


Genetic Diagnosis and Reproductive Biology of.....in The Toba Like, North Sumatra (Kaban, S. & A. Wibowo)



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GENETIC DIAGNOSIS AND REPRODUCTIVE BIOLOGY OF INTRODUCED *MYSTACOLEUCUS MARGINATUS* IN THE TOBA LAKE, NORTH SUMATRA

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ABSTRACT

After *M. marginatus* has been introduced to Toba Lake, North Sumatra, the status of *M. marginatus* stocks and their biological properties is unknown. In this study, we examine the genetic patterns of the *M. marginatus* in Toba Lake, North Sumatra Province. Employing the cytochrome *c* oxidase subunit I (COI) gene of the mtDNA, we profile the genetic variation within *M. marginatus* in Toba Lake. We also analyzed reproductive characteristic and commercial catches of *M. marginatus*. The result shown *M. marginatus* is a synonym for *M. marginatus*, in total, 1 polymorphic sites (represent singleton dimensions) was identified and phylogenetic reconstruction reveal low levels of genetic diversity with no clear pattern of haplotype-partitioning. Nucleotide diversity analysis infer the present of two lineages. The N_e value of *M. marginatus* (1,936 to 3,878), the population has not experienced population growth/expansion as expected and the total production of *M. marginatus* in Toba Lake of the year for 2013 has ranged between 3,347.05 – 6,694.1 ton. *M. marginatus* in the Toba Lake share similar life history traits include maximum body size, longevity, age at maturity, and fecundity (the number of eggs produced). *M. marginatus* is categorized as the opportunistic strategy consisted of fishes with short generation time, low batch fecundity, and low investment per offspring. This life history traits approach of *M. marginatus* are important for guide fisheries management for its sustainability. More over the information can be use as pilot data and apply it to data-poor species.

Keywords: *M. marginatus*; Reproductive; DNA; Toba Lake

INTRODUCTION

Mystacoleucus is a distinctive genus in the Cyprinid subfamily Barbinae, characterized by 6–10 branched rays in the anal fin, which is quite different from the other genus in this subfamily, whose anal fins normally have 5 branched rays (Wu *et al.*, 1977). *Mystacoleucus*, has a wide distribution in Java, Borneo, Sumatra, Malaya, and Thailand (Smith, 1945) and has seven species (Wu *et al.*, 1999). Roesma (2011) proposed that *M. marginatus*, is a new synonym for *M. marginatus* under the Indonesian common name wader.

M. marginatus is a source of protein and of relatively high commercial value important for many local communities in the West Sumatra (Singkarak Lake). Reported price of *M. marginatus* has increased from IDR. 59.042/kg (2012) to over IDR. 80.000/kg in 2014 (DG Fisheries, 2012). *M. marginatus* has been introduced to Toba Lake, North Sumatra intentionally to maximize their potential in 2003 (Kartamihardja & Sarnita, 2008).

There is no doubt that *M. marginatus* population in Toba Lake have experienced a very drastic decline since our last survey in 2013. However, limited information had been provided to understand the species ability to recover after declining to make informed conservation decisions as there is growing interest in this species in some small industry markets. Fishing is an important source of income and food for many peoples in Toba Lake. Good resource management is vital if continued economic progress and poverty alleviation is to be achieved. In many parts of Indonesia inland fisheries resources have become depleted due to inadequate management but, in the case of *M. marginatus*, there exists a current opportunity to ensure sustainable use.

It is worth to know the characteristics of *M. marginatus* stock, as the basic information to predict the possibility of fish population bounces back in a few years after major population declines in recent year. Other locality groups could have been chosen-Danau Singkarak, for example but comparison with the Singkarak is not possible due to non availability

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samples. The use of the mitochondrial DNA (mtDNA) is a powerful tool in rapid genetic assessments to differentiate population stocks (Mudumala *et al.*, 2011). The cytochrome *c* oxidase subunit I (COI) gene is the most well-known molecular marker for the analysis of intraspecific and interspecific relationships in many fish and shellfish (Craig *et al.*, 2001). In this study, we examine the genetic patterns of the *M. marginatus* in Toba Lake, North Sumatra Province. Employing the cytochrome *c* oxidase subunit I (COI) gene of the mtDNA, we profile the genetic variation within *M. marginatus* in Toba Lake. We also analyzed reproductive characteristic and commercial catches of *M. marginatus*.

MATERIAL AND METHODS

Area Study and Sample Collection

Sampling campaigns were conducted at six sites, Figure 1. and three times between April 2013 and November 2013 around Toba Lake, North Sumatra Province. Sampling and collecting representative samples of the adult fish community and fish juvenile (total sample size of 1078 individuals) were carried out using various types of fishing gear, Sulangat (a complex local fishing gear equipped with lamps), scoope net and gill nets.

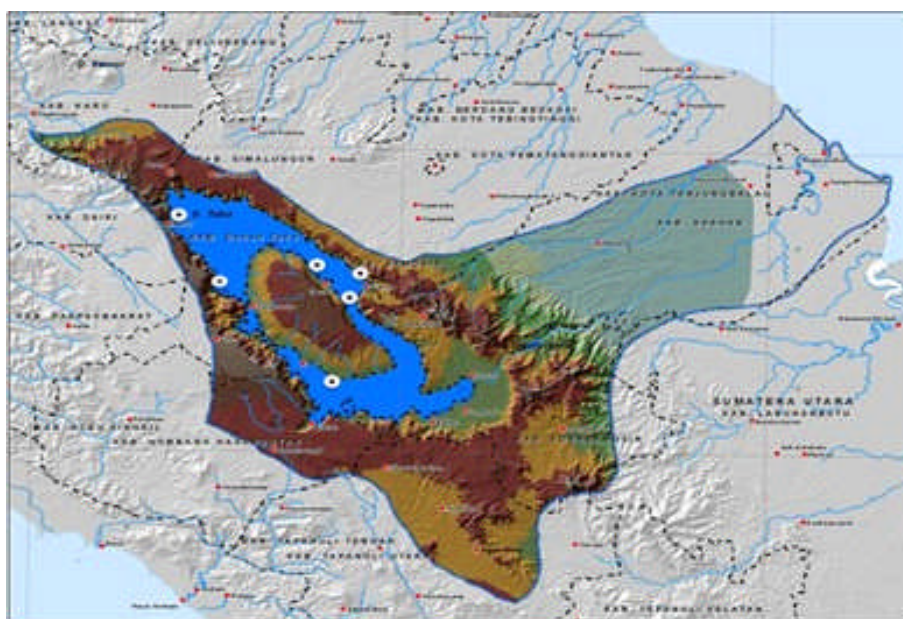


Figure 1. Sampling site in Toba Lake, North Sumatra Province (white dot).

Sample Preservation and DNA Analysis

Target species caught were identified to the species level following Kottelat *et al.* (1993). Immediately after fish was caught, tissue was taken from every individual and stored in absolute alcohol of 1.5 mL. In particular, at least 30 fishes collected for genetic study. Total genomic DNA was extracted from muscle tissue of each specimen using the DNA extraction kit procedure 'DNeasy Blood & Tissue' (Geneaid). The partial fragment of mitochondrial Cytochrome C Oxidase Subunit-1 gene (COI) was amplified using modified universal primers described by Ivanova *et al.* (2007):

Fish-COI-F (5'-ACT TCAAAC TTC CAY AAA GAY aty GG-3) and

COI-Fish-R (5'-TAG ACT TCT GGG TGG CCR AAR Aay CA-3 ').

Polymerase chain reaction (PCR) amplifications were made in a 50 μ L of reaction volume consisted of 5 μ L DNA samples, 16 μ L double distillate water, 2 μ L of each primer and 25 μ L of PCR ready mixture solution (KAPPA). PCR cycling parameters included an initial denaturation phase at 95°C for 10 min, followed by 35 cycles at 94°C for 1 min, 48°C for 1 min and 72°C for 1.5 min and ended with a final extension at 72°C for 7 min. Finally, all amplicons were automatically sequenced in both directions at MacroGen S.A. Korea (www.macrogen.com). The COI sequences of every analysed specimen were submitted to the GenBank database (MF062186.1 – MF062179.1).

Chromatograms were controlled and checked manually edited with BioEdit (version 7.0.4.1) (Hall, 1999) and multiple alignments were done using Clustal W (Thompson *et al.*, 1994). Sequence divergence was estimated using the Kimura two parameters (K2P) model of base substitution (Kimura, 1980). Phenetic

reconstruction was done using a distance based method, Neighbor-Joining (NJ), carried out in MEGA5 software (Tamura *et al.*, 2007) with the K2P model of substitution. Support for nodes in NJ analyses was assessed using non-parametric bootstrapping with 100 full heuristic pseudo-replicates. For comparative purposes, we used the several sequences of the freshwater fish species in Genbank (Accession Number view in figure) to root the tree.

We estimate of N_e to a benchmark value ostensibly important for conservation and management of *M. marginatus* in the Toba Lake Sumatra River. We also calculated the population history of *M. marginatus* and generated a *mismatch distribution* plot (Rogers, 1995) using DnaSP3.51 software (Rozas & Rozas, 1999). Detecting population expansion, past population growth or stability based on DNA sequences can be estimates employing a range of neutrality statistics (Ramos-osins & Rozas, 2002) such as Fu and Li's D^* test, Fu and Li's F^* , Fu's FS test (Fu & Li, 1993) and Tajima's D test (Tajima, 1989). All of calculations were computed using DnaSP 3.0 software.

Reproductive Examination

In event level *sampling*, we go through our sampling frame selecting at least 30 *individuals* for each of sampling site. We measured the standard length (SL) and total length (TL) of collected *M. marginatus* to the nearest 1 mm, weighed the body (to nearest 0.01 g). The sex of each individual was identified and the gonads were assigned a developmental stage based upon morphology and macroscopic examination. Ovaries and testes were allocated a developmental stage according to the criteria modified Cassie (Effendi, 1979). Gonads from both sexes were subsequently preserved and stored in 10% formalin.

Histological preparations were made from four ovaries and two testes representing each stage of development to check the accuracy of macroscopic staging and to better discriminate between mature and immature fish. Such a small and incomplete sample stage from the population due merely to change. Histological preparations were also made if a fish's sex or developmental stage was uncertain. To prepare a gonad for histological examination, a small sample (<0.5 cm thick) was taken from the central portion of the preserved left gonad, embedded in paraffin, sectioned to 6 μ m thickness, stained with Mayer's haematoxylin and eosin Y, and mounted on a microscope slide (Hinton, 1990). All lengths reported here are standard lengths. Sex ratio, expressed as

female:male, deviations from 1:1 null hypothesis were statistically tested by chi-squared test.

Total Fish Production Assessment

The assessment should include a census in time on selected sites (six sampling sites) using enumerator (the enumerators examined the fish caught) and comprehensive interview and count number of active Sulangat, the primary means of capturing *M. marginatus* to determine the total fish production.

RESULTS AND DISCUSSION

Results

Genetic Analysis

The PCR products of approximately 636-bp were obtained only from 11 *M. marginatus* Toba lake samples by amplification with *Cytochrome Oxidase Subunit I (COI)* universal primers, which spans sequence position 5537 – 6225 in GenBank HM 536913.1. In total, 1 polymorphic site (represent singleton dimensions) was identified in the sequence analysis of the 636-bp fragment among a total of 11 *M. marginatus* individuals from Toba Lake (Figure 2). Based on these polymorphic sites, two haplotypes were identified (Table 1), haplotype 1 was most frequent (90%) and the frequency of the remaining haplotypes was very low and resulted in a low nucleotide diversity of 0.00029 and low haplotype diversity 0.182. Variation of *COI* gene sequences of samples was summarized in Table 2. Based on our estimate of theta (θ) at 0.341 in *M. marginatus*, the effective female population size (N_{ef}) was calculated to be 1,936 to 3,878. Given that effective population size inferred from the mitochondrial DNA is half of overall effective population size, and then we assume the effective population size (N_e) would be in between of 3,872 to 7,756.

Neighbor-Joining tree kimura 2 parameter (Figure 3) reconstructed from 15 sequences (11 sequences *M. marginatus* samples and 4 sequences samples as comparison). Base on phylogeny tree, common haplotypes of Toba Lake samples monophyletic with *M. marginatus*. Sequence analysis of the PCR products showed over 98% sequence identity to the *COI* gene of *M. marginatus*.

The population history of *M. marginatus* population was examined using two different methods. A pairwise distance matrix was constructed with the 11 sequences of the *COI* of the mtDNA. The mismatch curve is bell-shaped (Figure 4), graphs observed

mismatch distributions were compared with the expected ones. In the upper graphs expected curves were drawn by assuming constant population size whereas in the lower graphs expected curves were drawn under the assumption of population growth. The

mean difference values for haplogroup Toba Lake was 0.182. Expansion results are presented in Table 3. Fu's F_s test of neutrality was applied to further test the possible occurrence of population.

CCTTTATCTTGTATTTGGTGCCTGAGCCGGAATAGTAGGAACCGCCTTAAGCCTTCTTATTCG
 AGCCGAACTGAGCCAACCCGGATCACTTCTAGGCGATGATCAAATCTACAACGTCATCGTTACTG
 CCCACGCCTTCGTAATAATTTTCTTTATAGTAATACCTATCCTTATTGGAGGGTTCGGAAACTGAC
 TTGTACCACTAATGATTGGAGCCCCGACATAGCATTTCACGGATAAATAACATAAGCTTCTGAT
 TATTACCCCATCATTTCTACTACTATTAGCCTCATCTGGTGTGAAGCCGGAGCCGGAACGGGG
 TGAACAGTATATCCCCCTTTGCAGGGAACCTAGCCCACGCAGGAGCATCAGTAGACCTAACAA
 TTTTCTCACTCCACTTAGCAGGTGTATCATCAATTTTAGGTGCGATCAATTTTATTACTACAACCAT
 TAACATGAAACCCCAACCATCTCCCAATATCAAACACCCCTTTTCGTTTGATCCGTACTTGTAAAC
 CGCCGTACTACTTCTTCTATCACTACCCGTCCTAGCCGCGGGATCACAACTCCTAACGGACC
 GAAATCTTAACACCACATTCTTCGACCCGGCAGGGGGAGGAGACCCAAT

Figure 2. Nucleotide sequence of a partial DNA fragment (663 bp) of the *M. marginatus* COI gene. Single nucleotide polymorphism locations are designated in bold and underlined.

Tabel 1. Position of bp variation of *M. marginatus* COI gene

Haplotype	Polymorphic site	
	5555	6054
haplotype 1	T	C
haplotype 2	T	T

Table 2. Location, sample size and genetic data of *M. marginatus* base on COI gene

Location	S. size	sites plimrp	trans	transv	Indel	Nucleotides composition
Toba Lake	11	1	1	0	0	T = 27.4 C = 28.3 A = 26.7 G = 17.6

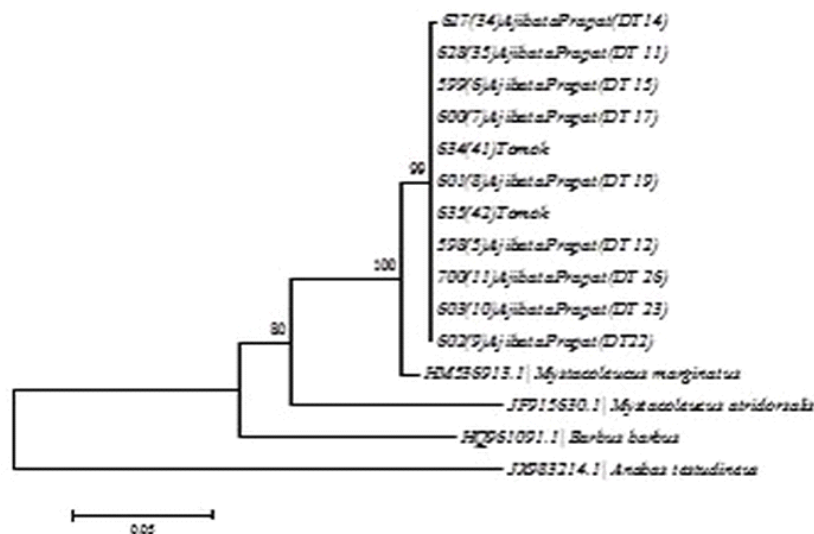


Figure 3. Neighbour-Joining phylogeny Kimura 2 parameter haplotype of *M. marginatus* base on COI gene.

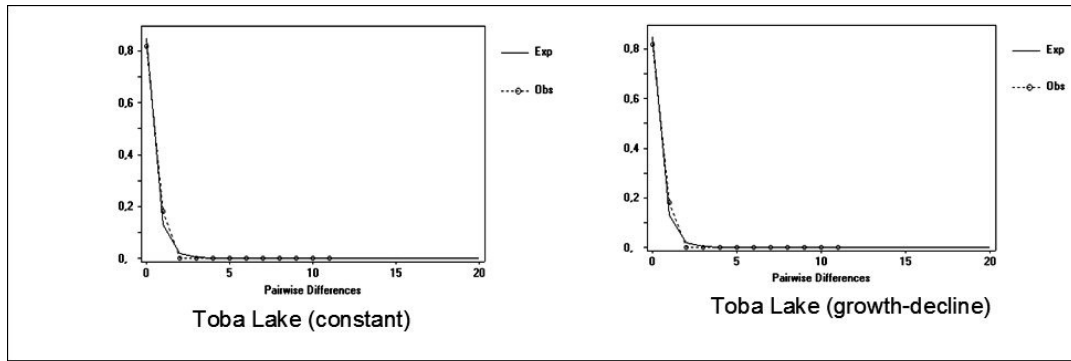


Figure 4. Observed and expected mismatch distributions for *M. marginatus* mtDNA COI sequences from Toba Lake under both constant and population expansion.

Table 3. Neutrality calculates for *M. marginatus* mtDNA COI sequences from Toba Lake. Not significant values in the data are indicated

	<i>Toba Lake</i>	<i>sig</i>
Tajima's (1989) D	-1.128	ns
Fu & Li's (1993) F*	-1.289	ns
Fu & Li's (1993) D*	-1.399	ns
Fu's (1993) FS	-0.400	
Not significant P > 0,10		

Aspects of Reproduction

Reproductive Characteristics

M. marginatus make downstream migrations to reach very specific spawning locations in Toba Lake. Sex ratio was significantly different from the expected 1:1 ratio, (3,65), sex ratio shows a dominance of males in the Toba Lake (males/846 individuals, females/232 individuals during sampling periods). *M. marginatus* has an asynchronous ovary containing oocytes at various stages of development, Figure 5. Ovarian maturity was classified into the following four stages based on the most advanced oocytes contained in the ovary. Stage I oocytes correspond to previtellogenic oocytes and are characterized by a small size, a basophilic homogenous ooplasm, central or sub-

central nucleoli and a high nucleoplasmic ratio. The developing phase (Stage II) includes oocytes in a primary to tertiary yolk stage and the mature phase (Stage III), ovaries much larger, occupying a significant part of the abdominal cavity. *Testis* histological observation produced the following results for III and IV maturation stage (Fig. 6)

Fecundity

Fecundity is the number of eggs in the female fish prior to release at the time of spawning fish. The fecundity is linked to longevity or weight of individual fish species (Effendie, 1979). The result showed that the Bilih fish fecundity with total length between 87 mm - 136 mm with the number of eggs 4,432-9,887 (7,125 ± 1,753).

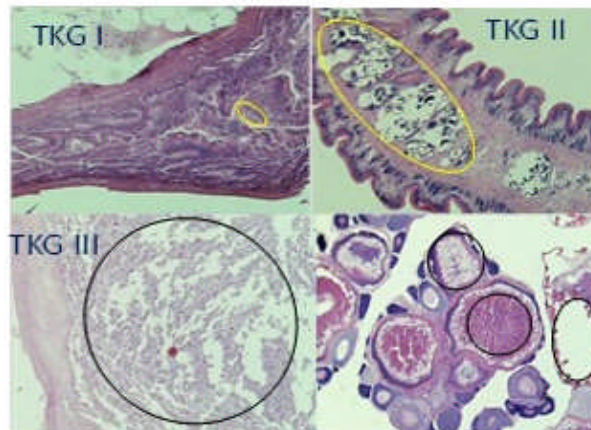


Figure 5. Histological maturation of the oocytes in *M. marginatus*.

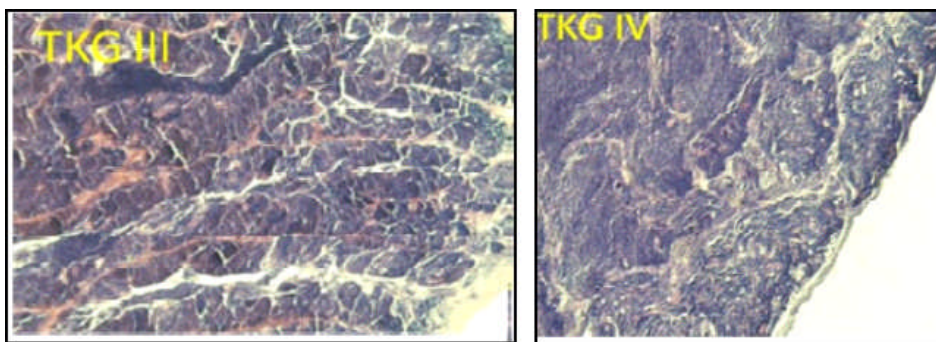


Figure 6. Testicular maturation stages in *M. marginatus*.

Egg Diameter

Egg diameter of *M. marginatus* range from 0.08 – 0.012 mm, the results showed that the diameter of fish eggs is the same and the number of eggs that very considerably. The condition of maturity in level III and IV founded in every location in large number, It's indicated that the Bilih fish will be spawning sustainable in Lake Toba.

Gonado Somato Index (GSI)

The highest male and female on maximum value of GSI Bilih fish found at the Tongging station (inlet of Lake Toba is located in Karo district) respectively at 12.23 and 12.79% (Table 4). The average value of the male gonad maturity index between observation stations based on Sapiro-Wilk test was not significantly different ($\alpha > 0.05$). The average value of GSI lowest male fish found in Paropo with value 2.77%. The average value of GSI female found in Tongging with value 7.22%. GSI female values at each station

were not significantly different ($\alpha > 0.05$). Based on the results of data analysis showed that the Variance of GSI are higher in male and female fish. The statement indicated that the fish can spawn almost all stations and sustainable spawn throughout the year.

Total Production

Based on our study by calculate the number of fishing gear (Sulangat) on Lake Toba, it was found as many as 917 units. It is found ed in nearly all districts in Lake Toba. The data showed that the range of the catches fish (*Mystacoleucus marginatus*) in Lake Toba by using sulangat obtained by the range of 10 - 20 kg / day. By converting the data is assumed that the potential of the *bilih* fish with sulangat amounted to 9.17 tons - 18.34 tons per day. (number of sulangat multiplied with production – 917x10-20 kg/day). However, total capture fishery of *bilih* fish production in 2013 in Lake Toba was 20,000 tons (Kartamihardja *et al.* 2015).

Table 4. GSI of *Mystacoleucus marginatus* Toba Lake 2013

Location	Male/ Female	N	GSI (%)			
			Min	Max	Mean	SD
Aji bata	♂	40	1.27	12.17	5.18	2.89
	♀	8	3.24	7.44	5.57	1.69
Panahatan	♂	18	1.13	5.50	2.96	1.03
	♀	18	1.25	12.51	5.82	3.67
Tongging	♂	16	1.05	12.23	4.31	2.60
	♀	5	3.42	12.79	7.22	3.92
Paropo	♂	10	1.13	4.47	2.77	1.07
	♀	12	3.46	8.41	5.57	1.32
Parapat	♂	8	1.05	5.98	3.80	1.49
	♀	9	3.77	6.33	5.43	0.90

The fisher using fishing gear (sulangat) in Lake Toba is done with the aid of light at night and in the morning they harvest the catch.

Discussion

This study used nucleotide sequences from the cytochrome *c* oxidase subunit I (COI) gene region to examine the genetic properties of *M. marginatus* among six sampling sites in Toba Lake with a total of 11 individuals. Sequence analysis showed over 98% sequence identity to the COI gene of *M. marginatus*, this suggest that *M. padangensi* is a synonym for *M. marginatus*. Analyzed specimens were included to specific taxonomic group only if they formed monophyletic group with maximum 3 % (K2P) sequences divergence (Hebert *et al.*, 2003). Using partial sequences of Cyochrome b sequence Roesma (2011) define that *M. marginatus*, is a new synonym for *M. marginatus*. The percentage of A + T base composition (54.1%) was much higher than C + G, which coincides with vertebrate protein-coding genes (Miller *et al.*, 2005).

Phylogenetic reconstruction of molecular variation between *M. marginatus* sampled from Toba Lake reveal low levels of genetic diversity with no clear pattern of haplotype-partitioning associated with a specific geographic location. However, when explored at the nucleotide diversity analysis infer the present of two lineages which is good for population longterm survival. Single-nucleotide polymorphism (SNPs) is important and now widely used for both, linkage analyses and biodiversity studies (Moorhead *et al.*, 2003; Pearson *et al.*, 2004). A single, intermixing population of *M. marginatus* across the sampled location. No significant structuring was observed among other pairwise comparisons, this indicates gene flow between geographically adjacent locations. These results have direct management implications in recommending that *M. marginatus* from Toba Lake be managed as single stocks. In general, the active movement of adult and passive movement of eggs and larvae would accelerate the occurrence of gene flow among populations (Slatkin, 1987).

It seems that the *N_e* value of *M. marginatus* (1,936 to 3,878) is sufficient to survive from a genetic viewpoint. The relatively high *N_e* reduces the probability of inbreeding among *M. marginatus*, potentially allowing the population to recover quickly (Zhang *et al.*, 2003). The population history of *M. marginatus*, suggest that the population has not experienced population growth/expansion as expected (Slatkin & Hudson, 1991). More sensitive tests Fu's *F_s* test of neutrality (Fu, 1997) was applied

to further test the possible occurrence of population expansion. However the Tajima's *D* were positive in both populations and the index of Fu's *F_s* test, was not significant at the 5% level for population for the pooled dataset. This is a weak indication of population expansion (Aris-Brosou & Excoffier, 1996). To our knowledge, this is the first study to explore the genetic variation of *M. marginatus* in the Toba Lake to single stocks. Further, our data indicate *M. marginatus* at each sampling location likely had not undergone recent population expansion based on mismatch distribution analysis and neutrality tests.

The same phylogeny means that *M. marginatus* in the Toba Lake share similar life history traits include maximum body size, longevity, age at maturity, and fecundity (the number of eggs produced). Referring to the organizing framework by Winemiller & Rose, (1992) which has evaluated patterns in fish life history traits across species, *M. marginatus* is categorized as the opportunistic strategy consisted of fishes with short generation time, low batch fecundity, and low investment per offspring. Each female fish has a finite amount of energy for reproduction. If all of this energy is used to create new fish biomass, then, at the extremes, it could be used to make one huge egg or billions of tiny eggs. Given these energy constraints, fish reproductive investment tends to be thought of as a spectrum of low investment in numerous offspring (Petrik, 2017). This life history traits approach of *M. marginatus* are important for guide fisheries management for its sustainability. More over the information can be use as pilot data and apply it to data-poor species.

It appears that the total production of *M. marginatus* in Toba Lake have been increased from 1,505 ton in 2010 (Fisheries Services, 2012) to at least 3,347 ton in 2013. This data based on local gear used for fishing called Sulangat, a complex fishing gear which uses lights attached to structure above water to attract both fish and members of their food. In every device, daily catches varied considerably between 10 – 20 ton and at least 917 Sulangat were deployed in Toba Lake. Our study provides some information on the reproductive insight (histology) and total fish production of *M. marginatus* that would be helpful in similar studies and contributed to fisheries management of this species.

CONCLUSION

The introduced fish species, *M. marginatus* is a synonym for *M. marginatus*. The phylogenetic reconstruction reveal low levels of genetic diversity with no clear pattern of haplotype-partitioning, while

nucleotide diversity analysis infer the present of two lineages. The Nef value of *M. marginatus* (1,936 to 3,878), the population has not experienced population growth/expansion as expected. *M. marginatus* in the Toba Lake share similar life history traits include maximum body size, longevity, age at maturity, and fecundity (the number of eggs produced). *M. marginatus* is categorized as the opportunistic strategy consisted of fishes with short generation time, low batch fecundity, and low investment per offspring. This life history traits approach of *M. marginatus* are important for guide fisheries management for its sustainability.

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