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## Mitochondrial DNA Diversity of Terubok (*Tenualosa toli*) from Daro and Mukah, Sarawak Inferred by Partial Cytochrome b (Cyt-B)

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### ABSTRACT

Terubok (*Tenualosa toli*) is one of Malaysia commercially important fish which is found mainly in Sarawak. Their numbers of catch have been declining in the past 15 years due to heavy exploitation. Hence, a study was done to determine the mitochondrial genetic diversity of *T. toli* from Daro and Mukah, Sarawak inferred by partial Cytochrome b gene. DNA extraction was done on 84 *T. toli* samples. PCR amplification using Cyt-b primers had been carried out and sequence of 469 bp length was obtained from each sample. Next, phylogenetic analysis was performed to study the relationship among the individuals. The genetic diversity of the population was determined through the haplotype and nucleotide diversity. Result of the study showed that the Haplotype Diversity (HD) of *T. toli* was relatively low for Daro (Hd = 0.232) and Mukah populations (Hd = 0.178). The nucleotide diversity was also low for both populations. Moreover, only nine haplotypes were identified from the 84 individuals. A single haplotype was shared amongst 76 individuals. These findings correlate with previous study that showed the number of the fish caught had declined drastically and might cause a genetic deprivation towards its population. Comparably, maximum likelihood analysis revealed that two *T. toli* individuals were separated from the main clade, suggesting that these two individuals might come from another Terubok population. Genetic diversity of the mitochondrial DNA Cytochrome b of the *T. toli* obtained in this study would be useful in the implementation of conservation and fisheries management of this species in Sarawak.

**Key words:** *Tenualosa toli*, terubok, mitochondrial DNA, Cytochrome-b

### INTRODUCTION

*Tenualosa* species or locally known as Terubok is one of the important commercially Clupeoid fish in Malaysia. *Tenualosa toli* is a protandrous sequential hermaphrodite that changes their sex from male to female and normally live for between two or three years (Anonymous, 1999; Blaber, 1997; Blaber *et al.*, 1996). They are migratory fish. Anonymous (1999) stated that adult

will spend most of their life in the estuary near to the sea. During spawning season, they will move to the lower reaches of the estuary which is low in salinity to spawn. After spawning, larvae and fry will move to the upper reaches of the estuary which is high in salinity.

*Tenualosa toli* is one of the species that can be found in Sarawak. It is in high demand in the local market and sometimes exported due to its meat and roe. It has been reported that the population of terubok in Sarawak is mainly found in the area of Daro and Mukah region. The studies of the current status had indicated that the Terubok population had been heavily exploited and due to this reason, the numbers had been declining drastically (Mohd-Shamsudin *et al.*, 2011). Such event had rendered this species to the declination in its number (Mohd-Shamsudin *et al.*, 2011). This may periodically affect its genetic diversity. However, the genetic data that indicates the variability of the genetics in a population in consistent with its current status is not well documented. This raises questions regarding the status of the *T. toli* population in Daro and Mukah region on its genetic diversity, whether it is highly diverse or less diverse.

In this study, it is hypothesized that the genetic diversity of the *T. toli* population in Daro and Mukah, Sarawak is less diverse because of the overfishing events that has render its number to be declining. Clearly, in a long run, a population structure that has a small genetic diversity within its members will be hard to manage. This is because as the heterozygosity decreases and together with the effects such as low fecundity, the population will not be able to produce enough stocks for the future population. This may render it to be extinct as it is concomitants to the severe effects of inbreeding depression. Thus, this will cause the lost of one of the most important fisheries resources within the Borneo's regions, severing the socio-economics of the local citizens. Such occurrence had been reported in Dusky Grouper (*Epinephelus marginatus*) in the Mediterranean Coast (Maggio *et al.*, 2006).

The use of molecular marker had been successful applied in fisheries sector to identify closely related fish species, determination of genetic diversity of fish population and species identification (Ponzoni *et al.*, 2010; Hurst *et al.*, 1999; Okumus and Ciftci, 2003). Among the most commonly marker used is mitochondrial DNA. The used of mitochondrial DNA markers offer several advantages such as maternal mode of inheritance and relatively limited recombination that make it effective in conducting studies regarding phylogenetics analysis (Hurst *et al.*, 1999). Moreover, the mitochondrial DNA markers are highly conserved with definitive functions, which is an excellent choice in conducting genetic population and variation studies (Howell and Gilbert, 1988; Lakra *et al.*, 2009). It is also known to have several advantages compared to nuclear DNA in the study of sequence divergence due to its rapid evolution, limited exposure to recombination, lack of introns and high copy number (Brown, 1985; Luo *et al.*, 2011). The study of genetic diversity had also been done to local Malaysian fishes such as the Peninsular Malaysian Pangasiid species (Tumiran *et al.*, 2011) and two indigenous Sarawak's Mahseers (Nguyen *et al.*, 2006) as well as in Asian Seabass, *Lates calcarifer* (Norfatimah *et al.*, 2009) and in Broadhead Catfish, *Clarias macrocephalus* (Nazia *et al.*, 2010). The use of molecular markers in these studies had successfully determined the genetic diversity within the fish species population. Currently, genetic study on *T. toli* in Sarawak has not been well documented and their genetic diversity is still unknown. Our preliminary study on *T. toli* population in Mukah region Sarawak showed that the population has low genetic diversity (Abdul-Hadi *et al.*, 2013). Therefore, further sampling was done on Mukah (with increasing number of samples) and another main landing site in Sarawak (Daro) to compare the genetic diversity between populations in Sarawak. Hence this study was conducted to determine the genetic diversity of the *T. toli* located at two different localities that is Daro and Mukah region in Sarawak.

Study on genetic diversity of *T. toli* in Sarawak will assist in the understanding of its population structure in Daro and Mukah region. The result from the data will contribute to help in the management and restoration of the fisheries resources. The information obtained from this study can be used to develop the genetic database of *T. toli* in Sarawak.

## **MATERIALS AND METHODS**

Samples of terubok, *T. toli*, were collected from the Daro and Mukah region of Sarawak with the assistance of the Department of Agriculture, Sarawak. A total of 84 samples (41 samples from Daro and 43 samples from Mukah) were collected. All samples were identified based on morphological feature using key identification of clupeoid species by Whitehead (1985). For DNA sample, the white muscle and fin clips were preserved in 95% ethanol and stored at -20°C. Genomic DNA was extracted using a commercial DNA extraction kit, the Cell/Tissue DNA Extraction Kit (Spin Column) (BioTeke Corporation, China).

Amplification of partial cytochrome b gene was done using universal primer based on Faria *et al.* (2006) which were the Cytb2 f (5'-CCTTCTAACATTTTCAGTCTGATG-3') and Cytb2 r (5'-AGGATTGTGGCCCTGCAATTAC-3'). A 25 µL reaction was prepared according to Abdul-Hadi *et al.* (2013) consisting of 2.5 µL of 10×PCR buffer, 1.25 µL of 25 mM MgCl<sub>2</sub>, 0.6 µL of 100 pmol of both primers, 0.2 µL of 5U *Taq* DNA polymerase (Vivantis, Malaysia), 0.5 of 10,000 uM dNTP and 2.0 µL of 100 ng DNA sample was prepared in a 25 µL reaction. The amplification was done in a Thermal Cycler (Eppendorf AG, Germany) with a cycling profile beginning with the initial step 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 40 sec, annealing at 54°C for 45 sec and extension at 72°C for 60 sec and finally subjected to the final extension at 72°C for 7 min. The amplified product (4 µL) was visualized in 1.5% agarose gel. The PCR products were purified using a DNA purification kit (Vivantis, Malaysia) according to the manufacturer protocol and then sequenced bi-directionally by First Base Laboratories Sdn. Bhd. using similar primers used for PCR amplification.

All sequences obtained were edited to remove any unwanted sequence using Chromas (Technelysium Pte Ltd.) Identification of the sequences were done using the Nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) at the National Center of Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>). The edited sequences were aligned together with other *Tenualosa* sp. sequence (*T. toli*, Accession number: KC405574.1 and *T. ilisha*, Accession number: EU552622.1) available in the Genbank database using CLUSTAL W. *Tetrapturus pfluegeri* (Accession number: DQ198010.1) and *Coris julis* (Accession number: HM049942.1) were used as outgroup. Phylogenetic trees of the haplotypes were constructed using Neighbour-Joining, Maximum-Parsimony and Maximum-Likelihood algorithms. Kimura-2-parameter was used as the best-fit DNA substitution model in NJ and ML analyses. Confidence level of the trees was tested with 1000 resamplings (Tamura *et al.*, 2011). DnaSP (Version 5.1, Universitat de Barcelona) was used to estimate the mitochondrial genetic variation including the haplotype and nucleotide variation between the samples and to determine the level of genetic variation within phylogenetic clades through the calculation of Tajima's test of neutrality.

**Statistical analysis:** Arlequin (version 3.5.1.2) (Excoffier and Lischer, 2010) was used to perform an analysis of molecular variance (AMOVA) to examine population structure of *T. toli* from Daro and Mukah. The significance of the fixation index was tested by 1000 permutations of the data set.

**RESULTS**

The amplification of partial Cytochrome b (Cyt-b) was performed on all *T. toli* samples collected from the Daro and Mukah region. These are the two main landing site for terubok. The amplification obtained a fragment with an approximate size of 500 bp for all the samples. Sequence analysis of the amplified fragment showed a total length of 469 nucleotides with all the 84 sequences having 100% similarity with the *T. toli* mtDNA cyt-b sequence in the Genbank Database (*T. toli* full mitochondrial genome, Accession Number: AP011600.1 and *T. toli* partial Cyt b, Accession Number: KC405574.1). The result confirmed that all samples collected were *T. toli*.

The study had identified nine haplotypes from the total 84 individuals (Table 1). Seventy-six individuals shared a single haplotype (Hap01) with the highest relative frequency of 0.9048. The other eight haplotypes (Hap02 to Hap09) showed distinctive unique composite nucleotides and only represented to a single individual with relatively low frequency of 0.0119, respectively. Five haplotypes (Hap02, Hap03, Hap04, Hap05 and Hap06) were found within the Daro locality and another three haplotypes (Hap07, Hap08 and Hap09) were detected within the Mukah locality. Composite sequence for the haplotypes discovered in Daro and Mukah population were shown in Table 2. Phylogenetic analysis showed all the 84 individuals (41 individuals from Daro locality and 43 individuals from Mukah locality) are clustered and mixed together within the same clade. NJ tree on haplotype showed evidence of multiple shallow haplotype divergent within the clade suggesting the isolation of some natural populations in the past (Fig. 1).

Table 1: Numbers of haplotype and relative frequency

Daro	Mukah	Haplotype	Frequency
<b>Samples</b>			
D1, D2, D3, D4, D5, D6, D7, D8	M2, M3, M4, M5, M6, M7, M8, M9	Hap01	0.9048
D9, D11, D12, D13, D14, D15	M10, M11, M12, M13, M14, M15, M16		
D16, D17, D18, D19, D22, D23	M17, M18, M20, M21, M22, M23, M24		
D24, D25, D26, D29, D30, D31	M25, M26, M27, M28, M30, M31, M32		
D32, D33, D34, D35, D36, D37	M34, M37, M38, M39, M40, M41, M43		
D38, D39, D40, D41	M44, M50, M59, M60		
D10		Hap02	0.0119
D20		Hap03	0.0119
D21		Hap04	0.0119
D27		Hap05	0.0119
D28		Hap06	0.0119
	M1	Hap07	0.0119
	M19	Hap08	0.0119
	M26	Hap09	0.0119

Represented haplotypes of partial Cytochrome b towards the individuals of *T. toli*. The Hap01 is represented to 76 samples with the highest frequency, whereas the other eight haplotypes represent to every single different individuals and low frequency, No shared unique haplotypes between the Hap02-Hap09 amongst the other individuals. The abbreviations D indicates that the samples were collected from Daro whereas M from Mukah

Table 2: Genetic diversity of 84 individuals of *T. toli*

Locality	Haplotype diversity (Hd)	Nucleotide diversity (B)	Tajima's D
Daro	0.232	0.00321	-2.65901 (p>0.05)
Mukah	0.178	0.00097	-1.76265 (P>0.05)

Haplotype diversity, nucleotide diversity and Tajima's D test for neutrality for the population of *T. toli* in both Daro and Mukah population. The haplotype diversity shown is the mean of genetic diversity

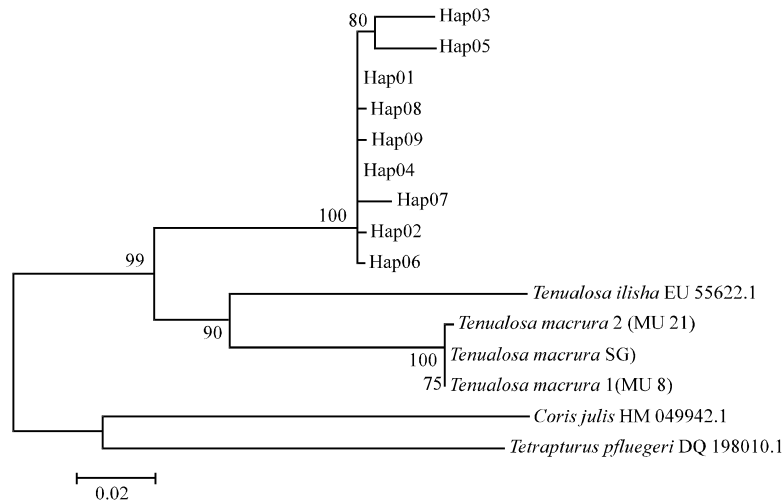


Fig. 1: Neighbour-Joining (NJ) phylogenetic tree of *T. toli* Cyt-b haplotypes and *C. julis* and *T. pfluegeri* as outgroup. Hap01 are shared among 76 individuals in both Daro and Mukah. Hap02, 03, 04, 05 and 06 are singleton haplotypes in Daro specimens. Hap07, 08 and 09 are singleton haplotypes in Mukah specimens

All 41 individuals of *T. toli* from Daro population had a relatively low haplotype ( $H_d = 0.232$ ) and nucleotide diversity ( $B = 0.00321$ ) (Table 3). The neutrality test using Tajima's D also showed a low value of  $-2.65901$  ( $p > 0.05$ ). Accordingly, all 43 individuals of *T. toli* from Mukah population also showed low haplotype ( $H_d = 0.178$ ) and extremely low nucleotide diversity ( $B = 0.00097$ ) (Table 3). Tajima's D also showed a low value of  $-1.76265$  ( $p > 0.05$ ). The insignificant result of Tajima's test in both localities indicates a population size expansion. The average  $F_{ST}$  value was  $-0.00381$  ( $p > 0.05$ ), suggesting non-significant genetic variation amongst the two localities. The pairwise  $F_{ST}$  data primarily demonstrates non-significance differentiation was detected between Daro and Mukah specimens.

## DISCUSSION

The use of mitochondrial Cyt-b gene to perform the analysis of genetic diversity and population genetic structure studies had been done in many closely related fishes (Norfatimah *et al.*, 2009; Pereyra *et al.*, 2010; Tseng *et al.*, 2011). The gene also holds viable information regarding the phylogenetic relationships within and between the individuals of the same population. Due to its molecular characteristics such as the genetically conserved regions within the genome and high evolutionary rate, the gene is suitable and a powerful molecular tool to observe the occurrence of genetic differentiation due to any events that might influence it such as founder effects or genetic bottlenecks reoccurrence. Present study conducted was the very first study regarding the genetic population of commercial important Clupeoid, *T. toli* from Sarawak, Malaysia. This species has high economic value but have been threatened towards extinction due to events of overfishing. Therefore, this study was done to gain some insight regarding the population structure of the *T. toli*.

Phylogenetic analysis was conducted to investigate the evolutionary relationship based on the haplotypes present amongst the 84 individuals of the *T. toli* from two different localities in

Table 3: Composite sequence for the haplotypes discovered in Daro and Mukah population

Haplotype	Composite sequence
Hap01	5'-AAACTTCGGGTCCTCCTGGGACTCTGCTTGGCATCACAAATCTTAACAGGACTATTCCTGGC CATGCACTACACCTCTGATATCGCAACCGCCTTTTCATCGGTTACACACATCTGCCGCGACGTCA ATTACGGATGATTAATTCGAAATGTGCACGCAAATGGTGCATCCTTTTTCTTCATGTGTATCTAT GCCCATATTGGCCGAGGACTCTACTACGGCTCTTATCTGTACAAAGAAACCTGAAACATTGGGG TTGTCCTACTGTTGCTGGTCATGATAACAGCCTTCGTGCGCTACGTCCTCCCTGAGGACAAATA TCCTTCTGGGGGGCCACAGTCATTACAAACCTACTGTCTGTGTGCTTACGTAGGAAACGAGC TCGTCCAATGAATTTGAGGAGGCTTCTCCGTTGATAATGCCACCCTTACCCGATTCTTCGCCTTC CACTTCCTATTCCCATTCC
Hap02	5'-G-3' 313
Hap03	5'-A A A A A G A A T A-3' 4 17 18 20 21 23 28 35 36 38 458
Hap04	5'-T T C T C A A T T -3' 434 436 438 439 440 442 445 446 447
Hap05	5'-T C T T A T C C A -3' 435 438 446 452 456 458 459 460 463
Hap06	5'-T-3' 409
Hap07	5'-G A A T -3' 7 8 23 25
Hap08	5'-T-3' 223
Hap09	5'-G-3' 17

Composite sequences for each of the haplotypes. The numbers indicate the position of the composite nucleotide from the 5' end of the sequence

Sarawak. The analysis also determined the possibilities in the existence of several *T. toli* sub-populations. The mixture of both the haplotypes present from Daro and Mukah might also suggest that these samples might have been assimilated together and there is little genetic differentiation between the two groups. A total of nine haplotype (one shared haplotype and eight unique haplotype) were found in the study indicating low genetic diversity. The number of haplotype was much lower compared to the study on Japanese flounder (*Paralichthys olivaceus*) wild population which recorded 65 haplotypes from 69 samples. Furthermore, Phylogenetic analysis amongst the haplotypes showed that there were no differences between the two localities. The tree topologies also indicated that all the samples from both localities formed a monophyletic group.

Neighbour Joining also showed that only two haplotypes (Hap03 and Hap05) are separated from the main clade and distinguished the two haplotypes as distinctive individuals. This divergence might indicate that these two individuals may have come from other *T. toli* population due to its migrating behavior (Blaber *et al.*, 1996). A study done in Asian arowana, *Scleropages formosus*, (Haslawati *et al.*, 2011) notably implied that low pairwise FST was not significant to differentiate the shallow divergence between the specimens of the same species. Therefore, samples with these haplotypes (Hap03 and Hap05) can be categorized within the same population but might have evolved to have little genetic differentiation, an onset of the occurrence of population expansion as stated by Grant and Bowen (1998).

Genetic diversity of the *T. toli* population was also determined through the haplotype and nucleotide diversity. The study showed that the haplotype diversity determined is low in both Daro

( $Hd = 0.232$ ) and also Mukah ( $Hd = 0.178$ ). This can be related with the shared identical haplotypes Hap01, amongst all the 36 Daro samples and 40 Mukah samples and only eight unique haplotypes were present. Both populations also exhibited low nucleotide diversity. Low diversity of haplotypes and nucleotides might be due to the heavy exploitation activities towards this species, which eventually cause the genetic bottleneck effects that may have deleterious effects upon the variability of the nucleotide sequence of the *T. toli* Cyt-b gene. Study on *Clarias macrocephalus* had shown that shared haplotypes of more than 50% frequency might suggest that the species might probably have a common origin or a result of a continuous gene flow (Nazia *et al.*, 2010). Grant and Bowen (1998) suggested that low haplotype and nucleotide diversity were the results of recent population bottleneck or the occurrence of founder event through mtDNA lineage, which was consistent with the present findings. They also implied that such damage on the genetic diversity was invoked through all the populations within the regions.

*Tenualosa toli* exhibits migratory behaviour. Adult *T. toli* can be found mostly in the estuary near to the sea and will migrate to the lower reaches of the estuary which is low in salinity during spawning season (Anonymous, 1999). Larvae and fry produced from the spawning will move to the upper reaches of estuary near to the sea. Heavy exploitation limits the gene flow into the population thus as numbers of individuals decreasing within the natural stock, the genetic diversity also decrease (Lacy, 1997; Gajardo *et al.*, 2002; Okumus and Ciftci, 2003). Such events might occur in *T. toli* population during their spawning migration which can reduce the number of spawners leading to low genetic diversity.

High genetic diversity signifies that the population has a large effective population size, which could sustain the events of exploitation as well as could be used to produce superior broodstock for the means of aquaculture. Low genetic diversity means that the population is an unstable population that cannot sustain any further events of exploitation, which might has the possibility of the occurrence of inbreeding depression. This raises concern about the natural population of Terubok, *T. toli* in both regions. Future uncontrollable exploitations may render this population's genetic diversity to be receded.

The presence of the singleton haplotypes indicates that there are genetic differentiations of the individuals amongst the population. In consistent to the low haplotype and nucleotide diversity, the Tajima's D showed a non-significant negative value for both Daro ( $D = -2.65091$ ,  $p > 0.05$ ) and Mukah ( $D = -1.76265$ ,  $p > 0.05$ ) localities, suggesting the occurrence of population size expansion, after the events of genetic bottleneck that has the probability to cause an excess of low frequency of polymorphism (Tajima, 1989). This expansion phenomenon happened with the possibility that natural selection occurred to purify negative selection and replenish the genetic diversity condition. This finding is concurrent to a study that had been done on Dusky grouper, *Epinephelus marginatus* (Maggio *et al.*, 2006). The study showed that the population structure of Dusky grouper had low haplotype and nucleotide diversity, followed by a negative Tajima's D. The findings further explained that the occurrence of population expansion within the population took place after the events of genetic bottleneck. Maggio *et al.* (2006) also stated that the factors that may result in low genetic diversity were overexploitation, habitat deprivation and genetic bottleneck or founder events.

The events of population expansion of *T. toli* might also suggest two possible theories. Firstly is that the unique and private haplotypes variability arise may also originated from the same-shared haplotypes, which in this case, it implies that haplotype Hap02, Hap03, Hap04,



Hap05, Hap06, Hap07, Hap08 and Hap09 might originate from Hap01, as seen in study done by Rahim *et al.* (2012) and might undergo the occurrence of evolution through natural mutation. Secondly, all the haplotypes that represent different individuals might also come from another population. Through natural selection of the events of fish migration, these distinctive individuals naturally entered both the Daro and Mukah populations to introduce new gene indicating gene flow into the gene pool. Further study should be done to determine the validity of population size expansion occurred for the current population. Such study involves the determination of genetic diversity of the population within time elapse of one to two years. Thus, the expansion of the population structure could be precisely determined.

In general, as the haplotype and nucleotide diversity of the *T. toli* in Daro and Mukah localities in Sarawak is low, it dictates that the mtDNA diversity of the population is diminishing. The results obtained here also indicated no significant heterogeneity in Cyt-b across Daro and Mukah specimens. Overall, low  $F_{ST}$  value was detected indicating a homogenous population of *T. toli* from both Daro and Mukah region. Panmixia of the species in these two localities is likely because of its migrating behavior (Blaber *et al.*, 1996) resulting in no or very weak population structuring. For a more comprehensive analysis of genetic variation in *T. toli*, the sampling design of future surveys should address a much broader geographic scale and utilize more rapidly evolving nuclear markers such as microsatellites or SNPs.

This information could be used in the implementation of conservation plan and efficient fisheries management for sustainable exploitation. The information presented also showed that there is still hope to save this commercially important species. Steps such as closed season for fishing should be implemented towards the migratory pathway of the *T. toli*. This is because of their migratory behaviour. Other Terubok from other populations might have the potential of introducing new gene variation that could replenish the genetic diversity of the *T. toli* populations in Sarawak. Thus, by doing so, in several years, the Terubok of Sarawak could be saved from severe genetic defects.

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