*Journal of Sustainability Science and Management*
*Volume 20 Number 1, January 2025: 78-100*
*eISSN: 2672-7226*


# GENETIC DIVERSITY AND POPULATION GENETICS OF TROPICAL SHADS (*Tenualosa toli*) FROM SARAWAK, MALAYSIA

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*http://doi.org/10.46754/jssm.2025.01.005*
*Received: 29 May 2024* *Accepted: 15 September 2024* *Published: 15 January 2025*

**Abstract:** The Tropical shads (*Terubok*), *Tenualosa toli* catch has been in decline for the past 15 years owing to the extensive exploitation and high commercial demand. There is limited genetic information on *Terubok* research using a molecular approach, leaving its population's genetic status unassessed. A total of 180 *T. toli* samples from Mukah, Daro, and Pusa in Sarawak, Malaysia were collected for this study. The DNA of samples was then amplified, sequenced, and analysed using BLAST and MEGA. The findings indicate that all the samples were *T. toli* with six haplotypes for *CO1* and 11 haplotypes for *CytB*. NJ and ML phylogenetic trees for the *CytB* gene revealed that all haplotypes formed only a single cluster. The population was discovered to have a low genetic diversity. The ($F_{ST}$) AMOVA and pairwise $\Phi_{ST}$ results indicate no significant genetic differences. The Gene Flow (Nm) also exhibits the presence of high Nm from the three populations. The IBD has proven insignificant, indicating that the *T. toli* populations came from similar origins. The neutrality test confirmed that all the samples experienced population expansion and the population is currently in equilibrium. This study provides comprehensive genetic information that can be used for the sustainability management of this valuable species in Sarawak.

Keywords: *Tenualosa toli*, Sarawak, population genetic, genetic diversity, population structure.

## Introduction

Tropical shads, *Tenualosa* spp. locally known as *Terubok* are one of the most economically vital fish species in Malaysia, especially in Sarawak. There are three species of *Tenualosa* recorded in Malaysia: *T. toli*, *T. macrura*, and *T. ilisha* (Abdul Aziz *et al*., 2015; Arjunaidi *et al*., 2016; Puvanasundram *et al*., 2018). *Empirit* is another name that refers to *Terubok*; however, this term is used for small-sized *T. toli* (Khairul Adha *et al*., 2014; Puvanasundram *et al*., 2018). *Tenualosa* species is considered an endemic species in Sarawak, inhabiting coastal areas (Blaber *et al*., 1996; Arjunaidi *et al*., 2016; Puvanasundram *et al*., 2018). Notably, *T. toli* belongs to the family Clupeidae, a protandrous hermaphrodite species. *T. toli* is also classified as an anadromous fish and spends most of its juvenile stage in freshwater. Furthermore, *T. toli* is categorised as a well-adapted fast swimmer among pelagic fish. In addition, this fish has a very thin ventral edge with a long caudal fin and a streamlined body that helps the species survive in waters with high levels of salinity. *T. toli* can be discovered in lagoons and estuarine waters and in shallow muddy water (Blaber *et al*., 1996; Ambak, 2010). Moreover, this fish is in high demand because of its unique taste and roe (Abdul Aziz *et al*., 2015; Puvanasundram *et al*., 2018).

Kasuma *et al*. (2019) highlighted the significance of *Terubok* fish in Sarawak as salted *Terubok* is considered a signature product of the state. The importance of *Tenualosa* spp. to the Sarawak economy can be observed since some salted *Terubok* fish are claimed to be able to sell for between RM3,000.00 and

RM4,000.00 daily. This is attributable to the local Sarawak *Terubok* fish that can command prices of between RM50.00 to RM70.00 per fish, while prices for some of the *Terubok* fish weighing between 800 g and 1 kg can reach RM150.00 a fish (Mohd Radzi, 2019; Nor Ain, 2021). Furthermore, Mohd Radzi (2019) and Nor Ain (2021) also reported that the roe of the *Tenualosa* spp. sells for between RM200.00 and RM500.00 per kilogram and has high demand from buyers in Singapore and Brunei. This can be observed by referring to the Fisheries Department (DOF) Malaysia (2021) fish landing statistics, where 2,168 tonnes of *Tenualosa* spp. were landed in the year 2020. The same report also stated that the fishing gear used to capture these fish include gill nets, bag nets, hooks, lines, and trawl nets.

However, there is another species of *Tenualosa* spp. in Sarawak which is *T. macrura.* This shad species has an almost identical morphology to that of *T. toli*, which can lead to misidentification, especially in the juvenile stages (Puvanasundram *et al*., 2018). It was also reported that the *Tenualosa* spp. population has been heavily exploited, including *T. toli* (Blaber *et al*., 1996; Khairul Adha *et al*., 2014). Consequently, this situation can affect genetic diversity by reducing the genetic diversity of the population (Kenchington, 2003; Gandra *et al*., 2021; Taboun *et al*., 2021).

Various molecular markers have been employed to assess the genetic diversity and populations genetic for both freshwater and marine species of fish (Madduppa *et al*., 2018; Panprommin *et al*., 2019; Zhai *et al*., 2019; Chen *et al*., 2020; Firidin *et al*., 2020; Sun *et al*., 2021; Prasertlux *et al*., 2022). Among the markers, mitochondrial genes such as Cytochrome Oxidase I (*CO1*) and Cytochrome B (*CytB*) were the genes used for the genetic diversity evaluation for marine fish species (Zheng *et al*., 2015; Moreira *et al*., 2019; Liu *et al*., 2020). *CO1* and *CytB* genes were used in the identification of marine fishes since these two genes are capable of identifying unambiguous sequences due to low intraspecific variation and high conservative region possessed by the two genes. Accordingly, it increases the accuracy of species delimitation in fish (Kochzius *et al*., 2010; Bingpeng *et al*., 2018; Naeem *et al*., 2020; Panprommin *et al*., 2023; Sout *et al*., 2023).

This study aims to identify the *Tenualosa* species from Daro, Mukah, and Paso, Sarawak, Malaysia using mitochondrial DNA (mtDNA) *CO1* and *CytB* genes. This study also aims to assess the genetic diversity and population structure of the identified *Tenualosa* spp. using the *CytB* gene. The outcome of this study will enable the establishment of *Tenualosa* spp. database from Sarawak waters, in addition to have baseline data on the genetic diversity and population structure of *Tenualosa* spp. in terms of its migratory patterns. This can later be used to conserve the habitat of this highly prized fish species.

## Materials and Methods

### *Samples Collection*

A total of 180 samples of *T. toli* (Figure 1) were collected from three locations of Sarawak River systems (Table 1, Figure 2). These locations include Mukah (n = 60), Daro (n = 60), and Pusa

Figure 1: External morphology of (A). Tenualosa toli, (B). Tenualosa macrura

Table 1: Sampling locations of *T. toli* in the Sarawak River system

| River | Sampling Site | Geographical Coordinate | Number of individuals (N) |
|---|---|---|---|
| Batang Saribas | Pusa | 01°37'31.0" N,111°15'38.4 E | 60 |
| Batang Lassa | Daro | 02°28'53.0" N, 111°27'07.1 E | 60 |
| Gigis River | Mukah, | 02°54'59.0" N,112°05'37.6 E | 60 |

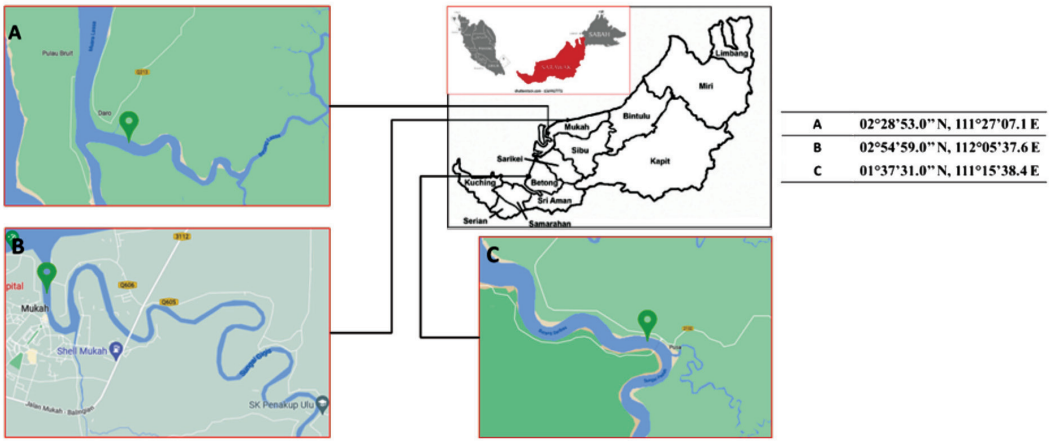


Figure 2: Sampling area of *T. toli* in (A) Batang Lassa River, Daro, (B) Gigis River, Mukah, and (C) Batang Saribas River, Mukah

(n = 60) and the samples were caught using a gill net. All specimens were morphologically identified using taxonomic keys and fish identification references from Whitehead (1985), Munroe *et al*. (1999), Narejo *et al*. (2008), and Hong *et al*. (2013). A small portion of pectoral fins and muscles from each individual was cut, preserved in 95% ethanol, and stored in a 1.5 mL centrifuge tube at 4°C until needed for further analysis.

### *DNA Extraction and Quantification*

Genomic material and DNA from the 180 samples was extracted using cfDNA Extraction Kit (BioTeke Corporation, China) following manufacturer protocol and the DNA was eluted into the final concentration of 70 µL. The concentration and purity of the extracted DNA stock was determined using a Bio Photometer (Eppendorf, Germany) and then stored at -20°C prior to amplification.

### *Polymerase Chain Reaction (PCR) Amplification and Sequencing*

Polymerase Chain Reaction (PCR) amplification of *CO1* was performed according to Lim *et al*. (2011), while *CytB* according to Faria *et al*. (2006). Polymerase reactions for both genes was prepared in 25.0 µL reaction volumes separately, which contained 1X of 10X PCR buffer with $MgCl_2$ (Merck Group, Germany), 0.008 mM of Thermo Scientific™ dNTP Mix (Thermo Fisher Scientific, United States), 0.24 mM of forward and reverse primer, respectively, 0.06 mM of Invitrogen Taq DNA Polymerase Recombinant (Thermo Fisher Scientific, United States), 8 ng of DNA template, and sterile deionised water filled up to 25.0 µL. The sequences of the primers used were FishF1 and FishR1 for *CO1* (Ward *et al*., 2005), while Alocytbf1 and Alocytbr1 for *CytB* (Faria *et al*., 2006). The thermocycling profiles for the *CO1* gene consisted of an initial denaturation step of 3 minutes at 94°C, followed

by 35 cycles of denaturation step at 94°C for 30 seconds, annealing at 54°C for 40 seconds, and extension at 72°C for 40 seconds, and final extension at 72°C for 10 minutes.

Meanwhile, the thermocycling profiles for the *CytB* gene consisted of an initial denaturation step of 5 minutes at 95°C, followed by 25 cycles of denaturation step at 94°C for 40 seconds, annealing at 54°C for 45 seconds, and extension at 72°C for 1 minute; and, final extension at 72°C for 7 minutes. The PCR products were visualised on a 1.2% (w/v) agarose gel stained with SYBR Safe and the clear and single bands of each product proceeded with the purification procedure using QIAquick® PCR purification kit (Qiagen, Germany) following the manufacturer's protocol prior to submit it to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing.

Samples were sequenced bi-directionally using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, United States). Sequencing reactions were conducted on an ABI 3730xl DNA Analyser (Thermo Fisher Scientific, United States).

### *Identification of Tenualosa spp. using mtDNA CO1 and CytB*

All *CO1* and *CytB* sequences were edited and aligned using the software Chromas (Technelysium Pty Ltd) and BioEdit (Ibis Bioscience). This process was essential to validate the sequences by removing the gaps, noise, and ambiguous sites. Confirmation of species identification was performed by comparing the clean sequences to the sequences in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the nucleotide Basic Local Alignment Search Tool (BLASTn) option to avoid misidentification of specimens collected. The highest percentages of identity, 97% and above were considered as maximum identification and most similar to the sample sequences (Aaron *et al*., 2018; Mwita and Chuhila, 2023). E (Expect) value for each sample was also provided in the result; the lower the E value or closer to zero, the more significant the alignment (Ghouri *et al*., 2020).

### *Data Analyses*

#### *Genetic Diversity*

The DnaSP software version 5.0.1.1 (Librado and Rozas, 2009) was first used to evaluate the presence of haplotypes in each of the three localities. Representative sequences from each of the haplotypes of *CO1* and *CytB* genes were deposited in the NCBI Genbank. Genbank (NCBI) accession number for *CO1* haplotypes was OQ380665 – OQ380670 while the haplotypes for *CytB* was OQ421124 – OQ421134. DnaSP was also used to calculate genetic diversity, including analysis levels of DNA polymorphism at each site, including haplotype or gene diversity (h) and nucleotide diversity ($\pi$). Nucleotide diversity is the average pairwise nucleotide differences per site among DNA sequences (Nei, 1987). Estimation of Gene Flow (Nm) was also determined using DnaSP software. Furthermore, DnaSP was again used to evaluate the mismatch distribution estimation of pairwise differences between all individual haplotypes with 10,000 parametric bootstrap iterations to provide a clear picture of previous population demography.

#### *Phylogenetic and Population Level Analyses*

The phylogenetic relationship among haplotypes was determined based on Neighbour-Joining (NJ) and the Maximum Likelihood (ML) methods implemented in MEGA 7.0 (Kumar *et al*., 2016). Both phylogenetic trees were constructed based on the suggested best-fit DNA substitution models. *CytB* gene analysis was conducted based on the Kimura 2 Parameter with Gamma distribution (K2+G) model with 1,000 bootstrap replications (Kumar *et al*., 2016). Next, all the *CytB* sequences were aligned with sequences of other *Tenualosa* species and some outgroup species available in the GenBank. All the sequences retrieved from the GenBank used for the phylogenetic tree construction were as follows: *T. toli* (KX859103.1), *T. thibaudeaui*

(MG958229.1), *T. ilisha* (MN748961.1), *T. macrura* (KY628753.1), and *Dussumieria elopsoides* (MH380250.1).

Population growth was assessed using two selective neutrality tests, Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) using the software Arlequin ver. 3.5 (Excoffier and Lischer, 2010) with 1,000 permutations under the assumption of neutral evolution in population equilibrium. Hierarchical Analysis of Molecular Variance (AMOVA) was also conducted using the same software. The $F_{ST}$ value was calculated by incorporating the variance value among groups within the total (FCT), populations within groups (FSC), and populations within the total (FCT). Minimum Spanning Network (MSN) was developed using Arlequin based on the calculation of *CytB* Haplotype Data Files of the three *T. toli* populations generated by DNAsp software. HapStar (ver 0.6; Teacher and Griffiths, 2010) was used to visualise the network (Elbrecht *et al*., 2014; Gwak *et al*., 2014; Osyczka *et al*., 2014; Williford *et al*., 2014). Isolation By Distance (IBD) was evaluated using the Mantel Test with 10,000 permutations as implemented in Arlequin ver. 3.5 software using the *CytB* sequence data. The association between geographical distances, measured as linear distance (km) between two sites using Google Earth and genetic distances across all groups was examined (Bohonak, 2002; Winters *et al*., 2010; Dong *et al*., 2012; Drew and Barber, 2012; Sá-Pinto *et al*., 2012; Orsini *et al*., 2013; Derycke *et al*., 2013; Li *et al*., 2013; Wang *et al*. 2013; Torquato *et al*., 2019).

## Results and Discussion

### *Identification of T. toli Using mtDNA CO1 and CytB*

A total of 141 and 108 individuals from three different populations were successfully sequenced for the partial mtDNA *CO1* and *CytB* gene, respectively. The percentage of similarities (BLAST) when compared with sequences from GenBank was 100% and 98.6% for *CytB* and *CO1*, respectively, which was above the 97% acceptance percentage. Therefore, this confirmed that all specimens collected were correctly identified as *T.toli*.

A precise species identification must include molecular analysis, as misidentification potentially occurs due to similarities and overlapping characteristics in the morphology of tropical shads (Arai and Amalina, 2014; Robert *et al*., 2019). In addition, correct identification is also crucial for the conservation and management of the organisms, as fishes exhibit various patterns of shapes, colours, and sizes (Omer *et al*., 2017). The samples from Daro, Mukah, and Pusa were confirmed as *T. toli*. The gene fragments *CO1* (499 bp) and *CytB* (469 bp) were successfully amplified and sequenced from all specimens extracted in this study. The efficiency of *CO1* and *CytB* was proven as a wise marker in species identification and have been extensively used in fish barcoding studies in the Barcode of Life Data System (BOLDSYSTEM) (Kochzius *et al*., 2010; Ratnasingham & Hebert, 2013; Fernandes *et al*., 2017; Puvanasundram *et al*., 2018; Panprommin *et al*., 2019; Basith *et al*., 2022; Zafar *et al*., 2024). It also indicates that at least 27 samples from each population were successfully amplified using *CO1* and *CytB* genes. The sampling size of a minimum of n = 25 was also used by several previous genetic population studies such as Hale *et al*. (2012), Villanueva *et al*. (2022), and Groeneveld *et al*. (2024), indicating the results obtained from such a sampling size is considered as valid and acceptable.

Past research has revealed that the taxonomy of Clupeiformes has been extensively studied (Whitehead, 1985). However, the molecular phylogenetic relationships are still poorly understood, indicating that the genetic characteristics such as the genetic species concept had never been tested (Baker and Bradley, 2006; Puvanasundram *et al*., 2018). In parallel with previous research, findings from this study on the *CytB* gene and *CO1* gene reported the genetic relationship between *T. toli* and *T. macrura* (Puvanasundram *et al*., 2018) and *T. toli* with *T. ilisha* (Arjunaidi *et al*., 2016). According to Blaber *et al*. (1999)

and Puvanasundram *et al*. (2018), *T. macrura* closely resembles *T. toli* but has a smaller head and longer tail compared with the latter. Meanwhile, according to Whitehead (1985) and Puvanasundram *et al*. (2018), *T. toli* resembles *T. ilisha* which has a shorter caudal fin (25% to 31% standard length), longer head (28% to 32%), more gill rakers, and spots along its flank. Therefore, it was suggested that molecular genetic analysis should always be included for precise species identification and confirmation of shad species (Robert *et al*., 2019).

### *Genetic Diversity of Tenualosa toli Inferred by mtDNA CytB*

*Haplotypes Distribution*

A total of 6 and 11 unique haplotypes were identified for *CO1* (Table 2) and *CytB* (Table 3), respectively. In *CO1* sequence analysis, Hap01 was identified as common haplotype shared by a total of 126 individuals with the highest relative frequency of 0.960. The other five haplotypes (Hap02 to Hap06) demonstrated distinctive unique composite nucleotides and only represented a single individual with a relatively low frequency of 0.008. Four haplotypes (Hap02, Hap03, Hap 04, and Hap05) were discovered within the Daro locality and another haplotype (Hap06) was recorded within the Mukah locality.

*CytB*, Hap01 is a common haplotype shared by a total of 98 individuals with the highest relative frequency of 0.910. The other 10 haplotypes (Hap02 to Hap11) exhibited unique composite nucleotides. They only represented a single individual with a relatively low frequency of 0.009. Two haplotypes (Hap02 and Hap03) were discovered within the Pusa locality and five haplotypes (Hap04, Hap05, Hap06, Hap07, and Hap08) were detected within the Daro locality. In comparison, another three haplotypes (Hap09, Hap10, and Hap11) were recorded within the Mukah locality.

*CytB* gene has been used widely in many studies on closely related fish species such as *Epinephelus coioides* (Waludin *et al*., 2018), *Channa striata* (Duong *et al*., 2019), and *Lates calcarifer* (Bakri & Esa, 2021). In addition, *CytB*

Table 2: Numbers of haplotype and relative frequency of CO1 of Tenualosa spp. from three study sites in Sarawak

| Sample | | | Haplotype | Frequency |
|---|---|---|---|---|
| **Daro** | **Mukah** | **Pusa** | | |
| D1, D2, D3, D4, D5, D6, D7, D8, D10, D12, D13, D14, D15, D16, D17, D19, D21, D22, D23, D24, D27, D28, D29, D30, D32, D34, D35, D36, D37, D38, D39, D41, D43, D44, D45, D46, D47 | M1, M2, M3, M4, M5, M6, M7, M9, M10, M11, M12, M13, M14, M15, M16, M17, M19, M22, M23, M24, M26, M27, M28, M29, M30, M32, M33, M34, M36, M38, M39, M40, M43, M44, M45, M46, M47, M48, M49, M50, M51, M52, M53, M54, M55, M56, M57, M58, M59 | P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16, P17, P18, P19, P20, P21, P22, P23, P24, P25, P26, P27, P28, P29, P30, P31, P32, P33, P34, P35, 36, P37, P38, P39, P40, P41, P42, P43, P44, P45, P46, P47, P48, P49, P50 | Hap01 | 0.960 |
| D9 | | | Hap02 | 0.008 |
| D20 | | | Hap03 | 0.008 |
| D26 | | | Hap04 | 0.008 |
| D31 | | | Hap05 | 0.008 |
| | M25 | | Hap06 | 0.008 |

Table 3: Numbers of haplotype and relative frequency of CytB Tenualosa spp. from three study sites in Sarawak

| Sample | | | Haplotype | Frequency |
|---|---|---|---|---|
| Daro | Mukah | Pusa | | |
| D1, D2, D3, D4, D5, D6, D7, D8, D9, D11, D12, D13, D14, D15, D16, D17, D18, D19, D22, D23, D24, D25, D26, D29, D30, D31, D32, D33, D34, D35, D36, D37, D38, D39, D40, D41 | M2, M3, M4, M5, M6, M7, M9, M10, M11, M13, M14, M15, M16, M17, M18, M19, M20, M23, M24, M25, M26, M27, M28, M30, | P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16, P17, P18, P19, P20, P21, P22, P23, P24, P25, P26, P27, P28, P29, P30, P31, P33, P34, P36, P37, P38, P39, P40 | Hap01 | 0.910 |
| | | P32 | Hap02 | 0.009 |
| | | P35 | Hap03 | 0.009 |
| D10 | | | Hap04 | 0.009 |
| D20 | | | Hap05 | 0.009 |
| D21 | | | Hap06 | 0.009 |
| D27 | | | Hap07 | 0.009 |
| D28 | | | Hap08 | 0.009 |
| | M1 | | Hap09 | 0.009 |
| | M22 | | Hap10 | 0.009 |
| | M29 | | Hap11 | 0.009 |

has also been considered the best mitochondrial gene for phylogenetic analysis (Farias *et al*., 2001; Megarani *et al*., 2020). *CytB* gene is ideal for examining the systematic diversity of phylogeny due to its slowly evolved codon position and variable domains (Kumazawa & Nishida., 2000; Farias *et al*., 2001; Shrestha *et al*., 2019; Megarani *et al*., 2020; Elvyra *et al*., 2020). MSN has indicated that the *T. toli* population across Daro, Mukah, and Pusa demonstrated that Hap01 (0.910) was the most dominant and shared by 98 individuals from all three populations. The other 10 haplotypes were unique to each population. There is the presence of unique haplotypes at each of the three localities, which are Daro (n = 5), Mukah (n = 3), and Pusa (n = 2). Daro population had the highest haplotype (0.232) and nucleotide (0.00321) diversities, followed by Mukah at 0.214 and 0.00095 and the Pusa population recorded the lowest value at 0.099 and 0.00021, respectively (Table 4).

Table 4: Genetic diversity for CytB gene in *T. toli* showing the number of individuals (N), number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π) and neutrality tests for each population Tenualosa spp. from three study sites in Sarawak

| Population | N | H | Genetic Diversity | |
|---|---|---|---|---|
| | | | ***h*** | π |
| Daro | 41 | 6 | 0.232 | 0.00321 |
| Mukah | 27 | 4 | 0.214 | 0.00095 |
| Pusa | 40 | 3 | 0.099 | 0.00021 |
| Total | 108 | 13 | - | - |

Genetic diversity can greatly influence the species or population's ability to cope and adapt to environmental conditions in new habitats (Frankham *et al*., 2002; Spielman *et al*., 2004; Gandra *et al*., 2021). The value of haplotype diversity (*h*) and nucleotide diversity (π) are vital indicators for genetic diversity, as a higher the values, the higher the genetic diversity in the populations (Liu *et al*., 2020; Zhang *et al*., 2023). The NJ tree is constructed based on the value of the distance of sequences. Meanwhile, the ML tree is constructed based on the maximum values of characters from gene sequences (Amiroch *et al*., 2018; Hong *et al*., 2021; Chen & Wang, 2022).

The sharing of haplotype at 91% frequency suggests that the species could probably have originated from a common origin and shared close relationships. The finding are supported by a study by Lee *et al*. (2021) which proposed that the sharing of haplotypes at 81% frequency might come from similar origins. The other 10 haplotypes were unique to each population. There were five, three, and two unique haplotypes of Daro, Mukah, and Pusa, respectively, that were private to single localities. The result of haplotype diversity, *h*, and nucleotide diversity, π from this study were as follows: Daro ($h = 0.232$; $\pi = 0.00321$), Mukah ($h = 0.214$; $\pi = 0.00095$), and Pusa ($h = 0.099$, $\pi = 0.00021$). These results overall indicated that both haplotype diversity and nucleotide diversity from the three localities have low genetic diversity ($h < 0.5$, $\pi < 0.005$) (Grant & Bowen, 1998; Fang *et al*., 2022; Trang *et al*., 2022; Yao *et al*., 2022). Findings from this study were consistent with previous studies on *Tenualosa* species indicated by low values of both haplotype and nucleotide diversity in *T. toli* species at Sarawak rivers, Daro ($h = 0.232$, $\pi = 0.00032$) and Mukah ($h = 0.178$, $\pi = 0.00097$) (Abdul Aziz *et al*., 2015). The other study on *T. ilisha* species from Indian waters also demonstrated relatively low haplotype and nucleotide diversity in Indian rivers, which were Ganga River ($h = 0.064$, $\pi = 0.00020$) and Hooghly River ($h = 0.051$, $\pi = 0.0002$) (Brahmane *et al*., 2013). Low values of haplotype and nucleotide diversity have also been reported in populations of several other different species of fish using the *CytB* gene such as in *Macruronus* species (Olavarría *et al*., 2006), *Macropodus ocellatus* (Zhang *et al*., 2020), and *Konosirus punctatus* (Liu *et al*., 2020). Abdul Aziz *et al*. (2015) also mentioned that the low value of both haplotype and nucleotide diversity might be associated with sharing identical haplotypes (Hap01) among all the samples. As in this study, only 11 haplotypes were detected among 108 individuals with Hap01 being the most common haplotype shared by 98 individuals.

The large number of individuals sharing the same haplotype, Hap01 from the three populations of *CO1* and *CytB* genes can indicate there is gene flow between Daro, Mukah, and Pusa. This condition can lead to population homogenisation, leading to many individuals from all three localities sharing the same haplotype (Zhang *et al*., 2019; Boulanger *et al*., 2021). The presence of gene flow can be explained by the migratory behaviour of the *T. toli* itself, which is an anadromous fish when it migrates to the river during spawning seasons (Blaber *et al*., 1996; Roomiani *et al*., 2014; Puvanasundram *et al*., 2018; Aiman *et al*., 2020). This situation can cause the gene flow from the three localities, causing the sharing of a single haplotype among many individuals (Puvanasundram *et al*., 2018; Baetscher *et al*., 2019).

Both NJ and ML trees inferred from *CytB* suggested that all 11 haplotypes formed a single cluster. The possible reason for the clustering might be that these 11 haplotypes came from a single population despite the fact that the samples collected were sourced from three different locations. The reasons can be supported by the study conducted by Arai *et al*. (2019) on *T. ilisha*, who discovered that all the haplotypes were grouped into a single clade and deduced that the samples originated from a single population. Abdullah *et al*. (2021; 2022) also reported that the same pattern was discovered as all the samples were grouped into a single clade and mentioned that the individuals possibly came from the same population.

The outcome of the phylogenetic tree, low haplotypes, and nucleotide diversity suggested that the individuals of *T. toli* originated from a single population. However, this population has undergone a bottleneck effect recently, which may have been caused by human activity (Zhang *et al*., 2020). Moreover, habitat destruction and overfishing might be the two possible reasons for this finding (Chong *et al*., 2010; Abdul Aziz *et al*., 2015; Mohd Yusoff *et al*., 2021). Chong *et al*. (2010) and Hon *et al*. (2016) also reported that there were some peat swamp areas in Sarawak that have been transformed into oil palm plantations, leading to the possibility of habitat destruction of some local species, including *T. toli*. This was since *Tenualosa* spp. inhabits foreshore areas, estuaries, brackish water, lakes, and freshwater rivers (Roomiani *et al*., 2014; Hon *et al*., 2016). In addition, *T. toli* also exhibits migratory behaviour in that it migrates to fresh water sources for spawning but feeds and grows mainly in the sea (Roomiani *et al*., 2014; Hon *et al*., 2016). In addition, overfishing events can possibly occur during the spawning migration, which can reduce the number of spawners, leading to low genetic diversity (Abdul Aziz *et al*., 2015). The impact can reduce the population size, leading to inbreeding and loss of genetic diversity and at worst can result in the extinction of populations (Lakra *et al*., 2007; Verma *et al*., 2016).

Phylogenetic trees of NJ and ML were constructed to resolve the relationship amongst 108 *CytB* sequences of *Tenualosa* species. All haplotypes were clustered in one clade supported by 100% bootstrap value in both trees (Figure 3). The phylogenetic tree revealed that the haplotypes of *T. toli* formed only a single cluster.

### *Population Structure of T. toli Inferred by mtDNA CytB*

An AMOVA of *T. toli* was almost perfect within the population variations (100.02%) and low variations were detected among the populations (-0.02%). There were no variations among groups due to the lack of populations used (only three). All hierarchical group analyses were insignificant at $p > 0.05$ (Table 5).

Pairwise $\Phi_{ST}$ values between the three populations ranged from -0.00599 between Daro and Mukah to 0.00877 between Pusa and Mukah. According to Chanthran *et al*. (2020), pairwise $\Phi_{ST}$ values are often used to infer gene flow, in which a lower $\Phi_{ST}$ value indicates low genetic divergence and higher gene flow. The highest gene flow was between Mukah and Daro (40.93) and the lowest was between Pusa and Mukah (25.28). The average gene flow for all three locations was 26.47.

IBD, based on the Mantel test, discovered that there was an insignificant correlation between genetic distance (pairwise $\Phi_{ST}$ values) and geographic distance (km) (Figure 4). This indicates that the genetic distance (pairwise $\Phi_{ST}$ values) was not influenced by geographic distance (km). Overall, the findings on genetic structure indicated panmixia with high gene flow among all three populations of *T. toli* in Sarawak Rivers (Daro, Mukah, and Paso).

The genetic structure of all three populations (Daro, Mukah, and Pusa) was examined by several methods, which were AMOVA F-statistics ($F_{ST}$), Pairwise $\Phi_{ST}$ values, and Gene Flow (Nm), IBD, and MSN. The amount and distribution of genetic variation within and between populations is defined as genetic structure (Pastorino & Marchelli, 2022). Efficiently identifying the genetic structure and related factors of a species can lead to an understanding of the evolutionary history of the species (Ju *et al*., 2019).

The AMOVA $F_{ST}$ result suggested no significant genetic differentiation ($F_{ST} = 0.42620$, $p > 0.05$) between all three populations. Pairwise $\Phi_{ST}$ values further confirmed no significant genetic differentiation for each population. This condition was suspected due to the presence of gene flow (Yin *et al*., 2013; Parmaksidz & Ekma, 2017; Sarker *et al*., 2021). Gene flow analysis proved that there was high gene flow between the three locations. Cheng *et al*. (2020) mentioned that the values of gene flow ($Nm < 1$)

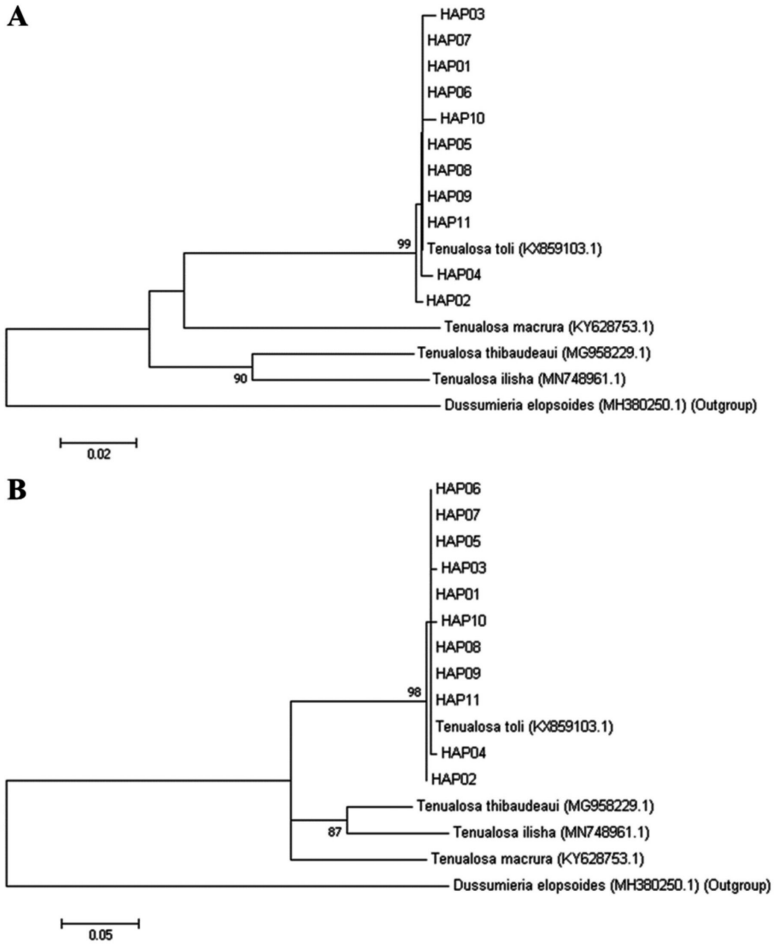


Figure 3: Phylogenetic analysis of CytB haplotypes with T, toli, T. thibaudeaui, T. macrura, and T. ilisha as sister group and Dussumieria elopsoides as outgroup (A) Neighbour-Joining (NJ) (B) Maximum Likelihood (ML)

Table 5: Pairwise ΦST (below diagonal) and gene flow (Nm) (upper diagonal) between populations of *T. toli* and AMOVA and F- Statistics

| **Pairwise ΦST and Gene Flow** | | | | **AMOVA and F-Statistics** | | | |
|---|---|---|---|---|---|---|---|
| **Populations** | **Mukah** | **Daro** | **Pusa** | **Variance components** | **% Variations** | ***F*-statistics** | ***P*-value** |
| **Mukah** | - | 40.93 | 25.28 | Among populations | -0.02 | -0.00023 | 0.42620 |
| **Daro** | -0.00599 | - | 34.94 | Within populations | 100.02 | | |
| **Pusa** | 0.00877 | 0.00321 | - | | | | |

Note: All pairwise comparisons are not significant at P > 0.05.
Note: All P-values are not significant at P > 0.05.

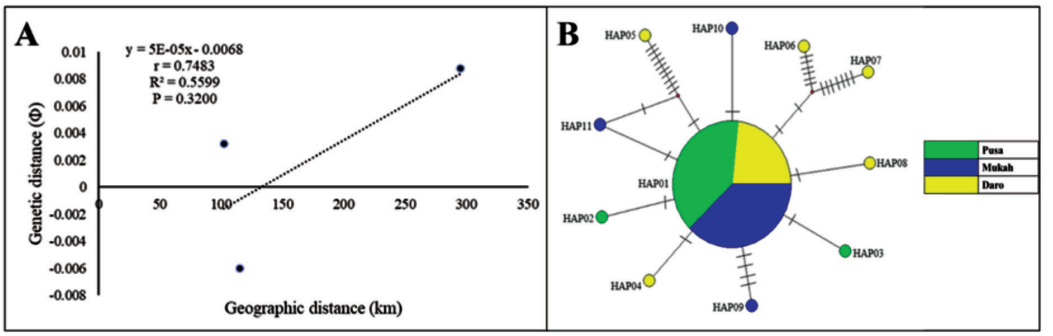


Figure 4: *T. toli* population structure analysis (A) Isolation By Distance (IBD) correlation between the genetic and geographic distance of *T. toli*. Note: All pairwise comparisons are not significant at P < 0.05. (B) Minimum Spanning Network (MSN) of *T. toli* across Daro, Mukah, and Pusa. The size of each circle indicates the frequency of the corresponding haplotype in the whole dataset

is interpreted as low to no Nm. The high Nm from this study was consistent with a previous study that indicated high to infinity Nm on *T. ilisha* between the Ganga and Hooghly rivers in India and in populations of *Rhabdosargus sarba* from Taiwan. This suggests that the free flow of the gene pool and the populations should be considered as one homogenous population (Brahmane *et al*., 2013; Hsu *et al*., 2020).

Another study by Cheng *et al*. (2020) revealed similar results as the Nm, valued between 9.00 and 24.00, indicating a high Nm rate. Previous research highlighted that the reason that led to the absence of genetic structure and population differentiation in the *T. toli* population was the migration behaviour of the *Tenualosa* species, increasing the chance for *T. toli* to migrate and spawn at other Sarawak rivers (Blaber *et al*., 1996; Roomiani *et al*., 2014; Abdul Aziz *et al*., 2015; Aiman *et al*., 2020). This is attributable to the fish's natural behaviour, leading to local population clusters or a mixture with other populations (Alvarado *et al*., 2005; Sarker *et al*., 2021).

In addition, the presence of high Nm between the three locations supported the findings of sharing the single haplotype by many individuals between the three populations above. IBD results from this study were consistent with the above findings, as there was no significant correlation between the genetic distance (pairwise $\Phi_{ST}$) and the geographic distance (km). The clustering of the MSN also indicates that the juvenile *T. toli* originated from similar origins and shared close relationships among the individuals, explaining the non-significant pairwise value $\Phi_{ST}$ and high gene flow rate. Similar results were obtained by Yu *et al*. (2016) and Nazia *et al*. (2010), where the Hyporhamphus sajori and Clarias macrocephalus, respectively, came from a single population due to the absence of obvious philographic structure of the MSN.

### *Demographic History of T. toli*

Two neutrality tests, Tajima's D and Fu's test revealed that each population achieved the following values: Daro (-2.62384; -0.03533), Mukah (-2.06816; -1.56486), and Pusa (-1.48662; -2.72950), respectively. Tajima's D values demonstrated a significant negative for all populations at $p < 0.05$. Meanwhile, in Fu's test, only Daro was insignificant at $p < 0.05$ (Table 6). Significant negative values of Tajima D and Fu's Fs would indicate a significant population expansion (Tajima, 1989; Fu, 1997).

Population mismatch distribution estimation of pairwise differences revealed that the population has a multimodal distribution pattern, as in Figure 5. A unimodal distribution pattern indicates that the population has undergone recent expansion, while a bimodal and multimodal distribution pattern indicates

Table 6: Neutrality tests for *T. toli* populations from Daro, Mukah, and Pusa

| Population | N | H | Neutrality's Test | |
|---|---|---|---|---|
| | | | Tajima's D | Fu's Fs |
| Daro | 41 | 6 | **-2.62384** | -0.03533 |
| Mukah | 43 | 4 | **-2.06816** | **-1.56486** |
| Pusa | 35 | 3 | **-1.48662** | **-2.72950** |
| Total | 119 | 13 | - | - |

Note: Bold indicated significant at $P < 0.05$.

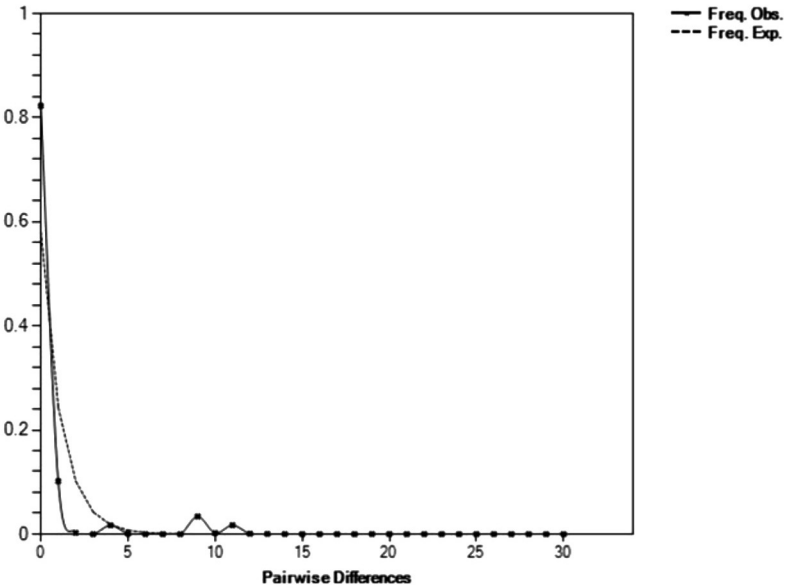


Figure 5: Multimodal mismatch distributions (pairwise differences) of *T. toli* showing the expected and observed pairwise differences between sequences with the respective frequencies

that a population is in an equilibrium state (Rogers & Harpending, 1992; Jenkins *et al.*, 2018).

In addition, the MSN had a star-like shape. The finding further supports the occurrence of a recent demographic expansion in the population.

Tajima's negative D values suggest a balanced selection and the recent directional selection of the population has increased with an excess of uncommon alleles (Tajima, 1989). Based on Ewens' sampling distribution, Fu's $F_{ST}$ is a sophisticated test that leverages information on haplotype distribution to detect previous population size fluctuations (Ewens, 1972; Ramos-Onsins & Rozas, 2002). Under an excess of mutations, a significant negative value of Fu's Fs statistics is considered proof of population expansion.

Consistent with haplotype and nucleotide diversity, Tajima's D values were significantly negative for all populations at $p < 0.05$. Meanwhile, in Fu's test, only Daro was insignificant at $p < 0.05$. Significant negative values of Tajima D and Fu's Fs would indicate a rapid, significant population expansion in the past (Tajima, 1989; Fu, 1997; Gui *et al.*, 2021). However, for Daro, since this site has a significant negative value for Tajima's D but an insignificant negative for Fu's Fs value, the

results reveal that the population of *T. toli* in this area has not expanded recently. However, the expansion that was experienced before was probably caused by the bottleneck effect that leads to a small effective early population size (Kashani *et al.*, 2021). Furthermore, the results were supported by population demographic mismatch distribution analysis of *T. toli* as it matched the multimodal distribution.

Kai *et al.* (2014) and Shi *et al.* (2014) stated that a population with multimodal distribution indicates that the population is demographically equilibrium. In addition, the result from the MSN demonstrates the presence of a star-like shape structure. The star-like shape in the MSN suggests that there is an occurrence of significant population expansion due to the bottleneck effect (Yu *et al.*, 2016; Landvik *et al.*, 2017; Supmee *et al.*, 2020; Zhou *et al.,* 2023). This bottleneck effect might be caused by habitat loss due to the changing sea temperature, current, and coastline morphology during the late Pleistocene (the past 2.5 million years ago) (Tschá *et al.*, 2016; Yu *et al.*, 2016; Divya *et al.*, 2017).

## Conclusions

In conclusion, this study revealed that all the samples taken from the three localities were confirmed as *T. toli* using *CO1* and *CytB* genes. The genetic diversity of *T. toli* in Sarawak is relatively low and consists of one homogeneous population from three different population. This suggested the species could probably have originated from a common origin and shared a close relationship. Genetic structure analysis indicates non-significant genetic differentiation and pairwise $\Phi_{ST}$ with high gene flow. IBD also exhibits a non-significant correlation between the pairwise $\Phi_{ST}$ and the geographical distances (km). MSN had similar results as ML and NJ phylogenetic trees, which is the *T. toli* came from a similar origin. The neutrality test confirmed that all the samples have experienced population expansion and the population is currently in an equilibrium state, probably due to bottleneck as a result of habitat loss during the late Pleistocene period.

It is suggested that collecting research data on genetic diversity and population dynamics is crucial and could serve as a monitoring tool for the sustainable management of resources. The genetic pools for a sustainable harvest must be preserved and replenished, which requires intensive management of fishery resources. Collecting more samples from different regions would make it possible to study the population genetics of a migratory species dispersed over regional boundaries such as the *T. toli*, if we can start working together, primarily with the related agencies like the DOF and Department of Agriculture Sarawak (DOA). A comprehensive strategy uses a wide range of complementary methods and more sensitive and adaptable markers like microsatellites for more conclusive results. Various programmes can be implemented such as a comprehensive conservation plan, fisheries management programmes, and foundation for sustainable fishery activities, together with other related information.

## Acknowledgements

The author would like to thank the Faculty of Fisheries and Food Science and Universiti Malaysia Terengganu for providing the research facilities. The authors would also like to express their gratitude to the Department of Agriculture, Sarawak for assisting in the collection of samples. Thank you also to Dr. Shahreza Md. Sheriff, Project leader for Fundamental Research Grant Scheme (FRGS) Vot no. 59245 and the Ministry of Higher Education Malaysia for funding the research. The authors would like to thank Prof. Dr. Muhammad Ikhwanuddin Abdullah@ Polity Ak Kria for his valuable comments on the manuscript. Last but not least we are deeply grateful and would like to appreciate the late Mr. Awang Alim Awang Kassim for his valuable contributions to the success of his project.

## Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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