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Isolation and Development of DNA Microsatellite Markers for the River Catfish (*Mystus nemurus*)

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Abstract

The microsatellite markers for *Mystus nemurus* were isolated using the 5' anchored PCR procedure. A degenerate primer was designed for the construction of the genomic library. Fourteen clones were sequenced and revealed a total of 45 microsatellite repeats. From these, 20 specific primer pairs were designed and employed to characterize unrelated fishes from six populations of *M. nemurus*. Thirteen loci were found to be polymorphic. The allelic frequencies ranged from 0.007 to 0.800, while the levels of heterozygosity ranged from 0.0563 (MnBp5-1-115b) to 0.8714 (MnBp5-1-12a).

Introduction

The river catfish (*Mystus nemurus*) is one of the most economically important indigenous freshwater fish in Malaysia and it has gained popularity among consumers in the country, as well as in the Southeast Asian region. Its good flesh quality and high nutritional value contributes to its high market price compared to other freshwater fish species in the country.

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However, commercialization and intensive farming of this species are still not widely established. Genetic information on *M. nemurus* is rather limited (Siraj et al. 1998; Chong et al. 2000; Leesanga et al. 2000; Usmani et al. 2001). Due to its inability to breed in captivity, the supply of *M. nemurus* is largely from the wild. Hence, finding ways to increase production efficiency through genetic studies should be the immediate goal of the local river catfish aquaculture industry.

The ultimate goal of aquaculture breeding program is to improve or alter economically important quantitative traits in a predetermined way. In order to achieve this, it is important to maintain the genetic variation present within and between stock populations, as poor breeding programs result in the loss of genetic variation of the cultured livestock (Taniguchi 2003). Genetic linkage mapping plays an important role in selection programs through the use of Marker Assisted Selection (MAS), or isolation of economically important genes.

Microsatellite markers have become the markers of choice for the study of various genetic problems. The popularity of these markers is mainly due to their presence in all the prokaryotic and eukaryotic genomes studied to date, besides being specific, codominant, highly polymorphic and easy to score. These have led them to be more informative than dominant markers such as RAPD and AFLP. Hence, microsatellite markers have proven to be tremendously powerful tools for constructing genetic linkage maps in many organisms (Gates et al. 1999; Waldbieser et al. 2001).

The isolation of microsatellite markers, however, has been the major drawback as prior genome sequences of any newly studied species are often necessary. Furthermore, their development is time consuming, laborious, expensive, and result in extremely low yields (Zane et al. 2002). Nonetheless, many of these problems are solved through the application of several enrichment protocols (Ender et al. 1996; Fisher et al. 1996). Several microsatelllite markers for *M. nemurus* have been reported earlier by Usmani et al. (2001). However, with the limited numbers of microsatellite markers available, a meaningful linkage map of this species could not currently be generated. Hence in this paper, another set of novel microsatellite markers, which were isolated and developed by using an enrichment protocol, namely 5' anchored PCR (Fisher et al. 1996) are reported.

Materials and Methods

Sample collection and DNA extraction

Samples of *M. nemurus*, each consisting of 15 individuals (minimum weight of 300g per fish) were collected from six locations in Peninsular Malaysia namely, Kedah, Perak, Selangor (hybrid), Johor, Terengganu and Sarawak (Fig. 1). Genomic DNA was extracted either from whole blood or muscle tissue based on the protocol of Taggart et al. (1992). The isolated DNA samples were quantified on agarose gel by running them alongside with known quantities of λ DNA. They were then diluted to similar concentrations (~50 ng μl^{-1}) to be used in the PCR reactions.

Fig. 1. Map of Malaysia indicating the locations of the sampling sites. The exact locations are indicated with red dots. Map was edited from www.lib.utexas.edu/maps

Construction of enriched genomic libraries

A degenerate primer was designed which contains the $(AG)_6$ microsatellite motif at the 3' end and three or more non-repetitive bases at the 5' end, as defined by Fisher et al. (1996), BP5: 5' NNNYYBMBMBMB$(AG)_6$ 3'; where N= A/C/G/T, S= G/C, Y= T/C, M= A/C and B= G/T/C, according to the IUB Code. The Ns were included into the 5'end in order to capture longer anchor sequences which allow the capture of more flanking sequences. This helps in designing the primer pairs flanking the microsatellite regions. The PCR amplifications were carried out in a total volume of 20 µl containing ~50 ng of template DNA, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 units of *Taq* DNA polymerase (Promega, USA), 50 ρmol of the degenerate primer, 0.2 mM each of the dNTPs (Promega, USA) and ddH_2O. The amplification

profiles consisted of 96°C (3 min), 40 cycles of 96°C (30 s)/57°C (20 s) and 72°C (30 s) and a final extension of 72°C (5 min). The PCR products were electrophoresed on 2% agarose gels in 1X TBE buffer to confirm the presence of bands.

Cloning, plasmid extraction and sequencing

The resultant products were cloned into the TOPO TA Cloning vector according to the manufacturer's instructions (TOPO TA Cloning Kit, Invitrogen, USA). Plasmids were isolated from positive clones of transformed *E. coli* cells using the method of Sambrook et al. (1989), and sequenced using the BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, USA). Sequences were resolved on the ABI PRISM 377 DNA Sequencer. Plasmids were sequenced twice in a single direction (5' – 3') to confirm the sequence. Vector sequences in the DNA sequences obtained were then removed and the true sequences were later submitted to the GenBank.

PCR amplification and product electrophoresis

Primer pairs were designed to amplify regions containing microsatellite repeats by using PRIMER 3 (Rozen and Skaletsky 1997) with the following criteria: the length of the primer pairs were designed to be within the range of 17 bp – 22 bp; possess GC contents of 40% - 60%; annealing temperature between 41°C – 60°C (optimum 55°C); and results in amplicons within the range of 150 to 300 bp.

Evaluations for polymorphisms and population characterization

The PCR amplification was performed in 10 µl total reaction volume containing 30 ng of genomic DNA, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100, 0.25 mM of each dNTPs, 10ρmols of each forward and reverse primers, and 1 unit *Taq* DNA polymerase (Promega, USA). The PCR conditions were as follows: predenaturation for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C (30 s), at the appropriate optimum annealing temperature (30 s), extension at 72°C (30 s) and a final extension at 72°C for 5 min. The PCR products were run on 4% Metaphor Agarose (BMA, USA) gel and visualized with UV light after ethidium bromide staining. In order to validate the banding patterns obtained, the selected PCR product was then run on an 8% non-denaturing polyacrylamide gel.

Data analysis

Statistical analysis was done using the POPGENE (ver 1.32) computer software (Yeh and Boyle 1997).

Results

Isolation of microsatellite loci

A strong background smear was produced even after a series of optimizations for the degenerate primer, BP5 (Fig.2). Nonetheless, construction of the genomic library was able to proceed. Of these, the 14 plasmids selected for sequencing successfully revealed a total of 45 microsatellites. Each clone contained terminal microsatellites at the 5' and 3' ends, except for MnBp5-1-05, MnBp5-1-02, MnBp5-1-115, MnBp5-2-27, MnBp5-1-30, and MnBp5-2-13 (Table 1). In these plasmids, the insert sequences were too long to be detected by a single direction automated sequencing. Each insert contained at least two microsatellite loci but an additional 17 inter-terminal microsatellite repeats was also obtained. The inserts obtained contained the expected GA/CT microsatellite repeats with the lengths ranging from 8 to 207 bp. The longest microsatellite sequence of 207 bp was isolated successfully from MnBp5-2-27, while MnBp5-2-05 contained four inter-terminal repeats.

Characterization of microsatellite markers

Fig. 2. Electrophoresis of the PCR products of BP5 (Lane 2). Lane M, 100 bp DNA ladder.

Of the 16 designed primer pairs, three were monomorphic (not reported) while the remaining 13 were found to be polymorphic in samples from the six populations characterized in this study (Table 2). The number of alleles per locus ranged from 3 (MnBp5-1-05b and MnBp5-2-05b) to 12 (MnBp5-2-02b), with an average of 6.23 alleles per locus. The allelic variations at one of these microsatellite loci are shown in figure 2. The heterozygosities were calculated to evaluate the variations across the 13 polymorphic markers. Locus MnBp5-1-12a revealed the maximum mean observed heterozygosity (H_o = 0.8714) while locus MnBp5-1-115b showed the minimum value (H_o = 0.0563).

Discussion

Isolation of microsatellite loci

The strong smearing background obtained for primer BP5 indicated that the presence of the GA/CT repeats is common in the *M. nemurus* genome. Several reasons supported this explanation. First, PCR amplification by this primer produced a distinct band in other taxa (e.g. green lipped mussels, Teh et al. 2002). Secondly, the microsatellite repeat lengths obtained varied in the number of repeats (8 to 207 bp). Thirdly, the plasmid DNA sequenced revealed a 100% success rate of producing unique microsatellite repeat sequences. In addition, previous findings had shown that dinucleotide motifs appeared frequently in fish genomes (Zane et al. 2002). The rapid method described in this study significantly reduced the time, effort and cost of microsatellite isolation. It requires minimum PCR optimization, yet produced promising results. While in the previously reported studies, the highest success rate of microsatellite isolation in fish was 8.92% (Zane et al. 2002), this study obtained an almost three fold increase of the total clones sequenced. Due to the effectiveness of this assay, colony hybridization, which usually requires the use of radio-isotopes was not necessary in this technique. However, there is a drawback to this technique, that is one may find it difficult to design specific primers flanking the microsatellite regions, due to the limited sequences available at the 5' or 3' terminal. Designing primers which are anchored on the repeat itself often lead to certain problems such as null alleles and mispriming. This problem may be resolved by inverse PCR (Shibata et al. 2003; Newton et al. 1997), a technique involving the amplification of the sequences that lie outside the boundaries of the known sequences. Comparisons on the microsatellite repeat unit lengths obtained were made with those from previous studies, and it was found that the lengths obtained in this study was similar to those previously reported for *M. nemurus*. Usmani (2002) found the longest perfect dinucleotide stretch to be 22 repeats while Chan (2003) found a GA microsatellite with 26 repeat units. In this study, the longest perfect dinucleotide repeat isolated is $(GA)_{24}$. The repeat unit length may be influenced by the mutation rate, different constraints on the maximum repeat unit length due to selection (reviewed in Ellegren 2000) or a bias from library screening (Schug et al. 2004). It is believed that the longer repeat unit lengths relative to the other catfish species (Sukmanomon et al.

Table 1. Microsatellite sequences identified in *M. nemurus* and their GenBank accession numbers

Clone	5' terminal repeats	Inter-terminal repeats	3' terminal repeats	GenBank Accession no.
MnBp5-1-05	$(CT)_7$	$(CA)_4$	-	AY205990
MnBp5-1-12	$(GA)_9AA(GA)_4$	$(GT)_4$	$(CT)_6$	AY205992
MnBp5-1-10	$(GA)_{10}$	-	$(GT)_4$	AY205993
MnBp5-1-02	$(GA)_{23}$	$(TG)_{11}$	$(CT)_9$	AF526561
MnBp5-1-115	$(GA)_8$	$(GT)_4$; $(GGTTA)_3$	-	AF544042
MnBp5-2-5	$(GA)_6$	$(GACA)_3$; $(GGAT)_4$; $(GGAT)_3$; $(TGGA)_4$	-	AY207448
MnBp5-2-6	$(GA)_9$	$(CAT)_7$	$(CT)_2GA(CT)_8$	AY207451
MnBp5-2-24	$(GA)_9$	$(AC)_4$; $(GA)_4TG(GA)_5$; $(GAAA)_2(GA)_6$	$(CT)_9$	AY207450
MnBp5-2-27	*Complex $(GA)_n$ microsatellite repeat* *	$(GT)_4$; $(CT)_4TT(CT)_4$; $(CT)_4CA(CT)_3N_{12}$ $(CT)_5$	-	AY207449
MnBp5-2-02	$(GA)_{10}N_5(GA)_5$	$(CA)_{10}$; $(A)_{11}N_5(A)_8(GA)_5$	$(CT)_9$	AF205994
MnBp5-1-30	$(GA)_8$	$(TG)_4$	-	AY852259
MnBp5-2-49	$(GA)_7(GGA)_2GAGGA$	-	$(CT)_6$	AY852258
MnBp5-2-38	$(GA)_7$	$(CAAA)_5GAACAAA$; $(TAAA)_3$; $(GAT)_2N_9(GAT)_3$	$(CT)_3CC(CT)_2CC(CT)_8N_2(CT)_2$	AY852257
MnBp5-2-13	$(GA)_9$	$(AT)_5$	-	AY804209

*Mnbp5-2-27:
$(GA)_8(CAGA)_2(CA)_3GACA(CAGA)_5(GA)_5(CAGA)_5CA(CAGA)_2(GA)_2GATA(GACA)_2(GA)_2CA(GA)_4$ $CA(GA)_2CA(CAGA)_2(GA)_5(GACA)_3(GA)_8GCA(AG)_4AC(AG)_8$

2003) may indicate a bias protocol being used for identifying microsatellites. The enrichment protocols are likely to be biased against the selection of microsatellites because of the nature of the hybridization reaction. In the conventional method, which involves colony hybridization, plasmids within the colonies that contain longer inserts usually produce a more intense signal on the membrane or X-ray film (Schug et al. 2004). Because of the chances of getting false signals, one would normally sequence colo-

Table 2. Characterization of 13 microsatellite loci in *M. nemurus*

Locus	Repeat motif	Primer sequence (5' – 3')	Exp. allele sizes (bp)	$MgCl_2$ (mM)	T_a (^oC)	N_a	H_o (H_e)
MnBp5-1-05a	$(CT)_7$	F: AACACACTCT CTCTCTC R: CCCTGGCTCTC CTCTACAAA	194	2.5	55	3	0.1733 (0.5090)
MnBp5-1-12a	$(GA)_9AA(GA)_4$	F: AGTTTGCCCG AGAGAGAG R: TTTTTGGAAA GCGGGATCTA	207	1.25	55	6	0.8714 (0.7487)
MnBp5-1-10b	$(GT)_4$	F: CCGGCAGAAC TAGGAGTGTC R: CTGTGTGAAC GCTTAAAGTC AA	231	2.5	55	5	0.2133 (0.4435)
MnBp5-1-02a	$(GA)_{23}$	F: GGTTTGAGCG CGAGAGAG R: GATTAGGGCC CGTGTCTAGC	196	1.25	50	7	0.2301 (0.7515)
MnBp5-1-115b	$(GT)_4$; $(GGTTA)_3$	F: TTTTGCTACTA GAGAGACTGA C R: TAGGCAAAAC GTGTACTTG	179	1.25	60	4	0.0563 (0.4782)
MnBp5-2-05b	$(GACA)_3$	F: CAAGTGCAAA GACAGACAGA R: TCTCTAAGGC TATCCATCCA	188	2.5	61	3	0.4800 (0.5962)
MnBp5-2-06b	$(CAT)_7$	F: CGTGTCCAGA CATGGTTAAT R: GAGTGGGCGA CTTTCAG	164	2.5	55	6	0.2566 (0.7294)

Table 2. Characterization of 13 microsatellite loci in *M. nemurus* (cont'd.)

Locus	Repeat motif	Primer sequence (5' – 3')	Exp. allele sizes (bp)	$MgCl_2$ (mM)	T_a (^oC)	N_a	H_o (H_e)
MnBp5-2-24b	$(GAAA)_2$ $(GA)_6$	F: GTCATATTTGC TTTGGCAGT R: GTGGTTTTGA ATGTTCTCTG	159	2.5	55	5	0.0778 (0.5512)
MnBp5-2-27b	$(GT)_4$; $(CT)_4TT(CT)_4$	F: TTATAACAGG GGAGTGAAGG R: GATGTCATCA AGTGGCAGT	270	2.5	55	4	0.1250 (0.3787)
MnBp5-2-02b	$(CA)_{10}$; $(A)_{11}N_5(A)_8(GA)_5$	F: ACACCAAAGA GATGACCATT R: TCTCTGTGAA ACGCTTCTTT	211	2.5	55	12	0.3103 (0.7946)
MnBp5-2-49a	$(GA)_7(GGA)_2GAGGA$	F: TTCTAGCGAG AGAGAGAG R: AGTTTTAAGT CTTGCGTGGA	229	2.5	55	5	0.2083 (0.4715)
MnBp5-2-38b	$(GAT)_2N_9(GAT)_3$	F: GGTTCATGCT GCTGTTTGTA R: GGCTGTTACA GTAAAATACA CG	189	2.5	55	10	0.1781 (0.7429)
MnBp5-2-13a	$(GA)_9$	F: TCCCCGAGCG AGAGAGA R: TCTGCAAGCC CTTTATAGAC	244	2.5	55	11	0.2778 (0.7896)

*N_a, Number of alleles; †H_o, Observed heterozygosity; H_e, Expected heterozygosity

nies with the most intense signals, which contain fragments with the longest microsatellites in the pool of putative microsatellite clones. In contrast, the enriched library particularly the one used in this study, provided clones

that were selected at random prior to any colony hybridization. Thus, there is no priority to any colony containing plasmids with microsatellites to be sequenced. By the nature of these differences, the enriched library protocol seems to produce shorter microsatellite repeats than the conventional protocols.

Juxtaposed microsatellite systems (JMS)

A Juxtaposed Microsatellite System (JMS) is defined as two microsatellite repeat arrays, separated by a flanking sequence of less than 200 bp (Estoup et al. 1999). This system is said to be useful in estimating the genetic admixture of two populations which are diverged from an ancestral population and came back into contact due to several environmental factors such as human action via stocking and introduction programs. Numerous studies on fish (*S. trutta*; Giuffra et al. 1996), insects (*A. meiiifera*; Lobo et al. 1989) and mammals (*Canis simensis*; Gotelli et al. 1994) have been reported. Although both *in situ* hybridization and genetic mapping have revealed a relatively even distribution of microsatellites over chromosomes, the frequent association of several microsatellites sequences in the same cloned insert is indicative of a clustering of microsatellites (Dietrich et al. 1994; Estoup et al. 1993). Estoup et al. (1999) had stated that a direct and essential screen for JMS would be the 5' anchored PCR technique. The advantage of a JMS for admixture study stems from the superior genealogical information carried by the two microsatellite sites to that carried by just one. Moreover, information regarding sharing or non sharing an allele between the alien and the admixed native population obtained at one microsatellite site is complemented by the sharing or non sharing of information at the second neighboring microsatellite site.

Characterization of the microsatellite markers

A comparison has been made between the metaphor gel electrophoresis and 8% non-denaturing polyacrylamide gel electrophoresis, and it was found that similar banding patterns were observed. Such comparisons had also been done in other studies (Bhassu 2002; Usmani 2002) with similar results. The percentage of polymorphic markers were detected was relatively high when compared to those of a previous study by Chan (2003). In the present study, 13 polymorphic loci revealed a total of 81 alleles in 90 unrelated fishes (average of 6.23 alleles per locus). This result was similar to the findings of Usmani (2002), who used 20 microsatellite markers and obtained 126 alleles (average 6.3 alleles per locus). This is in sharp contrast with the low level of alleles for isozyme markers (Na-Nakorn et al. 2004; Siraj et al. 1998), indicating the better sensitivity of

microsatellites for studying populations and the construction of genetic linkage maps.

The higher number of alleles in the loci could be attributed to high mutation rate which occurs at a particular locus or the loci undergoing selection, resulting from non-random mating.

The level of heterozygosities were in agreement with Chan (2003) and Usmani et al. (2003) but were found to be lower compared to other species (Rutten et al. 2004; Bhassu et al. 2004; So et al. 2006). Lower level of heterozygosities in general suggest the occurrence of unequal allele frequencies across the range, the presence of null alleles (O'Connell and Wright 1997), or the possibility of sampling error due to the natural shoaling behavior of the fish (Castric et al. 2002; Brown and Brown 1996; Pouyaud et al. 1999).

Conclusion

The findings of this study indicate the usefulness of the procedure that we used in identifying microsatellite loci in a much simpler and rapid way. These markers will eventually be used in the construction of a genetic linkage map, and in testing for associations with Quantitative Trait Loci (QTL) so that markers assisted selection (MAS) may become feasible in breeding programs of *M. nemurus*.

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References

Bhassu, S. 2002. Applications of DNA microsatellite markers in tilapia culture. PhD Dissertation, Universiti Putra Malaysia.

Bhassu, S., Yusoff, K., Panandam, J. M., Embong, W. K., Oyyan, S., Tan, S. G. 2004. The genetic structure of *Oreochromis spp*. (Tilapia) populations in Malaysia as revealed by microsatellite DNA analysis. Biochemical Genetics, 42:217-229.

Brown, G.E. and Brown G.A. 1996. Does kin-biased territorial behavior increase kin-biased foraging in juvenile salmonids? Behavior Ecology, 7:24-29

Castric, V., Bernatchez, L., Belkhir, K. and Bonhomme. F. 2002. Heterozygote deficiencies in small lacustrine population of brook charr *Salvelinus fontinalis* Mitchill (Pisces, Salmonidae): a test of alternative hypotheses. Heredity, 89: 27-35

Chan, S.C. 2003. Development and isolation of DNA microsatellite markers for the characterization and identification of *Mystus nemurus* (C&V) MSc thesis, Universiti Putra Malaysia, Malaysia.

Chong, L.K, S.G. Tan, K. Yusoff and S.S. Siraj. 2000. Identification and characterization of Malaysian river catfish, *Mystus nemurus* (C&V): RAPD and AFLP analysis. Biochemical Genetics, 38:63-76.

Dietrich, W. F., J.C. Miller, R.G. Steen. 1994. A genetic map of the mousse with 4,006 simple sequence length polymorphism. Nature Genetics, 7:220-255.

Ellegren, H. 2000. Microsatellite mutations in the germline: implications for evolutionary inference. Trends of Genetics, 16:551-558.

Ender, A., K. Schwenk, T. Stadler, B. Streit and B. Schierwater. 1996. RAPD identification of microsatellites in *Daphnia*. Molecular Ecology, 5:437-441.

Estoup, A., M. Solignac, M. Harry and J.M. Cornuet. 1993. Characterization of (GT)n and (CT)n microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. Nucleic Acid Research, 21:1427-1431.

Estoup, A., J.M. Cournet, F. Rousset and R. Guyomard. 1999. Juxtaposed microsatellite systems as diagnotics markers for admixture:Theoretical aspects. Molecular Biology and Evolution, 16:898 – 908.

Fisher, P.J., R.C. Gardner and T.E. Richardson. 1996. Single locus microsatellites isolated using 5' anchored PCR. Nucleic Acid Research, 24:4369-4371.

Gates, M.A., L. Kim, E. S. Egan, T. Cardozo, H.I. Sirotkin. 1999. A genetic linkage map of zebrafish comparative analysis and localization of genes and expressed sequences. Genome Research. 9, 334-347.

Giuffra, E., R. Guyomard and G. Forners. 1996. Phylogenetic relationships and introgression pattern between incipient parapatric species of Italian brown trout (*Salmo trutta* L. complex). Molecular Ecology. 5, 207 - 220.

Gottelli, D., C. Sillero-Zubiri, G.D. Applebaum, M.S. Roy, D.J. Girman, J. Garcia-Moreno, E.A. Ostrander and R.K. Wayne. 1994. Molecular genetics of the most endangered canid: the Ethiopian wolf *Canis simensis*. Molecular Ecology, 3:301 – 312.

Leesanga, S., S.S. Siraj, S.K. Daud, P.K. Sodsuk, S.G. Tan and S. Sodsuk. 2000. Biochemical polymorphism in yellow catfish *Mystus nemurus* (C&V), from Thailand. Biochemical Genetics, 38:1-9.

Lobo, J.A., M.A. del Lama and M.A. Mestriner. 1989. Population differenciation and racial admixture in the Africanized honey bee (*Apis mellifera* L.). Evolution, 43:794 – 802.

Na-Nakorn. U., W. Kamonrat and T. Ngamsiri. 2004. Genetic diversity of walking catfish, *Clarias macrocephalus*, in Thailand and evidence of genetic introgression from introduced farmed *C. gariepinus*. Aquaculture, 240:145-163.

Newton, C.R. and A. Graham. 1997. PCR: Introduction to biotechnology (2nd. Ed) Bios Scientific Publishers Ltd, Oxford, UK.

O'Conell, M. and Wright, J.M. 1997. Microsatellite in fishes. Reviews of Fish Biology and Fisheries, 7:332-363.

Pouyaud, L., Desmarais, E., Chenuil, A., Agnese, J-F. and Bonhomme, F. 1999. Kin cohesiveness and possible inbreeding in the mouth brooding tilapia *Sarotherodon melanotheron* (Pisces Cichiladae). Molecular Ecology, 8:803-813.

Rozen S. and H.J. Skaletsky. 1997. *Primer 3*. Code available at http:// www-genome.wi.mit.edu/genome_software/other/primer3.html.

Rutten, M.J.M., Komen, H., Deerenberg, R. M., Siwek M. and Bovenhuls, H. 2004. Genetic characterization of four strains of Nile tilapia (Oreochromis niloticus L.) using microsatellite markers. Animal Genetics, 35:93-97.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular Cloning: a laboratory manual (2nd, ed.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schug, M.D., E.E. Regulski, A. Pearce and S.G. Smith. 2004. Isolation and characterization of dinucleotide repeat microsatellite in *Drosophila ananassae*. Genetical Research, 83:19-29.

Shibata, K., K. Bandoh, N. Yaekashiwa, T. Matsuzaka and H.B. Tamate. 2003. A simple method for isolation of microsatellites from the Japanese aquirrel, *Sciurus lis*, without constructing a genomic library. Molecular Ecology Notes, 3:657-658.

Siraj, S.S., S.K. Daud, A. Othman and S.G. Tan. 1998. Population genetic structure of baung, *Mystus nemurus* (C&V) in Malaysia. M'sian J. of Appl. Bio., 27:77-82.

So, N., Maes, G. E., and Volckaert, F. A. M. 2006. High genetic diversity in cryptic populations of the migratory sutchi catfish *Pangasianodon hypophtalmus* in the Mekong River. Heredity, 96: 166 – 174.

Sukmanomon, S., S. Poompuang and M. Nakajima. 2003. Isolation and characterization of microsatellites in the Asian walking catfish *Clarias macrocephalus. Molecular* Ecology Notes, 10:1046-1047.

Taggart, J.B., R.A. Hynes, P.A. Prodohl and A. Ferguson. 1992. A simplified protocol of routine total DNA isolation from salmonid fishes. J. of Fish Bio, 40: 963-965.

Taniguchi, N. 2003. Genetic factors in broodstock management for seed production. Reviews of Fish Biology and Fisheries, 13:177-185.

Teh, C. H., B.H. Chua, S.Y. Yong, S.G. Tan and K. Yusoff. 2002. Development of single locus microsatellite markers for the green-lipped mussel, *Perna viridis* using a random amplified microsatellites (RAMS) based technique. Aquatic Resource and Environmental Studies of the Straits of Malacca, 351- 356.

Usmani, S. 2002. Isolation, characterization and application of microsatellite markers in the Southeast Asian river catfish *Mystus nemurus* (C&V). PhD. Dissertation, Universiti Putra Malaysia, Serdang.

Usmani, S., S.G. Tan, S.S. Siraj and K. Yusoff. 2001. Isolation and characterization of microsatellites in the Southeast Asian river catfish, *Mystus nemurus*. Molecular Ecology Notes, 1:264-266.

Usmani, S., S.G. Tan, S.S. Siraj and K. Yusoff. 2003. Population structure of the Southeast Asian river catfish *Mystus nemurus*. Animal Genetics, 34: 462-464.

Waldbieser, G.C., B.G. Bosworth, D.J. Nonneman and W.R. Wolters. 2001. A microsatellite-based genetic linkage map for channel catfish, *Ichalurus punctatus*. Genetics, 158:727-734.

Yeh, F.C. and T.J.B. Boyle. 1997. Population genetic analysis of co-dominant and dominant markers and quantitative traits. Belgium Journal of Botany, 129, 157.

Zane, L., L. Bargelloni and T. Patarnello. 2002. Strategies for microsatellite isolation: a review. Molecular Ecology, 11:1-16.

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