Indian J. Fish., 62(2): 29-32, 2015

Parentage determination in the freshwater prawn *Macrobrachium rosenbergii* De Man, 1879 using microsatellite markers

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ABSTRACT

Macrobrachium rosenbergii is an important cultured species in India and other tropical and sub-tropical countries. Selective breeding and genetic improvement of this species poses a big challenge, due to the problems associated in the identification of the pedigree. Physical tagging of the progeny for pedigree identification is costly, and it takes long time and induces bias in the estimation of genetic parameters. Microsatellites are commonly used to detect parents and their offsprings in wild and captive populations, to assign parentage. In the present study. a total of 1788 specimens comprising 160 offsprings and 18 female parents were used for pedigree assigning. Four *M. rosenbergii* microsatelIites were selected for parentage assignment and CERVUS-3.0 software was used to determine the parentage. The average number of alleles per microsatellite marker was 3, with the allele size ranging from 184 to 246 bp, the polymorphic information content (PIC) was 0.412 and the observed average heterozygosity was 0.548. The combined exclusion probabilities for the first and second parent were 0.422 and 0.640, respectively. The success of assigning the offsprings to one parent was 69.23%. Results of the present study indicates that microsatellites can be employed to identify the parentage in *M. rosenbergii*. However, the number of loci needed and the type of microsatellites, needs to be standardised further for enhancing the rate of success.

Keywords: *Macrobrachium rosenbergii,* Microsatellite markers, Parentage determination

Introduction

Giant freshwater prawn *Macrobrachium rosenbergii* is an important cultured prawn species of India. Even though *M. rosenbergii* is having immense potential for aquaculture, its production in India is not increasing as desired due to various reasons *viz.*, lack of readily available wild and pond matured broodstock and post-larvae, lack of understanding of genetic aspects of broodstock management and also due to misguided perceptions of a low potential for genetic gain. *M. rosenbergii* being one of the important farmed prawn species in India, the technology of captive production is sufficiently advanced to undertake genetic improvement program in this species.

For conducting appropriate selective breeding programs, pedigree information is the basic step and in *M. rosenbergii*, such information can be obtained if different families are reared in separate tanks, until they can be physically tagged. Management of families in separate tanks is cost intensive as the animals need to be reared family-wise till they attain a taggable size and this also induces bias in estimation of genetic parameters. Conventionally, physical tags, such as coloured elastomer implants, are being used to maintain family identity in aquacultured crustacean species (Jerry *et al*., 2001; Arce *et al*., 2003). However, their application to breeding programs involving shrimp has several drawbacks, *viz*., physical tagging requires animals to be reared family-wise, which requires infrastructure in terms of space. It is also size dependent and the tagged animal becomes commercially unsalable and that tagging is a costly and labour intensive process. All these factors limits the number of animals, from each family, that can realistically be tagged.

As an alternative to physical tagging, pedigree information from mixed families can be obtained through parentage assignment using highly polymorphic genetic markers such as microsatellites (Harris *et al*., 1991; Wright and Bentzen, 1994). Parentage assigning through DNA markers helps to rear all families in common environment which enables the estimation of breeding values without bias and it also provides an opportunity to tag more number of animals per family and also saves on time. A microsatellite is a simple DNA sequence that is repeated several times at various points in the organism's DNA. These are highly polymorphic, co-dominant and inherited in Mendelian fashion which makes them more suitable for pedigree analysis (O'Connell and Wright, 1997; Chistiakov *et al*., 2006). Several microsatellite markers have been identified and reported in *M. rosenbergii* (Chand *et al*., 2005;

Charoentawee *et al*., 2006; Divu *et al.,* 2008; Bhat *et al*., 2009; Min-See *et al*., 2009). The present study was envisaged to assess the feasibility of correctly assigning individuals of *M. rosenbergii* to their respective family groups using microsatellite markers.

Materials and methods

Biological samples

Matured *M. rosenbergii* samples, originating from natural population of Narmada River of Gujarat State, India, were collected and bred at the freshwater prawn hatchery, Central Institute of Fisheries Education (CIFE), Mumbai. Post-larvae (PL) were obtained from randomly selected berried females which were mass bred in the hatchery. Ten PL each from 16 single mother families were randomly collected and preserved in absolute alcohol (Sterling Chemicals and Alcohols Pvt. Ltd) for further analysis. Pleopods from 18 female brooders were also collected and each sample was preserved separately with proper labeling in absolute alcohol. A total of 160 PL and 18 female brooders were genotyped and used in the present study.

Genomic DNA extraction and microsatellite analysis

Genomic DNA was isolated from whole PL samples and pleopods of broodstock, according to the SDS phenol/chloroform method described by Sambrook *et al.* (2001). The quality of DNA was checked on 0.8% agarose gel and quantification was carried out in Biophotometer (Eppendorf, Germany). In the present study, eight microsatellite markers, reported by Bhat *et al*. (2009), having 4 to 5 alleles per locus and with the observed heterozygosity ranging from 0.78 to 0.86, were used. All the eight markers were amplified using a random sample of DNA from offspring and female parents. Out of eight, four microsatellite markers that showed proper amplification and polymorphism, were used for assigning pedigree (Table 1). Primers were commercially synthesised by Bioserve Biotechnologies Pvt. Ltd., Hyderabad. Genomic DNA was amplified in sterile 0.2 ml PCR tubes using BioRad PCR system. Amplified microsatellite DNA was separated by Polyacrylamide gel electrophoresis (PAGE) using 12% gel.

Parentage assignment

The allele frequencies and the expected heterozygosity for the four microsatellite markers were estimated from the genotype data of the offspring and 18 probable female parents using the POPGENE 1.31 (1999) software. Polymorphic Information Content (PIC) and parentage assignment in the form of paternity non-exclusion probability; NE-1P (First parent), NE-2P (second parent) and combined non-exclusion probability; NE-PP-combined non-exclusion probability (parent pair) and NE-I - combined non-exclusion probability (identity) were estimated with CERVUS 3.0 (2010) program (Kalinowski *et al.,* 2007). In the present study, female parents were used as candidate parents and tested for a parent-offspring relationship against offspring from 16 families. The log-likelihood (LOD) score for each paternity inference was estimated as the natural logarithm of the combined likelihood ratio obtained at each studied locus. The statistic delta (Δ), defined as the difference between LOD scores of the most likely female parent and the next most likely female parent, was calculated in order to discriminate among the non-excluded parents.

Using allele frequencies of the study population, a simulation module of the CERVUS 3.0 was used to generate criteria to estimate the Δ for assigning the maternity to the most likely female with a known level of statistical confidence. The parameters used for the simulation run were 10,000 replication cycles, a pool of 18 candidate maternal parents (100% of the candidate parents sampled and genotyped) and 90% of loci typed. Simulation results were then used to determine a threshold delta score, above which identified parent-offspring pairs can be considered as true relative at 80 and 95% confidence levels.

Results and discussion

Genetic diversity and characterisation of microsatellite loci

Four out of eight microsatellite loci assayed in this work showed appropriate amplification and polymorphism for parentage analysis in this population (Table 1). The average number of alleles per locus was three, the average

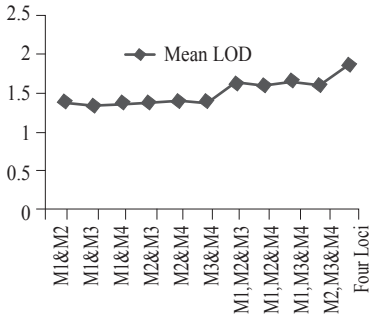
Table 1. Microsatellite primers and optimum annealing temperatures

Loci	Primer sequence	T_A(^{0}C)	K	Size (bp)	H_{exp}
Mr2-4	F-5' TTGCACTTCACACTTAACTGAA 3' R-5' GTTGGGCCTGTGGAGTTAGA 3'	56	5	190	0.605
Mr4-8	F-5' TCAGTGCTGGGGTGTGAA 3' R-5' TGTCCTAGGATGAGGAAAGCA 3'	54	5	219	0.688
Mr5-26	F-5' GGCTCAAGAACGCTATGAGG 3' R-5' TCAAAGACCCAATTACTGCTCA 3'	57	5	246	0.809
Mr1-38	F-5' TCTGCAAGCACCAATGTCTC 3' R-5' TTCAGGTATCTGGCTGAAGTGA 3'	59	5	184	0.777

Where T_A - Annealing temperature, K - Number of alleles, H_{exp} - Expected heterozygosity

observed heterozygosity was 0.548, the average expected heterozygosity was 0.570 and the mean PIC value was 0.412 (Table 2). The genotypes observed were used for genetic diversity analysis of the female parents and the offspring sampled. The families studied did not deviate significantly from the Hardy-Weinberg Equilibrium (HWE) for overall and for individual markers, except nine families, which showed significant deviation from HWE for the marker Mr5-26.

The four selected microsatellites showed moderate genetic variation in similar ways, to that previously described for this species for the same markers (Bhat *et al*., 2009). It has been reported that the number of alleles per microsatellite marker ranged from 2 to 26 and the observed heterozygosity ranged from 5 to 56% (Divu *et al.,* 2008; Min-See *et al*., 2009). Among the 4 markers studied, three exhibited HW equilibrium whereas Mr5-26 was in HW disequilibrium.



M1: Mr2-4, M2: Mr4-8, M3: Mr5-26, M4: Mr1-38

Fig. 1. Relationship between mean LOD score and the loci used for simulation

Table 2. Genetic diversity estimates for the four microsatellite markers

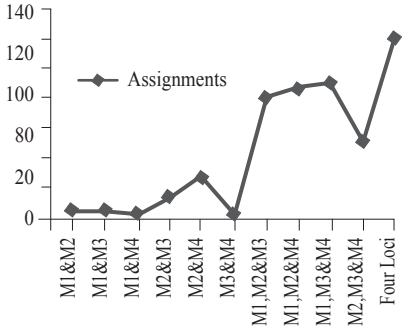
Locus	K	N	H_{obs}	H_{exp}	PIC	NE-1P	NE-2P	NE-PP	NE-I	HW
Mr2-4	3	178	0.489	0.517	0.438	0.867	0.750	0.618	0.312	NS
Mr4-8	3	178	0.478	0.494	0.391	0.879	0.793	0.685	0.359	NS
Mr5-26	3	178	0.747	0.51	0.405	0.871	0.783	0.672	0.345	***
Mr1-38	3	178	0.478	0.507	0.413	0.872	0.773	0.654	0.337	NS
Total	3	178	0.548	0.507	0.412	0.579	0.36	0.186	0.013	NS

N - Number of individuals; K - Number of alleles per locus; H_{obs} - Observed Heterozygosity; H_{exp} - Expected Heterozygosity, PIC - Polymorphic Information Content; NE-1P - Non-exclusion probability (First parent); NE - 2P - Non-exclusion probability (second parent); NE- PP - Combined non - exclusion probability (parent pair) NE-I - Combined non-exclusion probability (identity), HW - Deviation from Hardy-Weinberg equilibrium.

Parentage assignment

The polymorphic information content and combined non-exclusion probability were 0.412 and 0.013, respectively (Table 2). The CERVUS 3.0 with 10000 iterations gave a mean LOD score of 1.93 when all the four markers were used and critical LOD score was 5.25 (95% confidence levels) while the relaxed LOD score was 3.96 (80% confidence levels) and were found to be the best simulation parameters for parentage assigning in the present study. The PIC and the expected heterozygosity were moderate in nature and probably leading to a low non-exclusion probability. In the present study, a total of 69.2% offspring were assigned correctly to the 13 families and the assignment success for 160 progeny was 91%, which is much lower than reported for other species. The allocation of the parents increased as the LOD was relaxed and more allocation was seen when LOD was estimated with 80% confidence interval (Fig. 1). Varying degrees of accuracies for parentage assignment have been reported previously. Similar results, as in the present study, were reported by Perez-Enriquez *et al*. (1999) in red sea bream (*Pagrus major*) and by Olsen *et al.* (2001) in Chinook salmon (*Oncorhynchus tshawytscha*) using four and six microsatellite markers, respectively. However, very high allocation rates have been reported by Norris *et al*. (2000) and Jackson *et al*. (2003) in Atlantic salmon (*Salmo salar*) and in Atlantic halibut (*Hippoglossus hippoglossus*), respectively. A low allocation rate of 47% has been reported in Marsupenaeus(*Penaeus*) *japonicus* by Jerry *et al*. (2004).

In the present case, the accuracy of parentage allocation was maximum when all the four loci were used, which was followed by the combination of Mr2-4, Mr4-8 and Mr1-38 loci (Fig. 2). The success rate of allocating offspring to the parent is directly related to the number of markers used for pedigree analysis and the polymorphic status of the markers (Fishback *et al.,* 1999; Jackson *et al*., 2003). The obtained non-exclusion



No. of assignments at 80% confidence level

M1: Mr2-4, M2: Mr4-8, M3: Mr5-26, M4: Mr1-38

Fig. 2. Relationship between parentage assignments and the loci used for simulation

probabilities may be improved by incorporating more loci with high PIC. When the allocations were tested by using two, three and four markers it was observed that the non-exclusion probability increased with increase in the number of loci. The deviation of HWE in Mr5-26 locus may be due to sampling and inclusion of more number of families and progeny per family may help to reach the equilibrium. There is also need to verify the independent inheritance of the selected markers for more effective use in parentage assignment.

The ability to infer pedigree structure using molecular markers could greatly help to overcome the limitations of physical tagging and may help to extend the application of selective breeding to a number of aquacultured species. The present study indicates that microsatellites may be employed to identify parentage in *M. rosenbergii*, However, for enhancing the accuracy, the number of markers needed and the type of microsatellites, needs to be further standardised.

Acknowledgements

The Authors are grateful to Dr. W. S. Lakra, Director and to Dr. Dilip Kumar, former Director, Central Institute of Fisheries Education (CIFE) Mumbai for the facilities provided to carry out the work. The first author acknowledges the financial support provided by CIFE, Mumbai in the form of fellowship.

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Date of Receipt : 25.10.2012
Date of Acceptance : 27.10.2014