

# Cross-Species Amplification of Microsatellite DNA Markers and Comparison on the Sequence of Microsatellite Regions Among Southeast Asian Catfish Species

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## Abstract

Thirty microsatellite markers developed for River catfish (*Hemibagrus nemurus*) were screened for cross amplification in six species of catfish. Out of 30 primers, only 3 (10%) to 6 (20%) produced successful amplifications in six catfish species. Six different loci could be amplified in *Pangasius pangasius* and *Clarias batrachus*, 5 loci in *Clarias gariepinus*, 4 loci in *Pseudomystus siamensis* and 3 loci in *Clarias macrocephalus* and *Pangasius nasutus*. The low rate of amplification in these 6 closely related species proved the primers are locus-specific. Sequence analysis of locus MnRm7-1 showed differences in the flanking region and repeat motif in certain species, suggesting the existence of indel mutations in catfish species in that particular region. The examined DNA sequence revealed that the repeat motif is conserved within Pangasiid catfishes and not conserved within Clariid catfishes.

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## Introduction

Catfish are an important fishery resource both as a source of income and an alternative protein for most Southeast Asian nations<sup>1-3</sup>. Indeed, in the most recent accounting, catfish species made up for 67% of total freshwater aquaculture production in Malaysia<sup>4</sup>. Catfish have also been used as an animal model in reproductive, physiological and genetic studies. Species from three major families – namely, Bagriidae, Clariidae and Pangasiidae – are of particular commercial importance and widely cultured in Southeast Asia.

The study of genetics has been of major importance for the aid aquaculture sector and assist conservation programme. Microsatellite DNA markers are short tandem repeat motifs with high levels of polymorphism<sup>5,6</sup>, and are distributed in both coding and non-coding regions<sup>7</sup>. These markers have been

a subject of great interest due to their co-dominant characteristics, which make them more informative than dominant markers such as RAPD and AFLP. In aquaculture, microsatellites have become one of the most useful molecular markers in fish population studies and have supplemented conventional markers such as allozymes and mitochondrial DNA that show totally low overall levels of variation<sup>8</sup>.

Microsatellite markers also show high allelic variation that makes them effective as markers in many genetic studies. Microsatellite markers are widely applied in studies on population genetic structures and genetic diversity of species. However, the development of microsatellite markers is very time-consuming and labor-intensive. As a potential alternative, some microsatellite markers have been shown to be conserved within related taxa<sup>9,10</sup>. Through cross-species amplification, microsatellite markers

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developed for source species can be amplified in target species<sup>11,12</sup>. Cross-species amplification in catfish species has been tested by many researchers, and has led to the identification of polymorphic microsatellite loci in target species<sup>13-15</sup>.

In the present study, we examined cross-species amplification of primers developed for the river catfish *H. nemurus* in 6 catfish species collected in Malaysia. Our objective was to identify potential microsatellite markers that could be used in target species.

## Materials and Method

### 1. Fish Samples

A total of 210 samples of 7 catfish species were collected from 7 locations in Peninsular Malaysia. Fresh samples were identified based on previous study<sup>16</sup>, labelled, and immediately frozen at  $-20^{\circ}\text{C}$  in order to preserve their condition. Details of each sample are represented at Table 1.

### 2. DNA Extraction

The muscle tissues were taken from fresh samples for the DNA extraction process. The DNA extraction was performed using commercial Promega® Wizard Genomic DNA Purification Kit. DNA quantification was performed and DNA maintained at a temperature below  $-20^{\circ}\text{C}$  until further use. Thirty microsatellite primer pairs developed<sup>17-20</sup> were tested on 7 species in this study (Table 2).

### 3. PCR Conditions and Electrophoresis

Each primer selected was tested for cross-amplification in every species using Touchdown PCR or normal PCR. The PCR conditions are as follows:  $95^{\circ}\text{C}$  for 3 min, followed by 5 cycles of  $95^{\circ}\text{C}$  for 30s

and annealing at different temperature (see Table 3) or following touchdown PCR for 45s, and  $72^{\circ}\text{C}$  for 60s and extension of  $72^{\circ}\text{C}$  for 15 minutes. The primers and its repeat sequence were represented at Table 2. Since several primers show problems during amplification, PCR touchdown (MJ Research, USA) was performed to screen for primers capable of amplifying the selected DNA region. The PCR touchdown with 5 different annealing temperatures (60,58,56,54,52,48) consisting 5 cycles for each annealing temperatures respectively.

A 10  $\mu\text{l}$  PCR reaction consisting of 1.5–2.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTP, 0.5  $\mu\text{M}$  of the forward and reverse primer, 1X of Buffer, 0.1 unit of Taq Polymerase and 0.1  $\mu\text{l}$  genomic DNA as template. Initially, 3 random samples from each species were used during the optimization. The primers that showed positive amplifications were further evaluated using larger number of samples. PCR product was resolved by electrophoresis on 2.5% metaphor agarose gels, stained with GelRed™ 3X (GENTAUR) and viewed under UV and photographed using Alpha imager Gel Documentation System (Syngene).

### 4. DNA Purification and Sequencing

The target PCR product from single individual of each species was excised from the gel and purified using GeneJET™ PCR Purification Kit-Fermentas, then used for sequencing analysis. DNA sequence obtained for each species was submitted to the online program WebSAT<sup>21</sup> (<http://wsmartins.net/websat/>) to identify tandem repeats in each sequence. Repeated sequence repeat was considered a microsatellite when the number of repeats was more than three. All sequences were deposited at DNA DATA Bank of Japan (DDBJ).

Table 1. Sample size of each species used in this study

Family	Species	Sample size	Collection Date	Location
Bagridae	<i>Hemibagrus nemurus</i>	30	March 2009	Tasik Banding, Perak
	<i>Pseudomystus siamensis</i>	30	April 2011	Jejebu, Negeri Sembilan
Pangasidae	<i>Pangasius pangasius</i>	30	March 2009	Kuala Kangsar, Perak
	<i>Pangasius nasutus</i>	30	June 2010	Perlok, Pahang
Clariidae	<i>Clarias macrocephalus</i>	30	Dis 2008	Maran, Pahang
	<i>Clarias batrachus</i>	30	Jan 2009	Yong Peng, Johor
	<i>Clarias gariepinus</i>	30	April 2009	UPM Hatchery, Selangor

Table 2. Information of microsatellite loci of *Hemibagrus nemurus* used in this study

No	Locus	Primer sequences	Expected size	Tandem repeat	Accession Number	Source
1	<i>MnBP5-1-115b</i>	F: TTTTGCTACTAGAGACTGAC R: TAGGCAAACGTGTACTTTG	179 bp	(GT) <sub>4</sub>	AF544042	Hoh <i>et al.</i> 2013 <sup>19</sup>
2	<i>MnBP5-2-05</i>	F: CAAGTGCAAAGACAGACAGA R: TCTCTAAGGCTATCCATCCA	186 bp	(TGGA) <sub>2</sub> N(AGAC) <sub>3</sub>	AY207448	Hoh <i>et al.</i> 2013 <sup>19</sup>
3	<i>MnBP5-2-05d</i>	F: CATGGTGGATGGATAGATTT R: TGTTATCATAACACCAGTCAC	168 bp	(TGGA)	AY207448	Hoh <i>et al.</i> 2013 <sup>19</sup>
4	<i>MnBP5-2-06b</i>	F: CGTGTCCAGACATGGTTAAT R: GAGTGGGCGACTTTTCAG	164 bp	(CAT) <sub>2</sub> CAC(CAT) <sub>8</sub>	AY671084	Hoh <i>et al.</i> 2013 <sup>19</sup>
5	<i>MnBP5-2-06c</i>	F: CCCACTCTTCCTTCTTATCC R: TGGTCTGAGCGCTAGAG	132 bp	(TCT) <sub>3</sub> (CT) <sub>7</sub>	AY671084	Hoh <i>et al.</i> 2013 <sup>19</sup>
6	<i>MnBP5-2-24b</i>	F: GTCATATTTGCTTTGGCAGT R: GTGGTTTTGAATGTTCTCTG	139 bp	(AAG) <sub>3</sub> (AG) <sub>6</sub>	AY207450	Hoh <i>et al.</i> 2013 <sup>19</sup>
7	<i>MnBP5-2-27</i>	F: TTATAACAGGGAGTGAAGG R: CAAGTGCAAAGACAGACAGA	270 bp	(CT) <sub>8</sub>	AY207449	Hoh <i>et al.</i> 2013 <sup>19</sup>
8	<i>MnBP5-2-2a</i>	F: GGTCGACAGCGAGAGAGAG R: TCCTGAACTGCTCAGATTTT	242 bp	(GT) <sub>12</sub> N <sub>5</sub> (GA) <sub>5</sub>	AY205994	Hoh <i>et al.</i> 2013 <sup>19</sup>
9	<i>MnBP5-2-2c</i>	F: GCCCAATACACTGAATGAAC R: TTCTCTGAGCGAAGAGAG	245 bp	(CT) <sub>8</sub>	AY205994	Hoh <i>et al.</i> 2013 <sup>19</sup>
10	<i>MnBP5-2-05c</i>	F: TGGATGGATAGCCTTAGAGA R: CCACCCAATCACTTATTTGT	215 bp	(TGGA) <sub>2</sub> N(AGAC) <sub>3</sub>	AY207448	Hoh <i>et al.</i> 2013 <sup>19</sup>
11	<i>MnBP5-2-06a</i>	F: GGTTGCGCCGAGAGAGAGAG R: CGGGGTTAGAACACACATCC	168 bp	(GA) <sub>3</sub>	AY804195	Hoh <i>et al.</i> 2013 <sup>19</sup>
12	<i>MnBP5-2-13b</i>	F: CCGCTTTTTATTAGTCCTCA R: CACAGAAAACAGGGTTTCAA	234 bp	(CTCAT) <sub>2</sub>	AY804209	Hoh <i>et al.</i> 2013 <sup>19</sup>
13	<i>MnBP5-2-22a</i>	F: TGTCTGAGCCAGAGAGAGA R: GTCTCTGATGGTGTGTTGCTT	365 bp	(GA) <sub>9</sub>	AY205998	Hoh <i>et al.</i> 2013 <sup>19</sup>
14	<i>MnBP8-4-26b</i>	F: GCAACTTGCACAGTATTT R: ATGCGAAATTTGCACAGA	174 bp	(T) <sub>8</sub> (TAA) <sub>4</sub>	AY860214	Hoh <i>et al.</i> 2013 <sup>19</sup>
15	<i>MnBP8-4-34a</i>	F: GCCTACTGTTGTTGTTGT R: GTGGCCAGAAAAGTGTAGAA	232 bp	(GTT) <sub>5</sub> (ATT) <sub>11</sub>	AY806222	Hoh <i>et al.</i> 2013 <sup>19</sup>
16	<i>MnBP8-4-26a</i>	F: TTTCTGTTGTTGTTGTTG R: GCACAAAATACTGTGCAA	138 bp	(GTT) <sub>7</sub> (TTA) <sub>3</sub>	AY860214	Hoh <i>et al.</i> 2013 <sup>19</sup>
17	<i>MnBP4-2-08</i>	F: GCCCAGGACACACACACA R: TATGGCTCCCTCACACA	183 bp	(AC) <sub>5</sub> (TG) <sub>7</sub>	AY804197	Hoh <i>et al.</i> 2013 <sup>19</sup>
18	<i>MnRmB1-2</i>	F: TTAGCTGACAGGATGCACTG R: GTTCCGTATGATGATGATGATG	160-230 bp	(CAT) <sub>6</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
19	<i>MnRmC8-1</i>	F: TGTGCGCGATGTGTGTGT R: GAAACTGCTGGTTTTGTCAGC	280 bp	(GT) <sub>8</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
20	<i>MnRm D11-1</i>	F: GATCCCGAAAGAAAATCCA R: GTTAGCGGATAGATAGATAG	180-240 bp	(CT) <sub>29</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
21	<i>MnRm7-1</i>	F: TTTTCTCTCGCTGTCTCTC R: GCAGAGTTTGGGTGACATAC	174 bp	(CT) <sub>5</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
22	<i>MnSC4-3b</i>	F: GCCAAGGAGCTATGAACTGG R: GACGCAACTATGTCCACCAC	208 bp	(GAT) <sub>6</sub> (TGG) <sub>6</sub>	-	Keong <i>et al.</i> 2008 <sup>20</sup>
23	<i>MnSC3-15b</i>	F: TCCCTTTGTTGGAGTTAGGG R: GGAGGAAAAACCACAGAGTC	166 bp	(GAC) <sub>4</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
24	<i>MnLR2-1-15b</i>	F: TACGGAATCCGAGGTCCTC R: AAGCGGGCGGCTCTCTCT	206 bp	(CT) <sub>17</sub> (GAATG) <sub>2</sub> (GA) <sub>5</sub> (AG) <sub>6</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
25	<i>MnLR2-1-17b</i>	F: GCAGTTTCCTTCTTCTACT R: GGGGGGCGCGCAACTCTCTC	132 bp	(TG) <sub>11</sub> (AG) <sub>12</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
26	<i>MnLR2-1-21a</i>	F: GGAAAGGGCGAGGCTCTC R: GTCGAGGGTGAAGAGGGGAAG	199 bp	(CT) <sub>6</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
27	<i>MnLR2-1-21c</i>	F: GGAAAGGGGAGGCTCTCTCT R: AGCTCAATAAGGTGCCATGC	177 bp	(CT) <sub>6</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
28	<i>MnB10-2-17</i>	F: CGGATACGTGTTGCTTTC R: GCTCCTGTGCGCGGCTCT	248 bp	(CTTTC) <sub>2</sub> (GA) <sub>26</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
29	<i>MnLR2-2-11</i>	F: GGAAGGCGCGAGGCTC R: GGGAGAAGGGCCTCTC	204 bp	(CT) <sub>9</sub> (T) <sub>9</sub> (A) <sub>8</sub> T(GA) <sub>6</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>
30	<i>MnLR2-1-52a</i>	F: TCCCTTTTTATTGCCATTC R: GGAACGAGGAGGGCTCTCTCT	189 bp	(GA) <sub>22</sub> (T) <sub>8</sub>	-	Chan <i>et al.</i> 2005b <sup>18</sup>

## Result

### 1. Cross-species Amplification

Thirty heterologous microsatellite loci were tested on 6 species of freshwater catfish in the cross-species amplification experiment. Only 3 (10%) to 6 (20%) primers produced successful amplifications in the 6 catfish species. The amplifications were considered successful when they produce amplicons in size ranges similar to those in *H. nemurus*. In the species in which the amplification was succeeded the amplicons were observed in all individuals. The numbers of alleles and annealing temperatures (Ta) of successfully amplified primers were listed in Table 3.

In *P. siamensis*, 4 primers (*MnBP5-2-24b*, *MnRm7-1*, *MnSC4-3b* and *MnRmC8-1*) were amplified and 3 of them showed polymorphism, with *MnSC4-3b* being the monomorphic. Six loci were amplified in *Pangasius pangasius* (*MnBP5-2-24b*, *MnRmB1-2*, *MnRmC8-1*, *MnRm7-1*, *MnSC4-3b* and *MnLR2-1-21c*) and 3 showed polymorphism (*MnRmB1-2*, *MnRm7-1*, and *MnLR2-1-21C*). In *P. nasutus*, only *MnBP5-2-24b* and *MnRm7-1* produced polymorphic bands while *MnBP5-2-24b* and *MnRmC8-1* were monomorphic. For Clariid catfish, 6 loci were amplified in *C. batrachus* (*MnRmC8-1*, *MnRm7-1*, *MnSC4-3B*, *MnLR2-1-15b*, *MnLR2-1-17b* and *MnLR2-2-11*), 3 in *C. macrocephalus* (*MnRmC8-1*, *MnRm7-1*, and *MnSC4-3b*) and 5 in *C. gariepinus* (*MnBP5-2-24b*, *MnRm7-1*, *MnSC4-3b*, *MnLR2-1-15b* and *MnLR2-2-11*). Only the primer for locus *MnRm7-1* was amplified in all 7 species. Because of metaphor agarose gels high resolution performance, a single band was assumed as monomorphic.

### 2. DNA Sequence of *MnRm7-1* in 6 Catfish Species

Locus *MnRm7-1* showed amplification in all species thus selected to get the sequence information of *H. nemurus* and other 6 catfish species. The variations in sequence were observed and cross-amplified microsatellite motifs were highlighted in different colours in Fig. 1.

In *P. siamensis*, the forward flanking regions (10 bases) exhibit high similarity to the source sequences. On the other hand, the sequences from the other 5 species were different from that of *H. nemurus*. Most of them seem to have undergone substitutions. In the source species, the tandem repeat was CT with 5 repeats. The same repeats were also observed in *P. siamensis* (4 repeats), *P. nasutus* (5 repeats) and *C. macrocephalus* (5 repeats). For *P. pangasius*, the tandem repeat was TC, and the number of repeats was 8. A compound simple sequence repeat for locus *MnRm7-1* was observed in *C. batrachus* and *C. gariepinus*.

## Discussion

Primer pairs developed for the river catfish, *Hemibagrus nemurus* could be successfully amplified in the other 6 catfish species selected for this study. Out of the 30 primers selected, 9 showed promising results. In previous studies, various groups have attempted cross-species amplification in freshwater fishes<sup>22</sup>. These attempts have generally involved obtaining amplicons from species closely related to the source species. To a certain extent, the loci that can be amplified in distant taxa, such as between loci in

Table 3. Number of alleles and annealing temperature (Ta) of amplified microsatellite loci in six catfishes

Species	<i>H. nemurus</i>	<i>P. siamensis</i>	<i>P. pangasius</i>	<i>P. nasutus</i>	<i>C. batrachus</i>	<i>C. macrocephalus</i>	<i>C. gariepinus</i>
Locus	Number of alleles and Ta (°C)						
<i>MnBP5-2-24b</i>	48	(2)Tc	(1)Tc	(2)48	-	-	(1)Tc
<i>MnRmB1-2</i>	55	-	(2)48	-	-	-	-
<i>MnRmC8-1</i>	54	(2)Tc	(1)48	(1)58	(1)48	(1)Tc	-
<i>MnRm7-1</i>	48	(2)60	(3)58	(2)60	(2)Tc	(2)58	(3)Tc
<i>MnSC4-3b</i>	50	(1)Tc	(1)Tc	-	(1)Tc	(2)Tc	(2)Tc
<i>MnLR2-1-15b</i>	48	-	-	-	(1)Tc	-	(1)Tc
<i>MnLR2-1-17b</i>	48	-	-	-	(1)Tc	-	-
<i>MnLR2-1-21c</i>	54	-	(3)Tc	-	-	-	-
<i>MnLR2-2-11</i>	54	-	-	-	(4)Tc	-	(2)48

Tc is Touchdown procedure.

Number of alleles is written in bracket.

<b>&gt;HEMIBAGRUS_NEMURUS_MNRM7-1&lt;174BP&lt;&gt;(CT)5&lt;&gt;ACCESSION NUMBER AB905430&lt;</b>	
1	GGGCTTTTCC CCCAAAAATG CAGCACCTCT CTGTCTTTCC CAGACGTCTT CCTTC <u>CTCTC TCTCT</u> GTGTGTC
71	TCTCTTTTCTC TGCAGCTGAA CTGAAATGAG GTAATTAAGA GAACAGGCTC TGAGCTCCAG TACTTCCTGC
141	CTCTCCTGCT GTATGTCACC CAAACTCTGC AAGT
<b>&gt;PSEUDOMYSTUS_SIAMENSIS_MNRM7-1&lt;178BP&lt;&gt;(CT)5&lt;&gt;ACCESSION NUMBER AB905431&lt;</b>	
1	GGGCTTTTCC CATCCTATGC ABBTAATTAT GTC <u>CTCTCTC TCT</u> GTGTCTCC AGTAGTTCTG GTTCCTCCTG
71	CTGTATGTCA CCCAAACTCT GCAYGAACTG AAATGAGGTA ATTAAGAGAA CAGTCTCTGA GCTCCAGTAC
141	TTCCGGCCTC TCTGTGTGA TGTACCCAA ACTCTGCA
<b>&gt;PANGASIUS_PANGASIUS_MNRM7-1&lt;187BP&lt;&gt;(TC)8&lt;&gt;ACCESSION NUMBER AB905435&lt;</b>	
1	GGGGTTTTTG TTTCCCTNCA TCTAATGCAG GGTGTCTCT GTCTNNCCA GACGTTCTCC TTTCTCTCTC
71	GCTG <u>CTCTC TCTCTCTCTC</u> TGCAGCTGAA CAGAAATNAG GTAATTAAGA GAACAGACTC TCAGCTCCGG
141	TACTTCTGCT CTCTCCTGCT GTATGTCACC CAAACTCTGC AGGNN
<b>&gt;PANGASIUS_NASUTUS_MNRM7-1&lt;191BP&lt; (&gt;CT)5&lt;&gt;ACCESSION NUMBER AB905436&lt;</b>	
1	GGGGTTCTCT CTCCCTCTGC CTCTCCANCT CCTCTCTGTC TTTCCAGAC GTTCTCCTTC <u>CTCTCTCTCT</u>
71	GTGTCTCTCT TTCTCTGCAG CTCAACTGAA ATGAGGTAAT TAAGAGAACA GGCTCTGAGC TCCAGTACTT
141	CCTGCCTCTC CTGCTGTATG TCACCCAAAC TCTGCAGGGA GAGNNNCN
<b>&gt; CLARIAS BATRACHUS MNRM7-1&gt; 191 &lt;&gt; (CTT)5(TC)5 &lt;&gt; ACCESSION NUMBER AB905432 &lt;</b>	
1	GGGGTTTTTT GTTCTCTCC AAATCCACAG CATCCTCTCT GTCTTTCCA GACGTTCTGC <u>TTCTTCTTCT</u>
71	<u>TCTTC TCTCT CTCTC</u> TGGCT CTTTCTCTCT GCAGCTGAAC TGAATGAGG TAATTAAGAG GACAGGCTCC
141	GAGCTCCAGT ACTTCTGCTC TCTCCTGCTG TATGTCACCC AACTCTGCA N
<b>&gt; CLARIAS_MACROCEPHALUS_MNRM7-1&lt; 176BP &lt;&gt; (CT)5 &lt;&gt; ACCESSION NUMBER AB905434 &lt;</b>	
1	GGGGGCCCTT CCCCCCCAC TTCTCTGCTC TCTCTGTCT TCCAGACGT TCTCCTTC <u>CT CTCTCTCT</u> GT
71	GTCTCTCTTT CTCTGCAGCT CAACTGAAAT GAGGTAATTA AGAGAACAGG CTCTGAGCTC CAGTACTTCC
141	TGCCTCTCT GCTGTATGTC ACCCAAACCT TGCAAA
<b>&gt; CLARIAS_GARIEPINUS_MNRM7-1 &lt; 182BP &lt;&gt; (TC)10(A)10 &lt;&gt; ACCESSION NUMBER AB905433 &lt;</b>	
1	GGGTGCCCCC CCCNCCCNCC ACCCCNCCN CCTCTCTCTC NNTTCCACAC ATTCTACTTC TTCT <u>TCTCTC</u>
71	<u>TCTCTCTCTC TCTCT</u> GTCTG NCGCTCTCTC TCNCTATGTC GAAACGAAGA GGAAAAAAA AAGNNTAAAC
141	ACACCTTCTGC CAAAACCCAC CTCCCCTCTC CTNCTTTCA AA

Fig. 1. Repeat motifs in microsatellite sequences of locus *MnRm7-1* in seven selected catfish species.

fishes, can also be amplified in arthropods.

The rate of success in cross-species amplification studies is also affected by the method adopted. The most commonly used method is to use the primers developed for various source species and to attempt to cross-amplify them in one or two target species<sup>23, 24</sup>. Another method is to diversify the target species and test the primer developed from a single source species<sup>14, 15</sup>. In this study, the second method was adopted because the number of target species was 6 and the primers developed for the source species had

already been tested for cross-species amplification in other species<sup>25</sup>.

The rate of successful cross-species amplification is normally predicted based on their relatedness in phylogeny<sup>26, 27</sup>. Indeed, one theory has suggested that the more evolutionarily distant a taxon, the lower the chance of successful amplification<sup>25</sup>. A species in the same genus is generally assumed to have a greater chance of success. However, our present result did not follow the phylogenetic relationship theory. The data obtained herein suggested that the

rate of successful cross-species amplification is locus specific, and independent of the genetic distance between source species and target species. In this study, *MnRmB1-2* and *MnLR2-1-21C* showed no amplification in *Pseudamystus siamensis* belonging to the family as source species but were amplified in *P. pangasius* from the Family Pangasiidae. The same phenomenon was observed for the loci *MnLR2-1-15b*, *MnLR2-1-17b* and *MnLR2-2-11* which were amplified in *C. batrachus* but not in *P. siamensis*. These loci showed their specificity only when amplified in certain species that are relatively distant from the source species. *MnSC4-3b* showed amplifications in 5 of the 6 other catfish species in this study, with the exception being *P. nasutus*, suggesting that the locus is conserved in certain catfish and cyprinid species. However, *MnLR2-1-17b* was not amplified in 5 of the 6 catfish species, with the exception being *C. batrachus*, suggesting that this locus is species specific. The results in this study suggested that the loci developed for *H. nemurus* are locus specific and conserved among various species without regard to genetic relationship with source species. The length of the primers and the degree of conservation in that particular locus appear to have a minimal effect on the probability of amplification<sup>28)</sup>.

The results of this study also disclose that the optimum annealing temperature (Ta) of the 6 species of catfish tested differs from that of in *H. nemurus* for each primer with except in the case of *MnBP5-2-24b* (Table 3), with the Ta in *P. nasutus* being similar to the Ta in *H. nemurus*. Some successfully amplified primers in this study showed lower annealing temperature than reported in source species while the other showed amplification when using touchdown PCR protocol. *MnRm7-1* showed higher annealing temperature in some sample species than in source species. A general trend that cross-species amplification has lower annealing temperature than original amplification<sup>20, 28)</sup>.

Cross-species amplification is a method of utilising primer pairs designed for a specific species (source species) in other species (target species) which normally closely related<sup>27, 29)</sup>. However, the data of all the PCR products generated in the target species have only been analyzed based on their banding patterns<sup>29)</sup>. There have been few analyses of the

successful cross-species amplification data at the sequence level<sup>30,31)</sup>. Thus, heterozygosity cannot be compared between species.

Previous study proposed that the PCR product generated from cross-species amplification to be analyzed at the sequence level to identify the homology between source and target species<sup>31)</sup>. In this study, the primer designated for the flanking region of locus *MnRm7-1* showed amplification in 6 species. Heterozygosity could be observed in the DNA sequences among the species.

Amplification products of target species were sequenced directly to confirm their identity whether the flanking sequences and repeat motifs were conserved in length. Each species showed sequences in flanking regions with slightly differed among the species, which means the possibility that heterologous or orthologous loci were amplified. Microsatellite primer pairs that can amplify loci in non-specific species are called orthologous or heterologous primers<sup>32)</sup>. The amplification of non-homologous loci has been observed in reptilia<sup>33)</sup>. In fish, however, cross-species amplification varies, showing a tendency to amplify homologous loci in species such as flatfish<sup>34)</sup> and non-orthologous loci in catfish<sup>31)</sup>. Heterologous microsatellites have been suggested for use in genetic polymorphism tests and genetic variability evaluations<sup>35, 36)</sup>. The present analysis also disclosed that the repeat motifs are conserved in Pangasiid catfishes but not in Clariid catfishes. A notable feature is that the repeat structure of this locus was slightly different among these species: (CT)<sub>5</sub> in the source species, *P. Siamensis*, *P. nasutus* and *C. macrocephalus*, (TC)<sub>8</sub> in *P. Pangasius*, (TC)<sub>10</sub>N(A)<sub>10</sub> in *C. gariepinus* and are (CTT)<sub>5</sub> and (TC)<sub>6</sub> in *C. batrachus*. The variation among species occurs with respect to the pattern of tandem repeats and the number of repeats in microsatellite DNA. The WebSat program uses the color orange to highlight overlapping pairs of SSRs (have something in common the base T). Thus, the last base (T) of the first SSR (CTT)<sub>5</sub> is also the first base (T) of the second SSR (TC). This contradicts the idea that the rate of base substitution in aquatic organisms is lower than that in terrestrial organisms<sup>9)</sup>. The amplification products in Clariid catfish provided evidence of indels in the flanking sequences and repeat motifs.

Amplification of non-orthologous loci will lead to obvious complications in phylogenetic inference, population genetics and evolutionary studies<sup>31</sup>. Therefore, we recommend that sequence analysis of cross-amplification products be performed prior to their application, as previously suggested<sup>31</sup>.

Cross-species amplification is a method for identifying potential DNA microsatellite markers in source species to be amplified in target species. The polymorphic marker in cross-species amplification can be used in the genetic study of target species. This obviates the need for developing new DNA microsatellite markers for target species which requires high skill, consumed more labour, time and money. However, the possibility of obtaining polymorphic loci is uncertain. In this study, the results showed uncertainty in the success of cross-species amplification, with few loci exhibit polymorphism. In the future, further studies of cross-species amplification in catfish and also another species will need to be performed with more closely related species to obtain the DNA sequences of successfully amplified loci. Since

the idea of close relationship having high success rate in cross-amplification is not applicable to this study, it is suggested that the markers that showed good performance in previous cross-amplification attempts should be used in the next study. Greater numbers of samples (100 specimens for species) and larger populations should be examined using more primer pairs in the screening process to increase the likelihood of obtaining polymorphic markers. Indeed, there are shortcomings in previous researches on the cross-amplification of non-homolog loci. However, cross-species amplification is not useful for the comparison of genetic diversity and differentiation among different species. It is necessary to identify the homology of DNA sequence in microsatellite DNA regions of species and/or local strains to compare.

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## River Catfish で開発されたマイクロサテライト DNA マーカーの 他魚種への応用と増幅領域の配列比較

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マレーシアをはじめとする東南アジアにおいて重要な産業対象種である *Hemibagrus nemurus* で開発された30マイクロサテライト DNA マーカーの他の淡水ナマズ 6 種への利用の可能性を検討した。30マーカーを調べた結果、3 から 6 マーカーにおいて 6 種のいずれかにおいて増幅が確認され、そのうちのいくつかにおいては多型が観察された。*MnRm7-1* は全ての種で増幅が観察され、全ての種で多形が観察された。*MnRm7-1* において増幅断片のシーケンスを行いリピート配列とその隣接領域の配列を種間で比較した。リピート配列は Pangasiid 内では保存され *Hemibagrus nemurus* と同じモチーフであったが、Clariid 内は種間で異なっており、*Hemibagrus nemurus* と異なるモチーフであった。また、隣接領域の配列は種間で異なっており、それぞれの種で異なる変異が蓄積されていた。