Isolation and Characterization of Polymorphic Microsatellite Loci from *Metapenaeopsis barbata* Using PCR-Based Isolation of Microsatellite Arrays (PIMA)

Tzen-Yuh Chiang 1,†, Tzong-Der Tzeng 2,†, Hung-Du Lin 3, Ching-Ju Cho 1 and Feng-Jiau Lin 1,*

1 Department of Life Sciences, National Cheng Kung University, Tainan 701, Taiwan; E-Mails: tychiang@mail.ncku.edu.tw (T.-Y.C.); lovego8@hotmail.com (C.-J.C.)
2 Department of Leisure, Recreation and Tourism Management, Shu-Te University, Kaohsiung 824, Taiwan; E-Mail: tdtzeng@stu.edu.tw
3 The Affiliated School of National Tainan First Senior High School, Tainan 701, Taiwan; E-Mail: varicorhinus@hotmail.com

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: fjlin@mail.ncku.edu.tw; Tel.: +886-6-2757-575 ext. 65527; Fax: +886-6-2742-583.

Received: 19 January 2012; in revised form: 19 February 2012 / Accepted: 21 February 2012 / Published: 1 March 2012

Abstract: The red-spot prawn, *Metapenaeopsis barbata*, is a commercially important, widely distributed demersal species in the Indo-West Pacific Ocean. Overfishing has made its populations decline in the past decade. To study conservation genetics, eight polymorphic microsatellite loci were isolated. Genetic characteristics of the SSR (simple sequence repeat) fingerprints were estimated in 61 individuals from adjacent seas of Taiwan and China. The number of alleles, ranging from 2 to 4, as well as observed and expected heterozygosities in populations, ranging from 0.048 to 0.538, and 0.048 and 0.654, respectively, were detected. No deviation from Hardy–Weinberg expectations was detected at either locus. No significant linkage disequilibrium was detected in locus pairs. The polymorphic microsatellite loci will be useful for investigations of the genetic variation, population structure, and conservation genetics of this species.

Keywords: microsatellite; management; PIMA; RAPD-PCR enrichment; *Metapenaeopsis barbata*
1. Introduction

The red-spot prawn, *Metapenaeopsis barbata*, is a widely distributed demersal species in Indo-West Pacific from the Gulf of Bengal to Japan and Indonesia [1–3]. It is one of the abundant prawns with high commercial values [2,4]. It was overfished from 1995 to 1996 around Taiwan [5]. Furthermore, the populations of this species declined in the past decade likely due to the effective benthic trawling [6] and ecological destruction. The life history, morphometric variation and fishery biology of these shrimps have been well studied [2,5,7–10]. Recently, Chu [11,12] investigated the population structure and historical demography of the whiskered velvet shrimp using one intron of elongation factor-1α gene and the mitochondrial DNA control region. For practicing conservation and understanding the genetic structuring across populations, codominant and highly polymorphic molecular markers are desired. Highly polymorphic microsatellite DNAs have been widely used in many aquacultural species to evaluate the genetic diversity [13,14], construct genetic maps [15,16], and determine species’ lineages [17], as well as for conservation and management in shrimps [16]. In order to protect and manage these overexploited wild resources of *M. barbata*, molecular markers are urgently needed to develop suitable strategies to maintain sustainable populations.

2. Results and Discussion

In total, 61 individuals of *M. barbata*, including 21 from Taichung, Taiwan and 40 from Fujian, China, were collected. Eight di-nucleotide SSR loci were isolated. The characteristics of the innovative microsatellite loci and variability measures across two populations are described in Table 1. The number of alleles per locus ranged from 2 to 4, with an average of 3.00 in Taichung and an average 3.25 in Fujian. The observed heterozygosities ($H_O$) and expected heterozygosities ($H_E$) ranged from 0.048 to 0.476 (averaged at 0.327) and ranged from 0.048 to 0.667 (averaged at 0.420), respectively, in Taichung; while the $H_O$ and $H_E$ ranged from 0.205 to 0.538 (average = 0.331) and from 0.283 to 0.654 (average = 0.392), respectively, in Fujian. No deviation from Hardy–Weinberg expectations was detected at either locus. There was no evidence of linkage disequilibrium between any pairs of loci. The locus-wise $F_{IS}$ for each population, shown in Table 2, was non-significant after Bonferroni correction [18], indicating heterozygote deficiency in all but one locus (MIMB03) in Fujian population. Microsatellite markers could be a good choice for the characterization of genetic diversity in *M. barbata* due to its reliable, informative, co-dominant nature and ease of exchange of data among different studies. The results suggest that the microsatellite DNA loci identified in this study are highly polymorphic, and that these markers can be useful for investigating the genetic structure and management of *M. barbata* populations.
Table 1. The forward (F) and reverse (R) primer sequences, repeat motif, size range and 
$T_m$, annealing temperature for eleven microsatellite loci of *Metapenaeopsis barbata*.

| Locus   | Primer sequence (5' to 3') | Repeat motif | Size range (bp) | $T_m$ °C |
|---------|-----------------------------|--------------|-----------------|--------|
| MIMB01  | F: CAATCGGCCTCTTACACTT (CA)$_9$ | 147–161      | 54              |
|         | R: GGCAAAAAAAGTGGTAATTGTT    |              |                 |
| MIMB02  | F: TCTATATGTGGTCCCGGTGT (TG)$_9$ | 168–182      | 54              |
|         | R: CATGTTCAGTATGTGTTCTATCG    |              |                 |
| MIMB03  | F: AAAACACGATTTCCAAACAGAA (TC)$_{12}$ | 204–218      | 57              |
|         | R: TGAAAATTGCGAATTTCCTTT    |              |                 |
| MIMB04  | F: TGATTGCGAAGGTCATCAAG (CT)$_{15}$ | 180–200      | 50              |
|         | R: TGAAAGGAAAGATTCGAGGAGA    |              |                 |
| MIMB05  | F: AGTTAACAGG CCTCCGGGAACTCC (AT)$_8$ | 175–193      | 50              |
|         | R: GGACAAGGGCGAGTACATA    |              |                 |
| MIMB06  | F: TTTAATGTGTATTGCGGTCTCC (GT)$_{11}$ | 149–155      | 60              |
|         | R: CATACACACCGGAGGAGGAGA    |              |                 |
| MIMB07  | F: TGCTGGACCTTTGGGTTTATAG (GT)$_{10}$ | 240–252      | 60              |
|         | R: CATACAAGGACAGGGCAGAATA    |              |                 |
| MIMB08  | F: TGGAGGAGATTGGGAGATTG (GT)$_{10}$ | 201–205      | 54              |
|         | R: GAATCGATTGACGGCTTGT    |              |                 |

Table 2. Genetic estimates of eight microsatellite loci for *Metapenaeopsis barbata* from 
Taichung, Taiwan, and Fujian, China. Number of alleles ($N_A$), allelic richness ($A_R$), 
expected ($H_E$) and observed ($H_O$) heterozgosities and significance of deviation from 
Hardy–Weinberg equilibrium ($P_{HW}$), fixation index ($F_{IS}$) and $P$-values of Chi-Square tests 
for fixation index ($P_{FIS}$) for microsatellite loci were estimated.

|          | Taichung | Fujian |
|----------|----------|--------|
|          | $N_A$    | $A_R$  | $H_O$ | $H_E$ | $P_{HW}$ | $F_{IS}$ | $P_{FIS}$ | $N_A$    | $A_R$  | $H_O$ | $H_E$ | $P_{HW}$ | $F_{IS}$ | $P_{FIS}$ |
| MIMB01   | 3        | 2.905  | 0.381 | 0.431 | 0.70525 | 0.118    | 0.074    | 3        | 3.260  | 0.300 | 0.303 | 1.00000 | 0.010    | 0.012    |
| MIMB02   | 3        | 2.905  | 0.381 | 0.431 | 0.70033 | 0.118    | 0.074    | 3        | 2.487  | 0.205 | 0.303 | 0.07629 | 0.325    | 0.183    |
| MIMB03   | 2        | 1.905  | 0.048 | 0.048 | 1.00000 | 0.000    | 0.000    | 3        | 2.929  | 0.500 | 0.482 | 0.18053 | -0.038   | 0.061    |
| MIMB04   | 3        | 3.000  | 0.286 | 0.429 | 0.60301 | 0.339    | 0.345    | 3        | 2.890  | 0.324 | 0.419 | 0.17474 | 0.228    | 0.139    |
| MIMB05   | 4        | 4.000  | 0.474 | 0.667 | 0.17390 | 0.296    | 0.381    | 4        | 3.998  | 0.538 | 0.654 | 0.21398 | 0.179    | 0.125    |
| MIMB06   | 3        | 3.000  | 0.476 | 0.563 | 0.07654 | 0.158    | 0.358    | 3        | 2.890  | 0.324 | 0.405 | 0.35083 | 0.201    | 0.102    |
| MIMB07   | 3        | 3.000  | 0.333 | 0.424 | 0.15270 | 0.218    | 0.070    | 4        | 3.510  | 0.229 | 0.283 | 0.13777 | 0.195    | 0.109    |
| MIMB08   | 3        | 3.000  | 0.238 | 0.368 | 0.08563 | 0.359    | 0.408    | 3        | 2.974  | 0.225 | 0.289 | 0.05880 | 0.223    | 0.234    |
| mean     | 3.00     | 2.964  | 0.327 | 0.420 | 1.00000 | 0.226    | 0.0392   | 3.25     | 3.067  | 0.331 | 0.392 | 0.9966  | 0.158    | 0.0476   |

3. Experimental Section

3.1. Isolation of Microsatellite Markers

In present study, we have developed eight polymorphic microsatellite markers that are specific for *M. barbata*. Genomic DNA was extracted from muscle tissues preserved in 95% ethanol by following 
standard phenol-chloroform procedure [19]. The enrichment of DNA fragments was carried out using 
the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) method [20] with a 
minor modification [21]. This PIMA (PCR isolation of microsatellite arrays) approach has been 
proposed [20]. It takes advantage of the fact that the RAPD fragments contain microsatellite repeats 
more frequently than random genomic clones [22]. Several RAPD primers were selected to amplify 
the target DNA fragments. The RAPD-PCR amplifications were performed in a thermal cycler (Bio-Rad) 
with a reaction mixture (50 μL) containing 20–100 ng DNA, 0.2 mM of each dNTP, 2 mM MgCl$_2$, 0.5 U 
Taq polymerase (Promega), and 5 pmol of one RAPD primer. The PCR program were as follows: initial 
denaturing for 3 min at 95 °C for 1 cycle, 40 cycles of 1 min at 94 °C, 45 s at 42 °C, 2 min at 72 °C,
followed by 10 min at 72 °C for an additional extension step. Approximately 100 ng of PCR product was ligated into a pGEM-T vector (Promega) according to the manufacturer’s instructions. The ligation mixture was then transformed into *Escherichia coli* competent cells to form the enriched microsatellite sequence library. Colonies of RAPD fragments were analyzed to verify existence of repetitive sequences. Clones were screened using repeat-specific and vector primers [20]. In positive clones, the repeat-specific and vector primers amplified DNA fragments that contain microsatellites, whereas no amplification was found in negative clones. Plasmid DNA from positives was purified using the High-Speed Plasmid Mini Kit (Geneaid). Both strands of the DNA insert were sequenced. DNA sequencing in both directions was conducted with an Applied Biosystems ABI3730 automated sequencer (Applied Biosystems). Primers for these loci were designed using PRIMER 3 software [23] and synthesized. Primers were designed according to the nucleotide sequences upstream and downstream of the repetitive DNA. A total of 8 primer pairs were designed from 8 sequences as the remaining sequences were too close to the cloning site. Polymorphisms of these microsatellite loci were assessed by 40 *M. barbata* individuals collected from Fujian Province, China and PCR conditions were optimized for each pair of primers. Reactions were performed in a total volume of 15-μL containing 10 ng of genomic DNA, 0.2 mM dNTP, 2 mM MgCl₂, and 0.12 μM of each primer. The PCR program consisted of 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 30 s at primer-specific annealing temperature (Table 1), 72 °C for 45 s and a final extension step at 72 °C for 5 min. Individuals were genotyped on 6% denaturing polyacrylamide gels stained with ethidium bromide straining and sized by comparison to a 10-bp DNA ladder standard (Invitrogen).

### 3.2. Data Analysis

Genotype data files were inter-converted for the various analytical software programs using CREATE [24] to minimize errors. The allele number, size range, number of bands per individual, expected (*Hₑ*), and observed heterozygosity (*Hₒ*) were quantified using the Arlequin version 3.5 [25]. Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using GENEPOP 3.4 software [26]. Results of tests for linkage and Hardy–Weinberg disequilibria were corrected for multiple comparisons by applying sequential Bonferroni corrections [18]. Inbreeding coefficient (*Fᵢₛ*) [27] and the significance of these values were calculated for each population with GenePop Web Version 4.0.10 [26,28].

### 4. Conclusions

The wild resource of *M. barbata* has declined in the last decade. Therefore, the development of microsatellites and their application, which can offer an effective tool for understanding genetic variation and population structure in *M. barbata*, is of vital importance. These eight polymorphic microsatellite loci reported here are the first microsatellite markers designed specifically for *M. barbata*, and the versatility of these new primer sets will provide for the further studies on the genetic structure, gene flow, sustainable management and molecular evolution of this susceptible species. The microsatellite loci are sufficient to perceive significant differences among populations of *M. barbata* in different fishing grounds of Asia in future studies.
Acknowledgments

The present work is a contribution from a research grant supported by the National Science Council, Taiwan, R.O.C. (NSC 99-2321-B-006-013-MY3).

References

1. George, M.J.; Muthu, M.S. On the occurrence of Metapenaeopsis barbata (De Haan) (Decapoda: Penaeidae) in Indian Waters with taxonomic notes on the genus. J. Mar. Boil. Ass. Inida 1968, 10, 286–291.
2. Wu, C.C. Survey of shrimp in Taiwan Strait and biological studies of thick shell shrimp (Metapenaeopsis barbata). Bull. Taiwan Fish. Res. Inst. 1984, 37, 67–82.
3. Chan, T.Y. Shrimps and Prawns. FAO Species Identification Guide for Fishery Purposes. In The Living Marine Resources of the Western-Central Pacific; Carpenter, K.E., Niem, V.H., Eds.; Food and Agriculture Organization of the United Nations: Rome, Italy, 1998; Volume 2, pp. 851–971.
4. Wu, C.C. Studies on the shrimp fishery and their fishing ground in Taiwan. Bull. Taiwan Fish. Res. Inst. 1985, 39, 169–197.
5. Tzeng, T.D.; Chiu, C.S.; Yeh, S.Y. Growth and mortality of the red-spot prawn (Metapenaeopsis barbata) in the northeastern coast off Taiwan. J. Fish. Soc. Taiwan. 2005, 32, 229–238.
6. Fisheries Agency, Council of Agriculture, Executive Yuan, Taiwan. 2010 Annual Report. Available online: http://www.fa.gov.tw (accessed on 24 February 2012).
7. Dall, W.; Hill, J.; Rothsliberg, P.C.; Staples, D.J. The Biology of Penaeidae. In Advances in Marine Biology; Blaxter, J.H.S., Southward, A.J., Eds.; Academic: New York, NY, USA. 1990; Volume 27.
8. Tzeng, T.D.; Yeh, S.Y. Growth parameters of red-spot shrimp, Metapenaeopsis barbata, from the adjacent waters off Taichung harbor. J. Fish. Soc. Taiwan. 1995, 22, 53–68.
9. Tzeng, T.D.; Chiu, C.S.; Yeh, S.Y. Comparison of multivariate allometric coefficients in red-spot prawn (Metapenaeopsis barbata) from adjacent waters off Taiwan. J. Fish. Soc. Taiwan 1998, 25, 85–92.
10. Tzeng, T.D.; Chiu, C.S.; Yeh, S.Y. Morphometric variation in red-spot prawn (Metapenaeopsis barbata) in different geographic waters off Taiwan. Fish. Res. 2001, 53, 211–217.
11. Chu, T.J.; Wang, D.; Haung, H.L.; Lin, F.J.; Tzeng, T.D. Genetic variations and expansion of whiskered velvet shrimp (Metapenaeopsis barbata) off China and Taiwan inferred from intron sequence. Biochem. Syst. Ecol. 2011, 39, 520–525.
12. Chu, T.J.; Wang, D.; Haung, H.L.; Lin, F.J.; Tzeng, T.D. Population structure and historical demography of the whiskered velvet shrimp (Metapenaeopsis barbata) off China and Taiwan inferred from the mitochondrial control region. Zool. Stud. 2012, 51, 99–107.
13. Pettay, D.T.; LaJeunesse, T.C. Microsatellite loci for assessing genetic diversity, dispersal and clonality of coral symbionts in “stress-tolerant” Clade D Symbiodinium. Mol. Ecol. Resour. 2009, 9, 1022–1025.
14. Ma, H.Y.; Bi, J.Z.; Shao, C.W.; Chen, Y.; Miao, G.D.; Chen, S.L. Development of 40 microsatellite markers in spotted halibut (Verasper variegatus) and the cross-species amplification in barfin flounder (Verasper moseri). Anim. Genet. 2009, 40, 576–578.
15. Guyomard, R.; Mauger, S.; Tabet-Canale, K.; Martineau, S.; Genet, C.; Krieg, F.; Quillet, E. A Type I and Type II microsatellite linkage map of Rainbow trout (Oncorhynchus mykiss) with presumptive coverage of all chromosome arms. *BMC Genomics* **2006**, *7*, doi:10.1186/1471-2164-7-302.

16. Zhang, T.; Kong, J.; Wang, W.; Wang, Q. Genetic variability assessed by microsatellites in the breeding populations of the shrimp *Penaeus* (Fenneropenaeus) *chinensis* in China. *Aquaculture* **2010**, *310*, 229–233.

17. McDonald, G.J.; Danzmann, R.G.; Ferguson, M.M. Relatedness determination in the absence of pedigree information in three cultured strains of rainbow trout (Oncorhynchus mykiss). *Aquaculture* **2004**, *233*, 65–78.

18. Rice, W.R. Analyzing tables of statistical tests. *Evolution* **1989**, *43*, 223–225.

19. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; ColdSpring Harbor Laboratory Press: New York, NY, USA, 1989.

20. Lunt, D.H.; Hutchinson, W.F.; Carvalho, G.R. An efficient method for PCR-based isolation of microsatellite arrays (PIMA). *Mol. Ecol.* **1999**, *8*, 891–893.

21. Chiang, T.Y.; Lee, T.W.; Lin, F.J.; Huang, K.H.; Lin, H.D. Isolation and characterization of microsatellite loci in the endangered freshwater fish *Varicorhinus alticorpus* (Cyprinidae). *Conserv. Genet.* **2008**, *9*, 1399–1401.

22. Cifarelli, R.A.; Gallitelli, M.; Cellini, F. Random amplified hybridization microsatellites (Rahm)—Isolation of a new class of microsatellite-containing DNA clones. *Nucleic Acids Res.* **1995**, *23*, 3802–3803.

23. Rozen, S.; Skaletsky, H. Primer3 on the WWW for General Users and for Biologist Programmers. In *Bioinformatics Methods and Protocols*; Krawetz, S., Misener, S., Eds.; Humana Press: Totowa, NJ, USA, 2000; pp. 365–386.

24. Coombs, J.A.; Letcher, B.H.; Nislow, K.H. CREATE: A software to create input files from diploid genotype data for 52 genetic software programs. *Mol. Ecol. Resour.* **2008**, *8*, 578–580.

25. Excoffier, L.; Lischer, H.E.L. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Res.* **2010**, *10*, 564–567.

26. Raymond, M.; Rousset, F. Genepop (Version-1.2) population genetics software for exact tests and ecumenicism. *J. Hered.* **1995**, *86*, 248–249.

27. Weir, B.S.; Cockerham, C. Estimating F-statistics for the analysis of population structure. *Evolution* **1984**, *38*, 1358–1370.

28. Rousset, F. Genepop’007: A complete reimplementation of the Genepop software for Windows and Linux. *Mol. Ecol. Res.* **2008**, *8*, 103–106.

© 2012 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).