Chapter IV

Discussion

Selection of microsatellite loci

Microsatellite markers exhibiting high level of polymorphism and heterozygosity are suitable for application to genetic studies in fisheries and aquaculture (O'Reilly and Wright 1995). In this thesis, eight microsatellite loci developed in our laboratory were examined for polymorphism. The number of alleles per locus and heterozygosity values revealed high levels of polymorphism of these microsatellite loci, except the locus CSCUPmo7 which showed heterozygosity of 0.21 when 43 individuals *P. monodon* from Trad were investigated. Low heterozygosity found at the CSCUPmo7 locus may possibly be resulted from null alleles (Koorey et al., 1993). The existence of null alleles may cause errors in genetic studies, thus, the locus CSCUPmo7 was not chosen for further study. Different microsatellite allelic patterns of the CSCUPmo3 locus within heterozygotes resulted in difficulty of allele interpretation. Therefore, only six polymorphic microsatellite loci, CSCUPmo1, CSCUPmo2, CSCUPmo4, CSCUPmo6, CSCUPmo9 and CSCUPmo11 which produced reliable allelic patterns were selected for DNA typing assays.

The allele differences between consecutive alleles of microsatellite loci used in this study depended on the repeat types. For the CSCUPmo1 (a perfect $(GAA)_n$ repeat) and the CSCUPmo11 (a compound trinucleotide repeat $(TAA)_n$ array that separated by TGA), consecutive alleles differ by 3 bp. For the CSCUPmo9 (a perfect $(TA)_n$ repeats) and other compound repeats of $(GATA)_n$ linked to different types of dinucleotide repeats (CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6 and CSCUPmo7), the alleles were different by multiples of 2 bases. This suggests that polymorphism detected should occur within (dinucleotide and the tetranucleotide) repeat arrays.

Usable microsatellites were very difficult to obtain in *P. monodon* owing to the large and complex repeat arrays found in this species. Tassnakajon et al. (1998) designed nine primer sets from *P. monodon* microsatellite containing clones and found only two primer sets yielded scorable amplification products. Most primer sets yielded multiple DNA bands, which may be due to the fact that these microsatellite loci are embedded in some kind of cryptic repeats dispersed throughout the genome (O'Reilly and Wright, 1995). Xu et al. (1999) has isolated usable microsatellites from *P. monodon* genome. Of which, 10 of 11 primer sets were successfully amplified and scorable, with alleles generally ranging in size from 139 to 410 bp. They reported that 87% of microsatellites contained unique flanking sequences for designing primers.

The level of polymorphism of each microsatellite depended on the number of repeats. Tri- and tetranucleotide microsatellites have been demonstrated to be highly polymorphic and are stable inherited in the human genome (Edwards et al., 1991). They are becoming increasingly popular markers because the allele differences are easier to distinguish than those of dinucleotide repeats and less stutter bands have been observed in their amplification products. The appearance of shadow or stutter bands has been shown to be due to slipped-strand mispairing during PCR (Hauge and Litt, 1993). The stutter bands tend to decrease with increasing unit length (Edwards et al., 1991). The deficiency of heterozygotes observed from microsatellite could be a result of small sample size (Lessios 1992) or the presence of null alleles (Pemberton et al., 1995). The sample size of 50, which is normally adequate for other DNA-based techniques, may not cover high number of alleles per microsatellite locus (Table 3.3).

Simple DNA typing with microsatellite markers

The use of microsatellite markers for DNA typing offers many advantages over other typing methods. Microsatellite loci can be amplified by PCR, thus, very small

amounts of genomic DNA are required for the analysis. In addition, microsatellite loci can be amplified from partially degraded DNA because most PCR amplified alleles are less than 400 bp long. In this study, several DNA extraction methods were used to isolate genomic DNA from pleopods and blood of adult *P. monodon* and the whole postlarvae. Of which, the alkaline extraction protocol modified from Rudbeck and Dissing (1998) is the most suitable method providing enough quality of genomic DNA for PCR amplifying of microsatellite alleles. This alkaline extraction method uses only two reagents, NaCl and Tris-HCl which are common in every laboratories. This method is appropriate when dealing with a large number of specimens because less time (10 minutes) was required for the incubation step. The DNA solution could be kept at -20 °C for a long period of time. In our experience, the DNA solution, kept over 6 months, could be used for PCR amplification. Although standard DNA extraction methods such as phenol-chloroform, salting-out yielded high quality DNA which can be kept for longer time at -20 °C, they involve the use of hazardous chemicals and more complex extraction. Therefore, they were not appropriate for use with a large number of specimens.

Multiplex analysis, either multiplex PCR in which two or more primer pairs are used together in one PCR reaction or a single loading of PCR amplified alleles of different loci, allowed more loci to be typed simultaneously. This minimized labor, materials and analysis time. In this study, three multiplex sets including one set of CSCUPmo1+2 multiplex PCR and two sets of the single loading of CSCUPmo4+ CSCUPmo9 and CSCUPmo6+CSCUPmo11, were successfully developed using radioisotopic detection methods. These microsatellite alleles were separated by 6% denaturing polyacrylamide sequencing gels. Allele sizes were compared with those of the DNA sequence of M13 mp18.

Three multiplex sets were selected based on the favorable display of the following criteria: 1) good and similar amplification product yields for different loci, 2) high levels of polymorphism, 3) little or no PCR artifacts arising from nonspecific amplification, 4) easy for allele determination and 5) non-overlapped allele size ranges (Sprecher et al., 1996; Henegariu et al., 1997 and Lins et al., 1996).

Typing of microsatellite markers requires separation of PCR products by denaturing polyacrylamide gel electrophosis. The radioactive-labeled method was commonly used for detection of microsatellite alleles by autoradiography after gel separation because of their sensitivity and efficiency. However, this method is expensive, hazardous and not appropriately used in some laboratories. The non-isotopic methods, for example the silver stain detection, have been recently used in substitution of the radiodisotopic method.

White and Kusukawa (1997) had reported the use of vertical 4% MetaPhor agarose gel electrophosis (24-cm-long) at 500 V for 4 hours, for separating microsatellite alleles that differ only in 2 bp when compared with 2 bp ladder and detected with the SYBR Green I stain. The stained Metaphor agarose gels were scanned on the FluorImager SI. The added advantage of the sensitive SYBR Green I stain allows cleaner amplification with fewer cycles. In that study, the theoretical separating ability of the minigels (10 x 10 cm, 1 mm thick) in average was 3.3 bp within the size range of 160-307 bp according to the calculation method of Lerma and Sinha, 1990 (cited in White and Kusukava, 1997).

Microsatellite alleles of loci CSCUPmo1 (3-bp different alleles) and CSCUPmo2 (2-bp different alleles), were separated in the horizontal 12-cm-long MetaPhor (4%) agarose gels (3mm thick) at 100 V for 6-8 hours and detected with ethidium bromide. Three bands that had sizes within their allele size range were found

(Figure 3.21). Microsatellite alleles of CSCUPmo1 and CSCUPmo2 loci which were separated in vertical 4% MetaPhor agarose minigels gave the same results (data not shown). In non-denaturing conditions, double stranded DNA at different amplified fragment size can bind one another with complementary base sequences and may migrate anomalously. There are non-specific bands that revealed between the authentic bands. Because of the ambiguous results of MetaPhor agarose gel analysis, this method was not studied further.

Denaturing polyacrylamide gels were used to separate microsatellite alleles that differ in 2-3 bp followed by silver-stain detection procedure (Bassam et al., 1990). This is a very sensitive method that allows rapid evaluation of amplified microsatellite fragments. As seen in Figure 3.22 and 3.23, this method sometimes reveals two fragments representing each allele as those of microsatellite loci CSCUPmo1, CSCUPmo2 and CSCUPmo11. This should be caused by differential strand migration for products of the same length having different sequences. The degree of differential migration of the opposing strands depends on specific amplified sequences being separated. Thus, it varies with the locus being amplified, with the distance the fragments have been subjected to electrophoresis, with the concentration of the gel matrix, to a certain degree and with the selection of primers for amplification.

Other two artifacts when detected microsatellite alleles by silver staining were repeat slippage (stutter bands) and a light band at one base below the primary allele fragment. The first described above, is seen at varying degrees with dinucleotide repeated loci (CSCUPmo2, CSCUPmo4, CSUPmo6 and CSCUPmo9) and some of the trinucleotide repeat loci (CSCUPmo1). The second artifact was seen with CSCUPmo1 (data not shown). Most likely, this is caused by the terminal transferase activity of the *Taq* DNA polymerase that add a single nucleotide at the 3′termini of amplified PCR products as described by Clark and also observed with STR amplification products by

Kimpton et al (Clark, 1988 and Kimpton et al., 1992 cited in Sprecher et al., 1996). These two artifacts (the production of extraneous high molecular weight bands associated with amplification of loci and the generation of several products shorter than the authentic allele that appear as extensive series of DNA fragments differing by one base) are not well understood. The first of these artifacts is often observed if too many cycles are included in the thermal cycling protocol, suggesting an illegitimate amplification event as the cause.

Using allelic ladders, further simplified DNA typing was carried out because the ladders contain fragments equal in length to several or all of the known alleles allowing rapid and precise assignment of alleles by matching band of unknown samples with standard alleles in the allelic ladder (Puers et al., 1994). Construction of allelic ladders were not successful in several loci because extra bands or artifacts appeared. These caused unresolvable banding patterns. Allelic ladders of CSCUPmo1 and CSCUPmo2 showed high background of artifacts and pooled alleles of these loci can not be re-amplified. Re-amplification of the mixture of known alleles from different samples is necessary to ensure unlimited supply of DNA standards. The allelic ladder of CSCUPmo11 could be constructed and re-amplified using the 1: 100 diluted amplified products with sterile distilled water. It gave the same easy-scoring results of allele sizes.

Most allelic ladders of microsatellite were constructed for microsatellite loci with 3-bp difference in allele sizes and have appropriate number of allele (Puers et al., 1994) used tetrameric tandem repeats microsatellite such as HUMF13A01 was used to constructed allelic ladders that are 4-bp difference in allele sizes and have 13 allelic bands that cover its allele size. Most of microsatellites in this study consisted of dinucleotide repeats and have the number of alleles greater than 20 alleles except the locus CSCUPmo11 (10 alleles). The allelic ladder of the CSCUPmo11 has 3-bp allelic

bands from 135 to 162 bp. The allelic band at 159 bp was not found and the 153 bp band had less intensity of DNA in the allelic ladder, thus this allelic ladders had 8 clear allelic bands.

The resolution of denaturing polyacrylamide gel electrophosis affects visualization of microsatellite alleles. High resolution was obtained with 8% polyacrylamide in sequencing gels (30x40 cm, 0.4 mm thick) which provided the good results for scoring allelic bands of the CSCUPmo11 (Figure 3.24). Lower resolution was obtained with minigels (10x10 cm, 1 mm thick). However, increasing the percentage of polyacrylamide to 15%, allelic fragment of the CSCUPmo11, which were 3 bp-different, can be precisely scored (Figure 3.28). The use of minigels for separation of alleles and allelic ladders of the CSCUPmo11 locus allows simpler and more rapid DNA typing at this locus.

Basically, highly polymorphic markers with abundance, even distribution throughout the genome and transmitting according to the Mendelian fashion are ideally required for wider applications. Microsatellites fall into these requirements, therefore they have been elevating used for population genetic studies in several taxa. Significant deviation from the Mendelian segregation found in the present study may be explained by several reasons including; first, null allele (non-amplifying alleles) at these loci may be present. This crucial circumstance is observed for amplification of microsatellites in human and other mammal studies (e.g. Callen et al., 1993; Koorey et al., 1993; Pemberton et al., 1995). Non-amplification alleles can be detected through mismatched patterns between parents and progeny whose their genotypes were already known (Pemberton et al., 1995). Null-alleles can underestimate heterozygotic levels as heterzygotes are scored as homozygotes resulting in non-Mendelian inheritance or significant deviation from Hardy-Weinberg equilibrium. Second, most dinucleotide

microsatellite show stuttered bands so that mis-scoring of a particular allele may be occurred (O'Reilly & Wright, 1995).

Segregation analysis of six microsatellites (CSCUPmo1, CSCUPmo2, CSCUPmo4, CSCUPmo6, CSCUPmo9 and CSCUPmo11) in *P. monodon* using 50 progeny from three representative full-sib families revealed non-significant deveations from expected gametic segregation for all loci except the loci CSCUPmo1 and CSCUPmo2 for families B10 and B26, respectively, indicating Mendelian inheritance nature of four microsatellite loci. Therefore, departures of Mendelian segregation pattern should be caused by the low number of sample sizes and sampling errors rather than from effects of selection in as much as no common allele (s) with the extremely high frequency was observed. Based on the basic biology of *P. monodon*, the important life-historical generalization may be drawn. The *P. monodon* females may spawn more than once a year. Each wild female may produce 248,000 to 811,000 eggs per single spawn (Motoh, 1981: cited in Solis, 1988). An enormous number of eggs may be sufficient to replace part of the entire adult population. Sampling errors could then be happened.

Finally, simple DNA typing kit of *P. monodon* could be developed for use in genetic studies of this economically important species. Simple DNA typing assays involve more rapid and simpler DNA extraction methods, multiplex analysis of selected paired of microsatellite loci, and non-isotopic detection of microsatellite alleles. The use of allelic ladders also enhanced the simplicity and reliability of DNA typing.