Newly Developed Microsatellite Markers of *Mystus nemurus* Tested for Cross-Species Amplification in Two Distantly Related Aquacultured Catfish Species

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ABSTRACT : The work reported here is an attempt to explore the possibility of DNA microsatellite loci transfer (cross-species amplification) to other economically important aquacultured catfish species other than its source species. A total of 25 new microsatellite loci developed for riverine catfish, *Mystus nemurus* were successfully cross-amplified in two distantly related catfish species within the suborder Siluroidei. Five out of the 19 loci that successfully cross-amplified in *Pangasius micronemus* were polymorphic, while for *Clarias batrachus*, cross-amplification was successful using 17 polymorphic loci. The observed heterozygosities were high for all the three catfishes. The results indicated that microsatellite loci could be as polymorphic in non-source species as in the source species. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 11 : 1513-1518)***

Key Words : Microsatellites, Cross-species Amplification, *Mystus nemurus*, *Pangasius micronemus*, *Clarias batrachus*

INTRODUCTION

Microsatellite is highly variable due to the high mutation rates in the non-coding regions of the genome. Owing to such characteristics, microsatellite markers are highly polymorphic and powerful tools for genetic variability, conservation and even for phylogenetic studies. However, the effort for isolating microsatellites is quite tedious, laborious and time consuming since the markers are species specific (Ashley and Dow, 1994). Furthermore, the cost of developing microsatellite markers for the targeted species of interest is costly. This has posed some problems for researchers who do not have the kind of financial means for this type of work.

M. nemurus is an economic important fish among the locals in Malaysia which falls within the catfish family together with two other equally important catfish species namely *Pangasius sp.* and *Clarias sp.* (walking catfish). These catfishes are cultured by farmers in small cages, rice fields and nets for local consumption and export purposes. Thus, the objective of this study is to test the ability of microsatellite loci transfer from *M. nemurus* to other catfish species, *P. micronemus* and *C. batrachus*.

MATERIALS AND METHODS

Samples and DNA extraction

The number of specimens used for *M. nemurus* was 30, for *P. micronemus* 5 and for *C. batrachus* 9. The DNA was extracted from the skeletal muscle using the standard phenol-chloroform extraction method of Taggart et al. (1992).

Microsatellites

The microsatellites were isolated based on a library-enrichment protocol using the 5' anchored PCR technique (Fisher et al., 1996). Microsatellite primers were designed using online software named Primer 3 (Rozen and Skaletsky, 1997). A total of 25 microsatellite markers newly developed for *M. nemurus* had been tested for cross-amplification in *P. micronemus* and *C. batrachus*. The annealing temperatures for the microsatellite markers were optimised independently for the three catfish species.

PCR conditions and product visualisation

All the PCR (MJ Research, USA) reactions were performed in 10 µl volumes containing approximately 50 ng genomic DNA, 1 U of *Taq* DNA Polymerase (Promega, USA), 1.25 mM of $MgCl_2$, 10 mM Tris HCl, 50 mM KCl, 0.1% Triton X-100, 0.25 mM of each nucleotide and 50 pmol of forward and reverse primers. The general PCR (MJ Research, USA) profile consisted of 40 cycles of 94°C for 30 s, 30 s at an annealing temperature specific for each primer pair (Table 1) and 68°C for 40 s. Before the cycles began there was 3 min incubation at 95°C and upon completion of the cycles 5 min incubation at 68°C was performed. Since some of the primers gave problems during amplification, a touch down PCR (MJ Research, USA) profile with either 2 or 3 different annealing temperatures were used. The PCR (MJ Research, USA) profile with 2 different annealing temperatures consisted of 8 and 30 cycles for the first and second annealing temperatures, respectively. For the PCR (MJ Research, USA) profiles

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Table 1. i) Microsatellite loci developed for *Mystus nemurus* which cross-amplify in two related freshwater catfish species

Locus	Repeat motif	Primers sequences (5'to 3')	*Mystus nemurus*		*Pangasius micronemus*		*Clarias batrachus*	
			T_a (°C)	Status[#]	T_a (°C)	Status[#]	T_a (°C)	Status[#]
MnLR2-1-15B	$(GA)_5$, $(AG)_6$	F: TACGGAATCCGAGGTCACTC R: AAGCGGGCGGCTCTCTCT	52/50	M	52	P	52	P
MnLR2-1-17B	$(TG)_{11}(AG)_{12}$	F: GCAGTTTCCTTCTCTTCACT R: GGGGGGCGCGCAACTCTCTC	50	P	50	M	50	M*
MnLR2-1-21A	$(CT)_8$	F: GGAAAGGGCGAGGCTCTC R: GTCGAGGGTGAAGAGGGAAG	48/47/46	P	56	M*	50	P*
MnLR2-1-21B	$(T)_8$, $(GA)_{16}$	F: TCACCCTCGACAGCTATCATC R: TGAGGACACGCGACTCTCTC	50	P	50	M	No amplification	
MnLR2-1-21C	$(CT)_6$, $(GT)_6$, $(GT)_4CTCT(GT)_6$	F: GGAAAGGGGAGGCTCTCTCT R: AGCTCAATAAGGTGCCATGC	48	P	50	M	48	P
MnLR2-1-21D	$(GT)_{11}(GA)_{12}$	F: TCTCTGTGTGTGTGTGCTG R: CGCCGGGCTCTCTCTCTC	50	M	No amplification		50	P
MnLR2-1-24C	$(TG)_4$, $(TG)_4CGCG(AG)_6$	F: TATTATGAACGTCGCGCTTG R: AGGCGCAGGGCTCTCTCT	48	P	48	M	No amplification	
MnLR2-1-52A	$(GA)_{22}$, $(T)_8$	F: TCCCCTTTTTATTGCCATTC R: GGAACGAGGAGGGCTCTCTCT	52/50	M	50	M	50	P
MnLR2-2-11	$(CT)_9$, $(T)_9$, $(A)_8T(GA)_6$	F: GGAAGGCGCGAGGCTC R: GGGAGAAGGGCCTCTC	48	P	48	M	48	P
MnB10-2-17	$(CTTTC)_2$, $(GA)_{26}$	F: CGGATACGTGTTGCTTTC R: GCTCCTGTGCGCGGCTCT	48/47/46	P	48	M*	48	P
MnSC3-15B	$(GGGGT)_2$, $(GGAG)_2$, $(GAC)_4$	F: TCCCTTTGTTGGAGTTAGGG R: GGAGGAAAAACCACAGAGTC	48/47/46	M	48	P*	46	P*

T_a: Locus specific annealing temperature in °C.

[#] Status of the amplified alleles: P = Polymorphic; M = Monomorphic.

* Occurrence of null alleles in the samples tested.

with 3 different annealing temperatures, the first two were done with 6 cycles each and 28 cycles were used for the third annealing temperature. The PCR products were run on 4% MetaPhor® agarose (FMC, USA) gel followed by ethidium bromide staining.

Statistical analysis

The calculation of heterozygosity values was performed by using computer software, PopGene 1.32 (Yeh and Boyle, 1997).

RESULTS AND DISCUSSION

In this study, microsatellites isolated for *M. nemurus* had been successfully cross-amplified in two distantly related catfish species. The successful cross-amplification of homologous microsatellites loci in species other than the targeted species showed a promising alternative for the problem mentioned above. Four microsatellite loci that gave unclear banding patterns in *M. nemurus* were excluded from band scoring (Table 1). Out of the 25 microsatellite markers tested, a total of 19 microsatellite markers were successfully amplified in *P. micronemus* with five of them showing polymorphisms (26.3%), while for *C. batrachus*, 17 out of the 18 microsatellite markers that were successfully amplified showed polymorphisms (94.4%). The high levels of polymorphisms indicated that microsatellite loci transferred well to other related species. This shows the microsatellite loci are as useful as they are in the source species since only nine individuals was sufficient for detecting polymorphisms in *C. batrachus*. Although the level of polymorphisms in *P. micronemus* was not as high as in its relative, increasing the number of samples will possibly increase the chances of getting more polymorphic microsatellite markers. This is in agreement with Sun et al. (2005) that detected polymorphisms in 49 samples of goats using microsatellite markers developed for sheep. The observed heterozygosity values for a microsatellite locus in the three catfish species were taken as an indirect measure for determining the polymorphism level for a particular locus. The highest observed heterozygosity in *M. nemurus* was 0.80 with the maximum number of alleles per locus being eight (Table 2). The maximum number of alleles per locus in *P. micronemus* and *C. batrachus* was three and six with observed heterozygosity of 0.75 and 1.00, respectively. The microsatellite markers that are capable of showing high heterozygosity values in non-source species should be useful in any kind of genetic variability and phylogenetic studies.

In the early years of the application of microsatellite

Table 1. ii) Microsatellite loci developed for *Mystus nemurus* which cross-amplify in two related freshwater catfish species

Locus	Repeat motif	Primers sequences (5' to 3')	*Mystus nemurus*		*Pangasius micronemus*		*Clarias batrachus*	
			T_a (°C)	Status[#]	T_a (°C)	Status[#]	T_a (°C)	Status[#]
MnSC4-1A	$(CCA)_7$	F: GCCAGCAACAAGGGGCCA R: CCTTGGATCGGAACTGGTC	46	P	46	M	No amplification	
MnRm7-1	$(CT)_3(GT)_2CTGT(CT)_4$	F: TTTTTCTCTCGCTGTCTCTC R: GCAGAGTTTGGGTGACATAC	52/50	P	56	P	50	P
MnRm27-1	$(GA)_{11}N_6(GA)_4GGG(GA)_4$	F: AGTTAGGGCTAAGCATTTCC R: GTATCTATGTGGTCGTGTGC	Unclear amplification		48	M	No amplification	
MnRmA6-2	$(CT)_4TTCTCTTT(CT)_{14}$	F: TTTTTACGCATTTGTTTGTG R: GGTTTTGAGAGAGAGAGAGAG	48	P	No amplification		48	P*
MnRmB1-1	$(ATG)_8$	F: TGTCCGAATGATGATGATGAT R: AAATTGCATGCTATAAACACTG	Unclear amplification		No amplification		56	P*
MnRmB1-2	$(CAT)_6$	F: TTAGCTGACAGGATGCACTG R: GTTCCGTATGATGATGATGATG	52/50	M	56	M*	56	P*
MnRmB11-1	$(CAT)_8CTCCT(CT)_3N_8(CAT)_{13}$	F: CAGCCTGAAAATGGCAAATC R: GGCTGGAATGATGATGATG	56/55/54	P	56	P	No amplification	
MnRmC3-1	$(TG)_{10}$	F: AGTGCGAGGTGTGTGTGTG R: GGTGGACCAGTGCCTCTAGT	48/47/46	P	52/50	M	No amplification	
MnRmC5-1	$(GT)_9$	F: TGGGAGACTGTGTGTGTGTG R: GATCTCGATGGCCTTCTTAGC	52/50	M	No amplification		50	P
MnRmC8-1	$(GT)_8$	F: TGTGCGCGATGTGTGTGT R: GAAACTGCTGGTTTTGTCAGC	48/46	M	56	M	48	P
MnRmCT6-1	$(CT)_{10}$	F: GAAGGTCGGCGCGGCTCT R: CGTAAAGGGGATGAAGGTTG	Unclear amplification		No amplification		52/50	P
MnRmD9-1	$(GATA)_6(GA)_{19}$	F: GTGCGCGGATAGATAGATAG R: GACGGAAAGACAGACTTGCAC	48/47/46	P	No amplification		48	P
MnRmD11-1	$(CT)_{25}$	F: GATCCCGAAAGAAATTCCA R: GTTAGCGGATAGATAGATAG	48	P	46	P	52/50	P*
MnRmR6-2	$(GA)_9$	F: CTACTTTGGCACACCGACCT R: TGACTCTTTCGGAGATCTCTCT	Unclear amplification		56	M	No amplification	

T_a: Locus specific annealing temperature in °C.

[#] Status of the amplified alleles: P = Polymorphic; M = Monomorphic.

* Occurrence of null alleles in the samples tested.

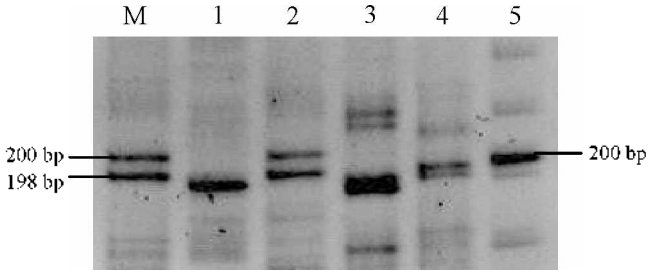

Figure 1. Microsatellite banding profile of cross-species amplification samples from *P. micronemus* using primer pair MnRmB11-1. Lane M: 20 bp DNA ladder; lanes 1-5: microsatellite profiles of samples from *P. micronemus*.

markers in genetic studies. it was believed that the markers are species specific (Ashley and Dow, 1994). Microsatellite markers developed for the species of interest was considered not applicable to other related species. However, this belief was not absolutely true in cases reported by some studies which showed that the markers were useful across different genera. families and even orders. It was reported that a small percentage of chicken microsatellite markers and a large percentage of turkey microsatellite markers were able to cross-amplify pheasant DNA by Baratti et al. (2001). Koskinen and Primmer (1999) in their study reported that microsatellite markers developed for salmonid fish were polymorphic in two *Thymallus sp.* which belong to a family from *Salmo sp.* Apart from animals. microsatellite markers in plant such as *Glycine max* was also reported to cross-amplify in other closely related species in the same genus (Hempel and Peakall, 2003).

Studies by FitzSimmons et al. (1995) and Rico et al. (1996) revealed that the ability for microsatellite loci transfer to other related species was due to the unusual high conservation of the unique sequences flanking the microsatellite region. Based on the studies carried out on turtles and fish it was suggested that aquatic organisms evolved at a slower rate than land animals (FitzSimmons et al.. 1995: Rico et al., 1996). Due to this. microsatellite loci have higher chances of successful cross-species amplification across higher taxa in aquatic species. The

Table 2. i) Amplification for *Mystus nemurus* and the two related freshwater catfish species. The number of alleles observed at each locus is reported in parentheses with observed and expected heterozygosity values (H_O: Observed heterozygosity; H_E: Expected heterozygosity)

Locus		*Mystus nemurus*	*Pangasius micronemus*	*Clarias batrachus*
MnLR2-1-15B	Allele size (bp)	110 (1)	230-260 (2)	140-200 (4)
	H_O	0.0000	0.5000	0.5556
	H_E	0.0000	0.4286	0.6993
MnLR2-1-17B	Allele size (bp)	115-170 (7)	250 (1)	105 (1)
	H_O	0.6000	0.0000	0.0000
	H_E	0.7910	0.0000	0.0000
MnLR2-1-21A	Allele size (bp)	190-215 (2)	225 (1)	200-255 (3)
	H_O	0.6000	0.0000	0.0000
	H_E	0.4308	0.0000	0.5455
MnLR2-1-21B	Allele size (bp)	190-225 (8)	205 (1)	-
	H_O	0.3684	0.0000	-
	H_E	0.8393	0.0000	-
MnLR2-1-21C	Allele size (bp)	180-185 (2)	190 (1)	165-230 (3)
	H_O	0.0000	0.0000	0.1111
	H_E	0.2032	0.0000	0.5425
MnLR2-1-21D	Allele size (bp)	115 (1)	-	125-150 (3)
	H_O	0.0000	-	1.0000
	H_E	0.0000	-	0.6209
MnLR2-1-24C	Allele size (bp)	280-300 (2)	280 (1)	-
	H_O	0.2667	0.0000	-
	H_E	0.2350	0.0000	-
MnLR2-1-52A	Allele size (bp)	145 (1)	180 (1)	140-190 (6)
	H_O	0.0000	0.0000	0.6667
	H_E	0.0000	0.0000	0.8627
MnLR2-2-11	Allele size (bp)	265-280 (2)	280 (1)	145-150 (2)
	H_O	0.8000	0.0000	0.1111
	H_E	0.4881	0.0000	0.2941
MnB10-2-17	Allele size (bp)	320-340 (2)	260 (1)	200-280 (4)
	H_O	0.5000	0.0000	0.4444
	H_E	0.4308	0.0000	0.6797
MnSC3-15B	Allele size (bp)	165 (1)	140-164 (3)	140-165 (3)
	H_O	0.0000	0.7500	0.2500
	H_E	0.0000	0.6071	0.2417
MnSC4-1A	Allele size (bp)	200-220 (3)	310 (1)	-
	H_O	0.2000	0.0000	-
	H_E	0.5712	0.0000	-
MnRm7-1	Allele size (bp)	195-200 (2)	190-195 (2)	205-225 (5)
	H_O	0.0333	0.0000	0.7778
	H_E	0.0333	0.5333	0.7712

results of this study agreed well with that suggestion because the three catfish species we studied belong to different families in the same suborder, Siluroidei. The three different families across the taxa are Bagridae for *M. nemurus*, Schilbeidae for *P. micronemus* and Clariidae for *C. batrachus*.

Microsatellite loci transfer to other non-source species serve as an alternative approach to study organisms without having to go through the technically demanding process of microsatellite isolation. Besides, it will save a great deal of time and money. Most of the known disadvantages of microsatellite which had been mentioned above are solved by microsatellite loci transfer system. However, there is still a drawback in microsatellite which is the occurrence of null alleles in source and non-source species.

The reported microsatellite markers can be further tested on any species of interest within the suborder Siluroidei if isolation of microsatellites is not an option.

Table 2. ii) Amplification for *Mystus nemurus* and the two related freshwater catfish species. The number of alleles observed at each locus is reported in parentheses with observed and expected heterozygosity values (H_O: Observed heterozygosity; H_E: Expected heterozygosity)

Locus		*Mystus nemurus*	*Pangasius micronemus*	*Clarias batrachus*
MnRm27-1	Allele size (bp)	-	150 (1)	-
	H_O	-	0.0000	-
	H_E	-	0.0000	-
MnRmA6-2	Allele size (bp)	158-165 (4)	-	180-190 (2)
	H_O	0.0000	-	0.7143
	H_E	0.7270	-	0.4945
MnRmB1-1	Allele size (bp)	-	-	90-115 (5)
	H_O	-	-	0.5000
	H_E	-	-	0.7500
MnRmB1-2	Allele size (bp)	160 (1)	230 (1)	150-165 (4)
	H_O	0.0000	0.0000	0.2000
	H_E	0.0000	0.0000	0.7778
MnRmB11-1	Allele size (bp)	200-240 (7)	195-200 (3)	-
	H_O	0.6538	0.2000	-
	H_E	0.6621	0.7333	-
MnRmC3-1	Allele size (bp)	245 (1)	240 (1)	-
	H_O	0.0000	0.0000	-
	H_E	0.0000	0.0000	-
MnRmC5-1	Allele size (bp)	130 (1)	-	130-145 (3)
	H_O	0.0000	-	0.5556
	H_E	0.0000	-	0.6209
MnRmC8-1	Allele size (bp)	280 (1)	270 (1)	85-100 (3)
	H_O	0.0000	0.0000	0.0000
	H_E	0.0000	0.0000	0.3922
MnRmCT6-1	Allele size (bp)	-	-	125-165 (4)
	H_O	-	-	0.5556
	H_E	-	-	0.6993
MnRmD9-1	Allele size (bp)	145-170 (5)	-	120-180 (5)
	H_O	0.1538	-	0.2222
	H_E	0.4005	-	0.6209
MnRmD11-1	Allele size (bp)	200-240 (5)	180-185 (2)	180-200 (2)
	H_O	0.5000	0.2000	0.6667
	H_E	0.7598	0.2000	0.5333
MnRmR6-2	Allele size (bp)	-	145 (1)	-
	H_O	-	0.0000	-
	H_E	-	0.0000	-

However, further optimisations of annealing temperatures and PCR profiles may be necessary for each species in order to get reliable PCR amplifications.

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