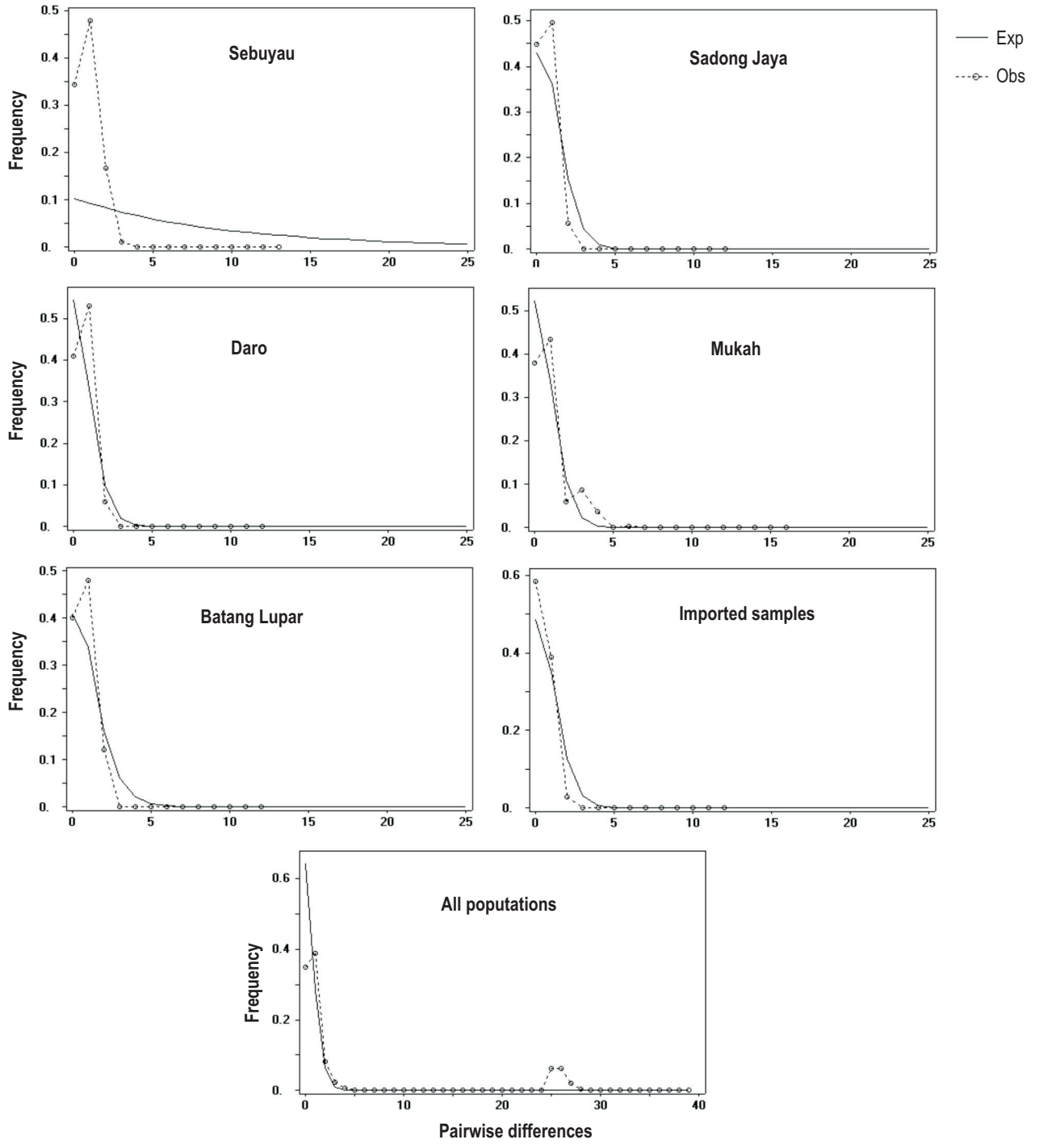


Fig. 4 : Observed and expected mismatch distribution for expanding population of *Tenualosa toli* for different and whole population

Table 3 : Below the diagonal: pairwise Tamura-Nei genetic distances among 6 populations of *Tenualosa toli*. Above the diagonal: population subdivision (Fst) values

	Sebuyau	Sadong Jaya	Daro	Mukah	Batang Lupar	Imported samples
Sebuyau		0.000*	0.000*	0.000*	0.000*	0.971***
Sadong Jaya	0.001		0.000*	0.000***	0.000**	0.978***
Daro	0.001	0.001		0.000*	0.000*	0.978***
Mukah	0.001	0.001	0.001		0.000*	0.967***
Batang Lupar	0.001	0.001	0.001	0.001		0.975***
Imported samples	0.030	0.029	0.029	0.030	0.029	

Upper diagonal population subdivision (Fst) values and probability test (Chi-square) for population differentiation based on 1000 permutations of the sequence data, significance levels (p<0.05=*, p<0.01=**, p<0.001=***)

Table 4 : Neutrality test (Tajima's D and Fu' FS) estimates (signifance level: p < 0.01) for selected populations of *Tenualosa toli* from Sarawak

	Tajima's D test				Fu's FS test		
Population	**Pi**	**Tajima's D**	**p-value**	**θ pi**	**Exp no of alleles**	**FS**	**p-value**
Sebuyau	0.844	-0.579	0.316	0.844	3.427	-1.326	0.127
Sadong Jaya	0.610	0.222	0.650	0.610	2.777	0.204	0.5000
Batang Lupar	0.721	0.393	0.373	0.721	3.001	0.748	0.268
Daro	0.652	-0.0475	0.428	0.652	2.517	-0.137	0.321
Mukah	0.980	-1.458	0.065	0.980	3.772	-0.890	0.270
Imported	0.444	-1.362	0.090	0.444	1.981	-1.081	0.048

whereas the lowest value was from haplotypes found in imported samples.

Haplotypes containing imported samples showed slightly higher genetic differences from all the other *T. toli* haplotypes (2.9%-3.3%), even though they belonged same species. This is based on the BLAST result showing that this haplotype is 99% identical to *T toli* sequences found in Genbank. As for the genetic distance between 2% to 11%, this could indicate conspecific populations (Baker and Bradley, 2001). According to the traders in the Satok market, these fish were imported from India, which would support the high genetic difference between imported and local samples. HTT7, HTT8 and HTT9 belong to a different gene pool or breeding group as unique haplotype portrays populations that belong to a geographically isolated population, but there were possible events of interbreeding leading to gene flow and low genetic differentiation (Nandeibam *et al.,* 2013). The difference between Sarawak and imported samples could be explained by several factors such as small population sizes, past bottleneck events or limited migration due to existence of physical barriers (Nguyen *et al.,* 2006). This raises a question as to the origin of *T. toil*. which could be resolved by collecting more imported *T. toli* samples from markets. Southeast Asia is believed to be mostly on the Eurasian plate and this area is actually surrounded in close proximity to the Indian-Australian, as well as Pacific and Philippine plates (Carpenter, 1998). This portrays the biggest concentration of plates in a continuous marine system leading to an assumption that all the surrounding plates disperse different species into Southeast Asian Sea leading to a huge species diversity (Carpenter, 1998). This could be one assumption as to why imported and local *T. toli* samples are genetically diverged even though they belong to the same species.

High genetic similarities and sharing of common haplotypes between *T. toli* samples from selected populations in Sarawak indicate a high level of gene flow and sharing of genetic material. This can be justified by their life history pattern as anadromous species living most of their life (especially mature stage) in estuaries, but spawn in rivers during breeding season (Whitehead, 1985). *T. toli* could be found in estuarine waters and feed along shoreline waters where their migration pattern ranges from Sematan to Lawas entering Lupar and Lassa rivers for spawning (Awang Alim *et al.,* 2012). Thus, it is predicted that migration behavior of *T. toli* during spawning season, between groups from different population might have resulted in the homogeneity of mtDNA haplotypes between them. The number of differences between two sequences increases as the time since this sequence diverged from their last common ancestor increases and measures of genetic differences between sequences is not reliable to indicate when they diverged because the rate of sequence evolution is not constant over time (Holder and Lewis, 2003).

High genetic differences and higher Fst values (0.967-0.979) between *T. toli* samples from Sarawak and imported

Table 5 : Mismatch distribution parameter estimates for selected populations of *Tenualosa toli* from Sarawak

Population	Ƭ (95% CI)	Mismatch distribution θ0	θ1
Sebuyau	2.482	0.000	3407.19
Sadong Jaya	2.115	0.056	3414.7
Batang Lupar	0.949	0.028	3427.2
Daro	0.000	0.113	6827.18
Mukah	1.824	0.028	3417.2
Imported	0.000	0.056	6822.18

samples could imply the absence of any recent migration or gene flow between these populations. This is further supported by the number of fixed haplotypes among imported samples. Within the populations in Sarawak, low mtDNA variations were observed among all the *T. toli* samples. High number of haplotypes (3-5), high haplotype diversity (0.552-0.656) and high polymorphic loci (2-7) but lack of significant differences in pairwise Fst values among population in Sarawak supports high level of intrapopulation variation compared to interpopulation variation. This result is similar to the study on *T. tambroides* (Nguyen *et al*. 2006). Low level of mtDNA variations could be due to availability of haplotype HTT1 and HTT2 in almost all of the populations. High haplotype and low nucleotide diversity might be an indicator of overfishing as all of these 5 populations are among the core terubok areas in Sarawak.

Haplotype diversity and nucleotide diversity value for Daro and Mukah observed in this study were different compared to the one observed in the study by Abdul Aziz (2015). Haplotype diversity in this study was higher for both Daro and Mukah whereas the nucleotide diversity for Daro was lower compared to the previous study. As for Tajima's D value, Sebuyau, Daro and Mukah population portrayed a negative value. A negative D value could be the result of recently occurred bottleneck leading to population size expansion (Tajima, 1989). Negative Tajima's D value implies excess of low frequency polymorphism which could be due to population size expansion. On the other hand, positive value could be due to population size reduction and balancing selection (Tajima, 1989). This could be seen in Sadong Jaya and Batang Lupar population. As for Fu's Fs-Statistical tests which were done for demographic history, a negative value is expected from a recent population expansion whereas a positive value is from a recent population bottleneck. This shows that recent population expansion occurred in Sebuyau and Batang Lupar.

The minimum spanning network (MSN) of 15 haplotypes of *T. toli* (Fig. 3) obtained from selected locality in Sarawak and imported samples showed high haplotype variability among samples collected in Sarawak. Haplotypes consisting of imported samples are clustered together. Most of the sequences are connected by at least one mutational step leading to the fact that there are no geographically based defined clades for *T. toli* population in Sarawak. Most of the sequences are mixed, hence leading to the existence of mixed haplotypes. MSN phylogram could not portray well defined separation of *T. toli* samples based on their geographical clades.

Pairwise Fst values for genetic differentiation among populations showed significant levels of genetic differentiation in all comparisons between imported *T. toli* population and local samples. However, there were no genetic differentiations in pairwise Fst values in most comparisons among populations from Sarawak (Table 3). Pairwise genetic distances calculated using Tamura-Nei model among *T. toli* populations in this study are shown in Table 3. The highest genetic distances were observed between imported and local samples (2.9%). There were minimum genetic similarities among samples from Sarawak and imported samples. On the other hand, low genetic distances were observed among all the locally collected samples (0.1%).

AMOVA results revealed that the majority of variance as well as percentage of variation were among populations. Inter-population variation showed a higher value, which about 86% is compared to within population variation with only about 14% variation. Mismatch distribution for expanding populations of *T. toli* portrayed a multimodal pattern (Fig. 4). Tajima's D value was negative for Sebuyau, Daro, Mukah and Imported samples. On the other hand, Fu's FS also showed negative value for Sebuyau, Daro, Mukah and imported samples (Table 4) but both analysis were not significant. Mismatch distribution value consists of θ0 and θ1 value which signifies growth rate of *T. toli* populations (Table 5).

The Tau and θ value for mismatch distribution in this study show similar pattern to *L. maculatus,* which portrayed large-scale expansion (Liue *et al*., 2006) and in accordance to neutrality tests. Sebuyau, Daro and Mukah portrayed negative Tajima's D value and Fu's FS value. Population expansion theory supported by unimodal pattern in mismatch distribution analysis, negative Tajima's D and Fu's FS value, high level of haplotype diversity and low level of nucletiode diversity (Chen *et al*., 2004).

The significance of this study on conservation could be the idea of selecting stocks for breeding program as it is believed that population, which portrays high intrapopulation variation could be used as base-line stocks for selective breeding (Nguyen *et al.,* 2006). This preliminary investigation has managed to provide a better insight on the phylogeny, genetic identity and level of genetic differences between the three shads. High genetic divergences and monophyletic status between *T. toli*, *T. macrura* and *T. ilisha* confirmed their taxonomic status as distinct species thus proves that Cyt b is a suitable gene for the purpose of species, identification. The rapid decline of local *terubok* population necessitates immediate study to quantify the remaining level of genetic variation in *T. toli* and *T. macrura* to assist in conservation and management of *terubok* in Sarawak.

So, it is recommended that more individuals of genus *Tenualosa* from different population should be included to provide a better insight to determine the phylogenetic relationship, as this study only focuses on few localities in Sarawak. Utilization of different types of genetic marker is recommended to obtain a more robust findings on the population of *T. toli*.

Acknowledgments

The authors would like to thank all the staff from Department of Aquaculture and lab mates from Fish Genetics and Breeding laboratory, who have assisted me in this project. The authors would also like to take this opportunity to thank Universiti Putra Malaysia for giving me an opportunity to conduct my research. This project was funded by Fundmental Research Grant Scheme FRGS/1/2014/STWN04/UPM/02/3.

References

Abdul Aziz, H.A., Z. Tarmizi, N.M. Ali, N.A. Ariffin, M.D.D. Abdullah, W.L. Lian, S.Y. Giat, T.N.A.M. Jaafar, A.M.A. Bolong, A.A.A. Kasim and S.M. Sheriff: Mitochondrial DNA Diversity of Terubok (*Tenualosa toli*) from Daro and Mukah, Sarawak Inferred by Partial Cytochrome *b* (Cyt *b*). *J. Fish Aquat. Sci.*, **10**, 92-101 (2015)

Arai, T. and R. Amalina: New record of a tropical shad *Tenualosa ilisha* (Teleostei: Clupeidae) in Malaysian waters. *Mar. Biodivers. Rec*., **7**, 1-4 (2014).

Avise, J.C.: Molecular markers, natural history and evolution. New York: London: Chapman and Hall (1994).

Avise, J.C.: Phylogeography: The history and formation of species: Harvard University Press, Cambridge, M.A. (2000).

Awang Kasim, A.A., P. Wong and Khairulniezawaymie. The terubok (*Tenualosa* spp.) rehabilitation integrated program (TRIP) and conservation in Sarawak. In: Proceedings of UMT 11th International Annual Symposium on Sustainability Science and Management, Universiti Malaysia Terengganu, pp. 1476-1479 (2012).

Baker, R. J. and R.D. Bradley: A test of the genetic species concept: Cytochrome *b* sequences and mammals. *J. Mammal*., **82**, 960-973 (2001).

Baker, R.J. and R.D. Bradley: Speciation in mammals and the Genetic Species Concept. *J. Mammal*., **87**, 643-662 (2006).

Bandelt, H.J., P. Forster and A. Rohl: Median-joining networks for inferring interspecific phylogenies. *Mol. Biol. Evol*., **16**, 37-48 (1999).

Barat, A., S. Ali, J. Sati and G.K. Sivaraman: Phylogenetic analysis of fishes of the subfamily Schizothoracinae (Teleostei: Cyprinidae) from Indian Himalayas using Cytochrome *b* gene. *Indian J. Fish*., **59**, 43-47 (2012).

Blaber, S.J.M.: Can the ecosystem approach improve management of tropical estuarine fisheries for diadromous species. *Am. Fish. Soc. Symp*., **69**, 649-661 (2009).

Blaber, S.J.M., D.T. Brewer, D.A. Milton, G.S. Merta, D. Efizon, G. Fry and T. Van der Velde: The life history of the protandrous tropical shad *Tenualosa macrura* (Alosinae: Clupeidae): Fishery Implications. *Estuar. Coast. Shelf Sci*., **49**, 689-701 (1999).

Blaber, S.J.M., D.A. Milton, J. Pang, P. Wong, O. Boon-Teck, L. Nyigo and D. Lubim: The life history of the tropical shad *Tenualosa toli* from Sarawak: First evidence of protandry in the Clupeiformes. *Environ. Biol. Fish*., **46**, 225-242 (1996).

Blaber, S.J.M., G. Fry, D. Milton, T. Van der Velde, O. Boon-Teck, J. Pang and P. Wong: The life history of *Tenualosa macrura* in Sarawak, further notes on protandry in the genus and management strategies. *Fish. Manag. Ecol*., **12**, 201-210 (2005).

Brown, T. A.: Genomes. 2nd Edn., Oxford Wiley- Liss (2002).

Carpenter, K.E.: An introduction to the oceanography, geology, biogeography and fisheries of the tropical and subtropical waters and central Pacific. *FAO species identification guide for fishery purpose:* The living marine resources of the western central Pacific Rome: FAO, pp. 1-9 (1998).

Chen, C.A., M.C.A. Ablan, J.W. McManus, J.D. Bell, V.S. Tuan, A.S. Cabanban and K.T. Shao: Population and genetic variability of six wrasse (*Thallasoma hardwicki*) in Northern South China Sea revealed by mitochondrial region sequences. *Marine Biotechnology,* **6**, 312-326 (2004).

Cook, B. D., M. Baker, T.J. Page, S.C. Grant, J.H. Fawcett, D.A. Hurwood and J.M. Hughes: Biogeographic history of an Australian freshwater shrimp, *Paratya australiensis* (Atydae): The role of life history in phylogeographic diversification. *Mol. Ecol*., **15**, 1083-1093 (2006).

Esa, Y.B., J.M. Waters and G.P. Wallis: Introgressive hybridization between *Galaxias* 262 *depressiceps* and *Galaxias sp* D (Teleostei: Galaxiidae) in Otago, New Zealand: Secondary contact mediated by water races. *Conserv. Genet*., **1**, 329-339 (2000).

Esa, Y.B., S.S. Siraj, S.K. Dud, J.R.R. Japning, A.R., Khairul Adha and S.G. Tan: Molecular systematics of mahseers (Cyprinidae) in Malaysia inferred from sequencing of a mitochondrial Cytochrome c oxidase I (COI) gene. *Pertanika J. Trop. Agric. Sci*., **31**, 263-269 (2008).

Esa, Y.B., R.R.J. Jeffrine, A.R. Khairul Adha, S.S. Siraj, S.K. Daud, S.G. Tan and S. Sungan: Phylogenetic relationship among several freshwater fishes (Family: Cyprinidae) in Malaysia inferred from partial sequencing of the Cytochrome *b* mitochondrial DNA (mtDNA) gene. *Pertanika J. Trop. Agri. Sci*., **35**, 307-318 (2012).

Esa, Y.B. and A.R. Khairul Adha: Genetic structure and preliminary findings of cryptic diversity of the Malaysian Mahseer (Tor *tambroides* Valenciennes: Cyprinidae) inferred from mitochondrial DNA and microsatellite analyses. *BioMed Res. Int.,* **2013,** 1-14 (2013).

Excoffier, L. and H.E.L. Lischer: Arlequin suite ver 3.5: A new series of programs to perform population and genetics analyses under Linux and Windows. *Mol. Ecol. Resour*., **10**, 564-567 (2010).

Felsenstein, J.: Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* **39**, 783-791 (1985).

Fu, Y. X.: Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **157**, 915-925 (1997).

Holder, M. and P.O. Lewis: Phylogeny estimation: Traditional and Bayesian approaches. *Nat. Rev. Genet*., **4**, 275-284 (2003).

Hsieh, H.M., H.L. Chiang, L.C. Tsai, S.Y. Lai, N.E. Huang, A. Linacre and C.I.L. James: Cytochrome *b* gene for species identification of the conservation animals. *Forensic Sci. Int*., **122**, 7-18 (2001).

Irwin, D., T. Kocher and A. Wilson: Evolution of the Cytochrome *b* gene of mammals. *J. Mol. Evol*., **32**, 128-144 (1991).

Jeffrine, R.J.R. and Y.B. Esa: Phylogenetic analysis of Hampala fishes (Subfamily Cyprinina) in Malaysia inferred from partial mitochondrial Cytochrome *b* DNA sequences. *Zool. Sci.,* **23**, 893-901 (2006).

Kamarudin, K.R. and Y.B. Esa: Phylogeny and phylogeography of *Barbonymus schwanenfeldii* (Cyprinidae) from Malaysia inferred using partial Cytochrome *b* mtDNA gene. *J. Trop. Biol. Conser.,* **5,** 1-13 (2009).

Khairul Adha, A.R., M. Hambali Tumiran, M.L. Shabdin, Y.B. Esa and A.A.S. Awang Husaini: The status of *Tenualosa toli* (Valenciennes, 1847) in the Southwest coast of Sarawak, Malaysia. *Kuroshio Sci*., **8**, 39-44 (2014).

Kimura, M.: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol., **16**, 111-120 (1980).*

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson and D.G. Higgins: Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947-2948 (2007).

Librado, P. and J. Rozas: DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451-1452 (2009).

Liu, J.X., T.X. Gao, K. Yokogawa and Y.P. Zhang: Differential population structuring and demographic history of two closely related fish species, Japanese sea bass (*Lateolabrax japonicas*) and spotted sea bass (*Lateolabrax maculates*) in Northwestern Pacific. *Mol. Phylogenet. Evol*., **39**, 799-811 (2006).

Nandeibam, S.S., B.K. Behera and A.P. Sharma: Population structure of *Puntius sophore* inferred from variation in mitochondrial DNA sequences. *Int. J. Res. Fish. Aquac*., **3**, 112-115 (2013).

Nguyen, T.T., B. Ingram, S. Sungan, G. Gooley, S.Y. Sim, D. Tinggi and S.S. De Silva: Mitochondrial DNA diversity of broodstock of two indigenous mahseer species, *Tor tambroides* (Cyprinidae) cultured in Sarawak, Malaysia. *Aquaculture*, **253**, 259-269 (2006).

Pääbo, S. and A.C. Wilson: Polymerase chain reaction reveals cloning artefacts. *Nature*, **334,** 387-388 (1988).

Rogers, A.R. and H. Harpending: Population growth curves in the distribution of pairwise genetic differences. *Mol. Biol. Evol*., **9**, 552-569 (1992).

Saitou, N. and T. Nei : The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., **4,** 406- 425 (1987).

Salini, J.P., D.A. Milton, M.J. Rahman and M.G. Hussain: Allozyme and morphological variation throughout the geographic range of the tropical shad, hilsa *Tenualosa ilisha*. *Fish. Res*., **66**, 53-69 (2004).

Stepien, C.A. and T.D. Kocher: Molecules and morphology in studies of fish evolution. In: Molecular Systematic of Fishes(Eds.: T.D. Kocher and C.A. Stepien). London, Academic Press, pp. 1–11 (1997).

Tajima, F.: Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585-595 (1989).

Tamura, K. and M. Nei: Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.,* **10**, 512-536 (1993).

Tamura, K., G. Stecher, D. Peterson, A. FIlipski and S. Kumar: MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.,* **30**, 2725-2729 (2013).

Chauhan, T. and R. Kumar: Molecular marker and their applications in fisheries and aquaculture. *Adv. Biosci. Biotechnol.,* **1**, 281-291 (2010).

Whitehead, P.J.P.: FAO species catalogue Vol.70. Clupeoid fishes of the world (suborder Clupeoidei). An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, shads, anchovies and wolf herrings. Part1- Chirocentridae, Clupeidae and Pristigasteridae, FAO Fisheries Synopsis, **125**, 1-303 (1985).