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Cross-Species Amplification and Variability of Microsatellite DNA Markers in Domesticated Indonesian Mahseer; A Case Study with *Tor soro, Tor douronensis*, and their Interspecific Hybrids

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ABSTRACT

Indonesian mahseer (*Tor* spp.) are freshwater species of high economic, cultural, and conservatory values. Owing to their high values and environmental degradation, the population of Tor fish gradually decreased, and domestication efforts have been made to conserve the population. This study was aimed to assess the cross-species amplification and microsatellite genetic diversity in Indonesian mahseer *Tor soro* (SS), *Tor douronensis* (DD), and their interspecific hybrids using primers developed for *Tor putitora*. Eleven primer sets were used to test for amplifiability and screen genetic diversity in 40 progenies derived from those three groups. Results showed that seven primer sets (64%) successfully amplified loci. Genetic screening using the three most consistently amplifying primers showed that the number of alleles in the three populations was low, ranging from 2 to 5 alleles. The observed heterozygosity (H_O) was high ranging from 0.650 to 0.789, and the fixation index (F_{IS}) was negative, indicating heterozygosity excess. In line with other parameters, the P-values of the HW parameter of several loci-population combinations were significantly departed from equilibrium (P <0.05). A few private alleles were observed in parental line DD and the hybrids. Overall, the cross-species primers developed from *T. putitora* were able to amplify loci in *T. soro*, *T. douronensis* and their hybrids and genetic diversity in the hybrid population was slightly higher than those in parental lines. Possible factors driving the phenomena and practical implications of these findings on the conservation measures are discussed.

1. Introduction

Indonesian mahseer fishes of the *Tor* genus, including *Tor soro T. douronensis*, are freshwater fish of Cyprinidae. They are large-bodied fishes with maximum bodyweight potentials that could attain over 50 kg Field (Haryono and Subagja 2008). They are important due to cultural value, economic prospects, or conservatory concerns. In relation to the conservation, no information is available regarding the conservation status of these two species (IUCN 2021). However, the high economic value of these species has led to the over-exploitation of these resources in nature. Additionally, degradation of their natural habitat has put these genetic resources in conservatory concerns (Wahyuningsih *et al.* 2012). As part of the conservatory and sustainable-use initiatives, several works dealing with genetic characterization and breeding for domestication have been initiated (Gustiano *et al.* 2013) using one or more types of molecular markers.

Microsatellite DNA markers have been recognized as a handy marker for many genetic studies (Wang *et al.* 2017). The markers, which are simple-sequence, tandem repeats of 1–6 bp, are becoming a marker of choice due to advantageous features such as highly polymorphic, evenly distributed in the genome, co-dominantly inherited and easily genotyped (Abdurakhmonov 2016; Bernardi *et al.* 2016). One of their advantageous features, namely higher resolution power, has allowed them to be applied in managing wild populations for conservation (Morin *et*

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al. 2010). Abdul-Muneer (2014) and King *et al.* (2012) have reviewed the application of these markers for conservation genetics and fishery management.

While the advantages of microsatellites as molecular markers have been widely acknowledged, they are not free from disadvantages. These include the need for existing molecular genetic information, laborious and tedious protocols for discovering the microsatellite loci, and flanking regions in the genome from which primers are amplifying the loci can be developed (Bernardi *et al.* 2016). One alternative approach to coping with this problem has been to carry out cross-species amplification. This approach, however, has indicated variable successes. A cross-species amplification study in golden mahseer *T. putitora* resulted in 0%, 43%, and 67% successful amplification when primer set developed from *Catla catla, Cyprinus carpio*, and *Barbus barbus*, respectively, were used (Mohindra *et al.* 2004). Therefore, exploration and preliminary studies of cross-species amplifiability of microsatellite loci are needed before being used in other species of interest.

The present study aimed to apply microsatellite DNA markers to conservation genetics of Indonesian mahseer fishes, namely *T. soro* and *T. douronensis*, by screening microsatellite allele polymorphisms within these species and their interspecific hybrids. Specifically, the study aimed to confirm the cross-species transferability of the microsatellite loci in Indonesian mahseer using primers developed for other congeneric species (*T. putitora*) and evaluate genetic diversity within these species and their interspecific hybrids.

2. Materials and Methods

2.1. Sample

The sample consists of three offspring groups derived from two groups of progenies representing parental species and one progeny group representing interspecific hybrids. Four mating designs of broodstock (female to male) were applied. They were *T. soro* x *T. soro* (SS), *T. douronensis* x *T. douronensis* (DD), and pooled progenies of reciprocal interspecific hybridization, namely *T. soro* x *T. douronensis* (SD) and *T. douronensis* x *T. soro* (DS). For each progeny group, three broodstock consisting of one female and two males were used. The *T. soro* were collected from the natural freshwater system of Subang district, West Java, in 1996, while *T. douronensis* were collected from Pagaralam district, South Sumatera, in 2014. They were introduced to the Research Installation of Freshwater Germ Plasm (RIFGP), Cijeruk, Bogor for domestication and breeding program. Total 40 fish seeds of 21 days old, measuring of 1.5-2.0 cm length representing both parental lines and hybrids, were sampled for microsatellite analysis.

2.2. Microsatellite Genotyping

Microsatellite genotyping consisting of genomic DNA extraction, Polymerase chain reaction (PCR), and microsatellite genotyping was conducted at the Laboratory of Physiology and Genetics, the Research Institute for Fish Breeding (RIFB), Subang, West Java, Indonesia.

The genomic DNA of the samples was extracted using a Genejet genomic DNA purification kit (Thermo Scientific). Briefly, 20 mg tissue of individual sample was minced and processed according to the manufacturer's protocol, including tissue lysis, DNA precipitation, DNA binding into a column, DNA washing, and DNA elution. The final product of these steps, the eluted DNA, was then used for PCR amplification. The PCR primers used in this study were adopted from Sahoo *et al.* (2013), who developed them from the genomic DNA of *T. putitora*. Eleven primers (Table 1) were initially chosen and were tested for their ability to amplify microsatellite loci in *T. soro, T. douronensis*, and their reciprocal hybrids. In this stage melting temperature of the primers was optimized, and the ones capable of consistently amplifying amplicon in all groups were used for further analysis. To ensure that the genome quality of the sample was good, PCR amplification using a cytochrome oxidase I (COI) primer, which consistently produced a good amplicon, was carried out as a control. Detailed technical description of PCR amplification, allele genotyping, and scoring in this study referred to Imron *et al.* (2015).

2.3. Data Analyses

Parameters of population genetic variability, including the number of alleles, private allele, allele frequency, observed and expected heterozygosity, Hardy-Weinberg equilibrium, and fixation index, were evaluated. Analyses of the parameters were implemented in the Genalex software (Peakall and Smouse 2012).

Table 1. List of primer sets used to amplify microsatellite loci in *T. Soro* (SS), *T. douronensis* (DD) and hybrids of SD and DS obtained from Sahoo *et al.* (2013)

Locus	Primer sequences	TM (°C)	Expected product size (bp)	Gen bank accession
TPM 02	F: GGCCCAGATGAGAGAGAA R: ATCAGCCCTCTACAAACAA	52	108–132	JX270776
TPM 21A	F: CCGTTCCATTCAGATGCC R: CGCTTGTGTCTTTGTGTGT	53*	122–142	JX270793
TPM 20C	F: AGCCTGTTTTAGCCTCTCTAA R: TACATCATGAATGTCTCCACA	52	154–188	JX270792
TPM 11	F: GTTGGAGAATGGCGTGTA R: AGGGGAAGAAGAGAGAAAA	51*	136–146	JX270784
TPM 20B	F: ATTTGCAGCGTGTGAGGG R: AGAGAGGCTAAAACAGGC	53*	132–148	JX270792
TPM 20A	F: AAACAGTTCCCCAATGACAA R: CCCTCACACGCTGCAAAT	50	106–124	JX270792
TPM 13	F: TTAAGATAAACCCATTCGACA R: GAAGCTATTGTGTTTTTCACG	52	130–146	JX270786
TPM 04	F: CTAGTAGGCTTGCTGCAATAG R: CGCGTTCAGTTTTAATTGTAG	52*	156–176	JX270778
TPM 15B	F: GGGAGAGATAGAGGATGAAAA R: TTTCATAGGGTCCAGAGAAAT	51*	150–166	JX270788
TPM 18A	F: AGCTATTGGGTGTGTTTGT R: CACATTTGCTCATCCTCT	50*	108–132	JX270790
TPM 18B	F: CAACAGCTATTGGGTGTGTT R: ACACTCACACACTCCAGCTT	55	150–172	JX270790

An asterisk next to the TM indicated that it was optimized in the present study

3. Results

3.1. Cross-species Microsatellite Amplification

Cross-species amplifiability of congeneric microsatellite loci in *T. soro, T. douronensis*, and their reciprocal hybrids were presented in Table 2. The genomic quality of all the samples being used for microsatellite analysis was considerably good, as indicated by total success in amplification with COI primer. Amplification with *T. putitora* primer, however, resulted in variable success. Three out of eleven primer sets in use, namely TPM 02, TPM 21A, and TPM 20C, consistently resulted in favorable products in all tested samples. Four primer sets of TPM 04, TPM 15B, TPM 18A, TPM 18B showed no amplification at all in all populations. The primer set TPM 13, while successfully amplified loci in two populations (DD and SD and DS), did not amplify loci in the SS population. The remaining three primer sets, namely TPM 11, TPM 20B, and TPM 20A, resulted in a variable degree of amplification success ranging from 65–95%. Only primer sets showing total amplification success in all populations were used for further analysis in this study.

3.2. Population Genetic Diversity in Parental Species and their Hybrids

Parameters of population genetic variability assessed using three loci in the populations of two parental species, *T. soro* and *T. douronensis*, and their interspecific hybrids were presented in Table 3. The number of alleles per locus for loci TPM 02, TPM 20C, and TPM 21A ranged from 2 to 5, 2 to 4, and 2 to 4, respectively. The lowest allelic number was found at the locus TPM 02 in DD and locus TPM 20C in SS, both parental species, while the highest allelic number was found at locus TPM 02 in the hybrid population. Averaged over all loci, the highest number of alleles (2.816) was found in the interspecific hybrid population, while the lowest allelic number (2.422) was found in the DD population. The alleles found in the populations consist of both shared and private alleles. Private alleles are those found only in a specific population. Averaged over three loci, the DD population possessed 1.3 private alleles, followed by the hybrids (1.0 private alleles). No private allele was identified in the SS population.

Levels of observed heterozygosity (H_0), averaged over three loci, across all genetic groups, ranged from 0.658 in SS to 0.724 in the hybrid populations. These figures mean that 60–70% of individuals within the respective group are heterozygous. More variable patterns emerged when the H_0 was looked at the locus wise in which highly variable levels of H_0 were seen. The H_0 at the locus TPM 02 ranged from 0.375 to 0.625, while those at the locus TPM 21A ranged from 0.500 to 0.700. The quite interesting finding concerning this feature was the presence of total heterozygosity at the locus TPM 20C in all individuals within all populations.

Table 2. Cross-species amplifiability of microsatellite loci in *T. soro* (SS), *T. douronensis* (DD), and interspecific hybrids (SD and DS) using primer set developed for *T. putitora* (Sahoo *et al.* 2013)

Loci \ Population	Amplifiability (%)		
	SS (n = 10)	DD (n = 10)	SD and DS (n = 20)
COI (control)	100	100	100
TPM 02	100	100	100
TPM 21A	100	100	100
TPM 20C	100	100	100
TPM 11	100	100	95
TPM 20B	90	80	80
TPM 20A	80	70	65
TPM 13	0	100	100
TPM 04	0	0	0
TPM 15B	0	0	0
TPM 18A	0	0	0
TPM 18B	0	0	0

Tabel 3. Summary of population genetic parameters in three populations of Indonesia mahseer, *T. soro, T. douronensis* and their hybrids as revealed by microsatellite markers

Parameter/loci	Parental lines		Hybrids SD and DS
	SS	DD	
TPM 02			
N	10	10	20
A	4.000	2.000	5.000
AP	0.000	1.000	1.000
H_O	0.375	0.625	0.471
H_e	0.648	0.492	0.478
HWE	0.052^{ns}	0.445^{ns}	0.042*
F_{IS}	0.422	-0.270	0.014
TPM 20C			
N	10	10	20
NA	2.000	3.000	4.000
AP	0.000	1.000	2.000
H_O	1.000	1.000	1.000
H_e	0.500	0.620	0.725
HWE	0.002**	0.019*	0.000***
F_{IS}	-1.000	-0.613	-0.379
TPM 21A			
N	10	10	20
A	3.000	4.000	3.000
AP	0.000	2.000	0.000
H_O	0.600	0.500	0.700
H_e	0.645	0.625	0.655
HWE	0.002**	0.061ns	0.000***
F_{IS}	0.070	0.200	-0.069
Overall			
N	10	10	20
A	2.554	2.422	2.816
AP	0.000	1.333	1.000
F_{IS}	-0.169	-0.228	-0.145
H_O	0.658	0.708	0.724
H_e	0.598	0.579	0.619

A: Number of alleles, AP: private allele, H_O: observed heterozygosity, He: expected heterozygosity, HWE: hardy-Weinberg equilibrium, and F_{IS}: fixation index

In comparison with the expected heterozygosity (He), namely the levels heterozygosity assuming population in the state of HW equilibrium. Table 3 shows that excluding the locus TPM 20C, a variable pattern of H_O distribution across populations was observed. For instance, a locus TPM 02 in the SS population showed a heterozygote deficit while it showed heterozygote excess in the DD population. A similar profile was seen for locus TPM 21A. In contrast to the previous loci, the locus TPM 20C consistently showed heterozygote excess in all populations due to complete heterozygote in all populations. Despite variable patterns based on locus-wise observation, averaged over all loci, Table 3 shows that more heterozygous individuals exist in all populations than homozygous individuals. This was reflected in the value of fixation indices (FIS) ranging from -0.169 to -0.258. The negative sign on the averaged FIS values within all populations indicates that heterozygote excess occurred in all populations. The P-values of HWE, as seen in Table 3, showed that six out of nine loci-population combinations were not in HWE. These included the TPM 20C in all three populations, TPM 02 locus in the hybrids, and TPM 20A in SS and hybrid populations.

4. Discussion

4.1. Transferability of Congeneric Microsatellite Loci

Conventionally, microsatellite primers were developed from a species through a laborious and time-consuming protocol (Bernardi *et al.* 2016). Currently, microsatellite loci identification and generation of corresponding primers have been allowed *in silico* using bioinformatic techniques and DNA sequence of genomic data (Wang *et al.* 2017). In addition to both approaches, cross-species microsatellite primer sets have been increasingly applied. This approach is particularly fit for situations in which technical or logistical shortages do not allow one to develop them. In the present study, we applied cross-species primers due to the need for markers that can cover genetic variability in congeneric species of the *Tor* genus.

The ability to amplify microsatellite loci in *T. soro*, *T. douronensis*, and their reciprocal interspecific hybrids using a subset of primers developed from the genome of *Tor putitora* suggest the potential use of cross-species primers for microsatellite loci amplification. This may indicate sufficient similarity

in the genomic region among these congeneric species. The relatively high genome similarity among the species under the present study with *T. putitora* is expected due to close taxonomic relationships. The *T. soro, T. douronensis*, and *T. putitora* are all congeneric species. This finding confirms the previous studies of (Keong *et al.* 2008), who successfully amplified microsatellite loci in *Tor tambroides* by using the primer of *Mystus nemurus*. In a review on microsatellite primers transferability, Barbara *et al.* (2007) found that taxonomically, marker transferability could increase among orders within a class. However, the level of success decreased along with the more distant taxonomic relationships.

4.2. Microsatellite Genetic Variability in Congeneric Species and their Hybrids

The number of alleles has been a sensitive parameter to detect genetic changes in a population (Allendorf *et al.* 2014; Greenbaum *et al.* 2014). The average number of alleles detected in this study, ranging from 2.442 in DD to 2.816 in the hybrid populations, was comparable to that found in *T. tambroides,* in which 2.86 alleles per locus was identified (Nguyen *et al.* 2007). However, the average allelic number in this study was quite low relative to those found in *T. putitora*, in which 5–8 alleles were identified (Sahoo *et al.* 2013). In general, genetic diversity in the hybrid population was higher than that in parental lines. The hybrid inherited and accumulated genetic diversity from both parental lines.

The most obvious difference of this study from that of Sahoo was the origin of the samples, particularly in terms of whether they represented the wild or captive populations. Many studies have documented the reduction in genetic diversity of captive populations relative to their wild counterparts, such as reported for farmed shrimp species, *Litopeanue vannamei* (Knibb *et al.* 2020), and cobia, *Rachycentron canadum.* While their study (Sahoo *et al.* 2013) sampled wild populations, the present study screened genetic diversity in seeds resulting from the spawning of only a few broodstocks per population. Hence, it is highly likely that alleles screened in the current study were only a subset of those that occurred in wild populations. The phenomena observed in the present study clearly exemplify the bottleneck or founding effect, namely the low number of individual broodstock contributing to establishing captive population and, in general, founding effect decrease genetic variability of populations (Greenbaum *et al.* 2014). The most apparent sign of genetic drift has been the number of alleles in the short term. The level of heterozygosity will also be reduced over time, but it took a long time to observed (Allendorf 1986; Allendorf *et al.* 2014). The genetic drift as manifested in the reduced allelic number in captive populations has also been documented in the selectively bred population of African catfish. A reduction in an allelic number of 35–80% was observed (Imron *et al.* 2011). A genetic screening using the same markers systems on wild populations counterpart of these species would clarify this hypothesis. While the number of alleles was markedly reduced, the same pattern was not observed in the levels of heterozygosity. This is because the expected reduction in heterozygosity is independent of the number of alleles and their frequencies (Allendorf *et al.* 2014). The average heterozygosity levels in all populations understudy were quite high, ranging from 0.650 to 0.789. Moreover, all populations displayed heterozygote excess, which might raise questions regarding the factors that resulted in those phenomena.

Heterozygote excess in populations may result from small randomly mated populations, natural selections, particularly when heterozygotes have a greater survival probability than the homozygote, and differences in allele frequency between sex chromosomes (Allendorf *et al.* 2013). While the possibility of the last two mentioned factors needs further verification, a small mating population (effective breeding number, Neb) seems to best explain the phenomenon in the present study. Under the condition of a limited number of breeders, the allele frequency in males and females will be slightly different, leading to heterozygote excess in the progeny relative to the Hardy-Weinberg proportion (Balloux 2004). A theoretical explanation for this phenomenon was well described by Allendorf *et al.* (2013), who used an extreme case in which progeny were produced from only one male and one female broodstock and two alleles. Under this simulated condition, there will be six mating combinations, three of which resulted in heterozygote excess. These mating combinations were between homozygous dominant and heterozygous, between homozygous recessive and heterozygous, and between opposite homozygous. The first two mating combinations would add 25% heterozygous individuals within

a population, while the last combination would result in 100% heterozygous individuals (Allendorf *et al.* 2013). The most extreme situation in which heterozygosity occurred in all individuals (100%) was also observed in this study, confirming that the founder effect was the major factor. This condition is possible in the mating of opposite homozygous. This explanation also holds for other parameters such as fixation indices (F_{IS}) and HWE, calculated based on heterozygosity levels.

The private alleles, namely alleles that occur in a specific population, have been used to estimate the number of migrants Field (Allendorf *et al.* 2013) and identify parental lines and hybrid in naturally hybridized populations (Cardoso de Carvalho *et al.* 2013). Diagnostic nuclear markers developed using single nucleotide polymorphisms, for instance, have successfully been applied to identify sturgeon hybrid in sturgeon populations of the Eastern Black Sea (Beridze *et al.* 2021). In the context of congeneric species, private alleles in a parental line can be used to identify and differentiate that species from others. In theory, private alleles found in the hybrid would be inherited from their parental lines. This study found private alleles in the hybrid and DD populations. However, looking at the parental lines, only the DD population possessed private alleles. The lack of private allele in the SS population could reflect the reality in that population or sampling error due to lower frequency of the allele in combination with founder effect and small sampling size in the progeny. Assuming the private alleles occurred in relatively high frequency, the presence of private alleles in this study could be used to identify the parental line of DD. Their use for hybrid identification, namely estimating parental species composing that hybrid, however, requires information of private allele of the other species.

4.3. Prospects and Implications for Conservatory Measures

The present study results provide several important information about the conservation of Indonesian Mahseer populations. Following a domestication period of almost 25 years for *T. soro* and six years for *T. douronensis*, both species have well adapted to the conditions at the research facilities, as indicated by their reproductive success. This success has allowed them to use their reproductive output to conserve their natural stocks. However, a thorough assessment, particularly from the perspective of a responsible genetic approach, is required to minimize ecological and genetic risks to wild populations (Grant *et al.* 2017).

The main objectives of stock restoration have been to restore the population undergoing depression to its previous level. To achieve these, several genetic benchmarks and corresponding broodstock management in the hatcheries need to be considered (Grant *et al.* 2017). The genetic similarity between the wild and hatchery origin stocks represents an important benchmark (Johnson and Friesen 2014). Accordingly, a survey on genetic diversity in natural populations is fundamental. This information will form the baseline genetic data that will be the basis for broodstock procurement and natural stock restoration.

Similarly applies to the captive population. Stocking hatchery stock into natural waters without considering the genetic composition of both the hatchery and the wild stocks may cause risk of reducing local adaptation, such as documented in Atlantic salmon (Jonsson *et al.* 2019; O'Sullivan *et al.* 2020), or changing genetic diversity of the existing wild stock (Wu *et al.* 2021). In most cases, the aforementioned genetic risks are tightly linked to hatchery management techniques, particularly broodstock origin, effective breeding number (Neb), and domestication. With respect to the origin of broodstock, conservation biologists recommend using individuals from the depressed populations themselves or from a genetically similar population (Grant *et al.* 2017). In terms of broodstock size, the general recommendation has been to use an effective breeding number (Neb) that can minimize loss of genetic diversity, at least Neb of 50 for short term and Neb of 500 for long term (Jamieson and Allendorf 2012). In addition, broodstock domestication in captive facilities (hatcheries) should be set at the shortest time possible to avoid genotype frequency and fitness changes.

The present study confirmed that producing progeny using a limited number of broodstock has severely affected genetic diversity in the progeny, as exemplified by the reduction in allelic diversity and heterozygote excess resulting from the bottleneck effect. To ensure that allelic diversity is maintained in a captive population, the maximum number of broodstock producing the progeny needs to be

applied. In addition, the domestication period, which has lasted for over 25 and 6 years for *T. soro* and *T. douronensis*, respectively, have also contributed to the profile of genetic diversity in the current hatchery population. Adopting the previously mentioned guidelines, the hatchery stocks of both *T. soro* and *T. douronensis* in the present study are less appropriate for restoring those in the wild. This is understandable given that the hatchery was particularly dedicated to supporting aquaculture development. However, breeding technologies of these two species that have been entirely under control can be implemented to develop conservation hatcheries (Fisch *et al.* 2015), specifically designed for conservation purposes by applying the previously mentioned genetic principles.

To conclude, the cross-species amplification of microsatellite loci in Indonesian mahseer *T. soro, T. douronensis,* and their reciprocal hybrid was successfully carried out using a subset of primers developed for *T. putitora*. Out of 11 primers tested, 30% were consistently amplified amplicon, 35% need further optimized, while the remaining 35% were not capable of amplifying. Microsatellite genetic variability in the hybrids was slightly higher than in the parental lines *T. soro* and *T. douronensis*. Microsatellite genetic variability in the hybrids was slightly higher than parental lines. More broodstock recently recruited from the wild was required to produce genetically healthy populations for conservation of Indonesian mahseer in natural populations.

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