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## The Use of Locus Specific Microsatellite Markers for Detecting Genetic Variation in Hatchery bred *Probarbus jullieni*

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**Abstract:** This study is to demonstrated that microsatellites markers developed for *Tor tambroides* can be used to amplify microsatellite loci in other family. It is assumed that microsatellite loci are more conserved for aquatic species compared to terrestrial ones due to aquatic environments are less mutagenic than terrestrial ones. Development of microsatellites still requires investment of time and resources. Thus using loci already developed in a related species may provide a cost-effective alternative to microsatellite isolation and development in a species of interest in present study, *Probarbus jullieni*. In this study we investigated the possibility of the conservation of microsatellite flanking regions among different species. Nine pairs of SSR primers, five gave very strong banding profile (SYK1, SYK 2, SYK 5 SYK 8 and SYK 9) which could be used for population studies by using the nested protocol. Results showed that SYK 2 and SYK 9 flanked the same (CA)<sub>n</sub> repeats and thus are highly conserved in a different species. The products of the SYK 5, 8 and 1 primer pairs showed differences in the microsatellite regions which they flanked in *Probarbus jullieni* when compared to those of the source species, *Tor tambroides*. The mean observed heterozygosity levels for all the primers ranged 0.23-0.81. The primers are all polymorphic with the mean number of alleles from 2-5.

**Key words:** Cross amplifications, *Probarbus jullieni*, microsatellites

### INTRODUCTION

There are nearly 700 fish species in Malaysia, the conservation status of them are not exactly known. *Probarbus jullieni* is a fish species found in the rivers of Peninsular Malaysia. The International Union for the Conservation of Nature and Natural Resources Red Data Book lists *P. jullieni* as endangered prompting conservationists to plan some conservation programs to rescue this species. Malaysia has imported 200 juveniles from Thailand in the late 80s and has been breeding *P. jullieni* in captivity since then. Conservation of genetic resources is an essential component of many species management programmes (Templeton, 1994; O'Connell and Wright, 1997). Hatchery-enrichment can potentially reduce the genetic diversity of natural stocks. An effective strategy for the conservation and restocking of a particular species is documenting its genetic make up in hatchery bred and non indigenous stocks to compare levels of genetic variation. Aquaculture practices may decrease the genetic variability present in farmed stocks by breeding among related individuals and use of small numbers of founding broodstock (Thai *et al.*, 2007). Thus

breeding programs may deliberately introduce divergent stocks and utilize crossbreeding programs to decrease diversity and productivity (Hulata, 1995). The ongoing development of a restocking program that had been initiated requires greater understanding of the genetic status of domesticated stocks. To this end, an initial study of genetic variation within and among populations using morphological and molecular markers showed that in Malaysia currently there are two distinct populations of *Probarbus jullieni*, those of Thai origin and the indigenous wild stocks. However, the degree of differences between these two populations showed that they belong to the same species. The resolving power of their genetic composition need to be evaluated further by using the more informative polymorphic microsatellite loci (Bhassu *et al.*, 2008). Microsatellites' potential applications in aquaculture include monitoring changes in genetic variation as a consequence of different breeding strategies, the investigation of interactions between wild and cultured populations, parentage assignment and estimation of relatedness between potential breeding pairs (Cross, 2000; Cross *et al.*, 2005; Davis and Hetzel, 2000; Liu and Cordes, 2004; Norris *et al.*, 1999).

Microsatellites or Simple Sequence Repeats (SSRs) are DNA segments in the genome that consist of a concatenation of very short sequence of nucleotides. Microsatellite segments consist of about 10-50 repeats of 2-6 nucleotides. (Weissenbach *et al.*, 1992). Because of their codominant inheritance, relatively high level of polymorphism and ease in analysis using Polymerase Chain Reaction (PCR), microsatellites (SSR) have been considered as a valuable molecular tool in genetic studies such as in the management and conservation of endangered species (Weber and May, 1989; Tautz, 1989) but development of microsatellites still requires investment of time and resources. Thus using loci already developed in a related species may provide a cost-effective alternative to microsatellite isolation and development in a species of interest. Cross-species amplification is only effective if primer sequences are conserved between species (Moor *et al.*, 1991; Peakall *et al.*, 1998). In this study locus specific primers that has been developed for *Tor* sp. was applied in this *Probarbus jullieni* population. If these primer pairs were found conserved among different species, the microsatellite loci developed will be very useful in the population studies of various species. Thus, in this study we investigated the possibility of the conservation of microsatellite flanking regions among different species and that these primer pairs are indeed flanking microsatellite sequences.

## MATERIALS AND METHODS

**DNA extraction:** All the fish used were reared in the Fisheries Research Extension Center in Perlok, Pahang, Malaysia where they are maintained as broodstocks for breeding and further possible restocking program. One scale was removed and stored in 70% ethanol and later kept at  $-80^{\circ}\text{C}$ . Genomic DNA from scales of *Probarbus jullieni* was extracted following the protocol recommended for the QIAamp DNA Mini Kit (Qiagen, Germany). The absorbencies of each of the extracted DNA sample were measured at 260 and 280 nm using a spectrophotometer to check on its quality and purity.

**PCR optimization:** The SYK primers (Bhassu *et al.*, 2008) were designed and synthesized for microsatellite DNA amplification of the masher fish, *Tor tambroides*. The initial PCR was carried out in a final reaction volume of 10  $\mu\text{L}$  containing 1x Promega reaction buffer, 1.5 mM  $\text{MgCl}_2$ , 200 mM each of dATP, dGTP, dCTP, dTTP, 1 U Promega Tag polymerase, 10 pmol of each oligonucleotide primer, 50 ng template. De-ionised distilled water was added to a final volume of 10  $\mu\text{L}$ . The amplification was



Fig. 1: PCR before optimization

performed by using an Eppendorf Mastercycler Gradient thermocycler programmed 3 min for pre-denaturation at  $95^{\circ}\text{C}$  followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec and annealing at  $58^{\circ}\text{C}$  for 40 sec and extension at  $72^{\circ}\text{C}$  for 45 sec and a final extension at  $72^{\circ}\text{C}$  for 7 min. The electrophoresis of the PCR products was done on 4% metaphor gel at 75 V. The gels were stained with  $0.5 \mu\text{g L}^{-1}$  ethidium bromide in water and visualized under ultraviolet light. The results are shown in Fig. 1.

A touchdown protocol was then used in an effort to generate clear and distinct PCR bands. The PCR reaction contained the following components in 10  $\mu\text{L}$  final volume: 1x Promega reaction buffer, 1.5 mM  $\text{MgCl}_2$ , 200 mM each of dATP, dGTP, dCTP, dTTP, 1 U Promega Tag polymerase, 10 pmol of each oligonucleotide primer, 50 ng template. De-ionised distilled water was added to a final volume of 10  $\mu\text{L}$ . The amplification was performed by using an Eppendorf Mastercycler Gradient thermocycler programmed 3 min for pre-denaturation at  $95^{\circ}\text{C}$  followed by 20 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec and annealing at  $58^{\circ}\text{C}$  for 1 min and extension at  $72^{\circ}\text{C}$  for 1 min and then another 20 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec and annealing at  $61^{\circ}\text{C}$  for 1 min and extension at  $72^{\circ}\text{C}$  for 1 min and a final extension at  $72^{\circ}\text{C}$  for 7 min. This was followed by electrophoresis of the PCR products on 4% metaphor gel at 75 V. The gels were stained with  $0.5 \mu\text{g L}^{-1}$  ethidium bromide in water and visualized under ultraviolet light. The size of the microsatellite alleles was determined by using a 20 bp DNA ladder. The results are shown in Fig. 2. The clear bands were extracted from the gel by using the QIAquick PCR Purification Kit and were then sent for sequencing. The sequences of the microsatellites are shown in Table 1.



Table 2: Blast analysis of the sequences of the three primers, SYK 9, SYK 8 and SYK 1 which showed conservation on chromosomes of *Danio rerio*

E value	Blast similarities	Primer
2e-29	Zebrafish DNA sequence from clone DKEY-285B22 in linkage group 1, complete sequence	SYK 9
3e-26	<i>Danio rerio</i> chromosome 13 genomic contig, reference assembly (based on Zv6_scaffold1897:1-848378)	SYK 8
1e-22	<i>Danio rerio</i> chromosome 1 genomic contig, reference assembly (based on Zv6_scaffold58:1-6153135)	SYK 1

pairs in *Probarbus jullieni*. However, no microsatellite sequences were flanked by SYK 6 and SYK 7 in *Probarbus jullieni* even though they flanked microsatellite repeats in *Tor tambroides*. When blast analysis was performed on all the SKY primers studied with the NCBI database, it was found that two primers SYK 1 and SYK 8 flanked microsatellite regions which are located on chromosome 1 of *Danio rerio* (Table 2) and the region of interest has an important role in host antiviral defense since the dinucleotide microsatellite regions are located in the oligoadenylate synthetase I (OAS1) and ribonuclease L (RNASEL) innate immunity genes (Rios *et al.*, 2007). The SYK 9 primer on the other hand flanked regions that code for Guanylate Cyclase Activating Proteins (GCAPs) which has a function in the regulation of the photoreceptor Guanylate Cyclase (GCs), expressed in the retina of *Danio rerio* fish (Imanishi *et al.*, 2004). Thus, the cross amplifications of *Tor tambroides* primers in *Probarbus jullieni* has indeed have saved a lot of time on the development of microsatellites and they can be used further in the population structure studies of hatchery bred stocks of the latter species.

A prerequisite for the amplification of microsatellite loci with the means of PCR is relatively expensive and time consuming, therefore isolation of microsatellites in different species may be applied other closely related species. It has been suggested that the successful amplification of microsatellite loci across species depends on the conservation of the priming sites within the flanking sequences (Weber, 1990). Therefore, the conservation of microsatellite primers in closely related species and slow evolution in the flanking regions permit cross-amplification (Moor *et al.*, 1991). The conservation of sequences flanking the microsatellites had been reported by Rico *et al.* (1996) who proposed a conservation of the microsatellite flanking regions in fish over a period of about 450 million years.

The estimated values of the observed and expected heterozygosity for all the populations are valued from minimum 0.23-0.81 and latter from the minimum 0.12-0.48 in hatchery populations. The Fis statistics value showed the level of heterozygosity in a population the positive value indicates deficit of heterozygosity. Therefore,

microsatellites as codominant and neutral genetic marker have been used to examination of genetic differentiation in many species, specially in aquaculture populations (Reilly *et al.*, 1999) and routinely used on studies of population subdivision, parentage analysis and shallow phenetic relationships and possibly gene flow (Adams *et al.*, 2004). It is shown that the microsatellites can be used for parentage analysis for this study.

## CONCLUSION

The significance of this study is the demonstration that it is possible to cross amplify microsatellite primer pairs not only across species within the same genus but also between species that belong to different genera. As some of the SKY primers have been demonstrated to be located in the conserved exon regions it is likely that they will be present over many generations of breeding programs or hatchery operations unlike random markers which can be lost due to genetic drift in such systems which usually start with small founder populations. Hence, they should be useful for parentage assignments at various stages of the breeding program or hatchery operation. This method is indeed efficient and cost effective to obtain new microsatellites for species of interest. Evidence has shown that microsatellite flanking regions are highly conserved and can be amplified across species and even across taxa.

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