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Evaluation of Published Microsatellites for Paternity Analysis in the Pacific White Shrimp *Litopenaeus vannamei*

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ABSTRACT: Selection of male and female shrimp broodstock for the production of high-quality fry is a primary goal of selective breeding programs of economically important species. Mating a single male with multiple females or a single female with multiple males following by determination of the performance of the progeny is one approach for the selection of suitable male broodstock. In these experiments, a single female of the Pacific white shrimp *Litopenaeus vannamei* was mated to two males using artificial insemination with a mixture of two spermatophores, one from each male. Paternity of 50 individual postlarvae was determined at the PL5 stage with microsatellite genetic markers, by comparing genotypes of the offspring with those of the males and female. The study was done in three crosses and using three microsatellite loci. Paternity of the offspring was successfully determined and it was also found that, in two out of three crosses, the percentage of offspring generated from different males was significantly different ($P<0.01$). The method could be relevant for future selection of male broodstock with desirable traits.

KEYWORDS: *Litopenaeus vannamei*; microsatellite; selective breeding; artificial insemination.

INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is one commercial species for the shrimp farming industry that has undergone a long history of genetic selection for performance improvement[1,2]. In selecting male and female broodstock for a breeding program, traits such as fecundity[3], growth and disease resistance[4] are evaluated. Male contributions to the performance of the progeny can be evaluated by mating a single female to several males. In shrimp, this has been achieved by mating a female to several males, one male at a time. However, these comparisons may be subject to wide experimental variation resulting from differences in the age, health, nutritional status, etc., of the female and the rearing environment of the progeny for individual spawning events. To remove some of these experimental variables, the female can be mated at the same time to two or more males, and the progeny, are produced and reared under the same environmental conditions. In this case, the paternity of individual offspring can be determined through microsatellite DNA-based identification of individual offspring. The technique has been successfully applied for family identification of many species[5,6], including oyster[7] and goat[8]. In the marine shrimp *Fenneropenaeus chinensis*, 4-5 loci are needed to assign parentage of offspring with accuracy of more than 90%[9]. Microsatellite identification of offspring and parentage has been performed routinely in the selective breeding program of the black tiger shrimp *Penaeus monodon*[10]. In the European lobster *Homarus gammarus*, microsatellite marking of communal larvae was used to identify families and different growth rates and survival of the offspring from different families were observed[11].

This study aimed to develop a method for male broodstock selection in *L. vannamei* by performing "single-sex multiple mating" and genotyping broodstock and offspring with microsatellite genetic markers. The results of this study are relevant to future selection of *L. vannamei* with desirable traits.

MATERIALS AND METHODS

Animals. L. vannamei broodstock were stocked in round maturation tanks 5 m in diameter and 0.5 m

deep with seawater at a 1:1 male to female ratio. Shrimp were tagged with colored visible implanted elastomer (NMT Inc., Seattle, WA) to facilitate individual identification. Females were unilaterally eyestalk ablated and maintained with a reverse artificial photoperiod (12:12 h, light/dark). Commercial pellets and fresh feed (adult *Artemia*, polychaetes and squid) were provided 5 times daily (0800, 1000, 1300, 1630 and 2200 h). Water qualities (temperature: 28 ºC, dissolved oxygen: 5 ppm, salinity: 34 ppt, pH 8.0) were monitored daily to assure an optimal environment for shrimp health.

One spermatophore was extracted manually from each of two male broodstock. The maturity of the spermatophores was confirmed by their appearance as a whitish mass at the opening of the gonopores. Gravid females were selected according to external observation of ovarian development. The sperm masses from the two spermatophores were gently mixed before applying to the thelycum of the female (Fig. 1). A single

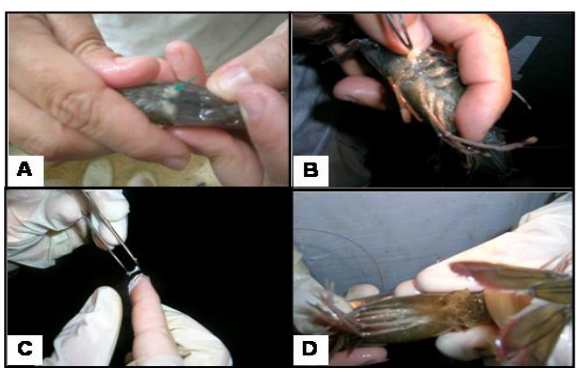

Fig 1. Artificial insemination process: (A) *Litopeneaus vannamei* male broodstock with fully developed spermatophores (arrow), (B) One spermatophore from two fathers was manually extracted, (C) The two spermatophores from two male were mixed together, (D) Mixed sperm were attached to the thelycum of the female.

piece of the last segment of pleopod from the female and two males of the same cross was removed and individually fixed in 95% ethanol for DNA extraction. Three crosses, A, B and C, were performed. At the time of spawning, the females were separated into individual spawning tanks. The amount of nauplii produced varied from 59,000-62,000 per female. Nauplii from the same female (cross) were stocked into each 200 liter larval rearing vessel. Larvae were cultured at the temperature and salinity of 32 ºC and 30 ppt, respectively. They were fed with the diatom *Thallasiosira weissflogi* during zoea 1-3 stages, followed by freshly hatched *Artemia* supplemented with commercial feeds during mysis and early postlarva (PL) stages. At 5 days post metamorphosis to PL (PL5), 50 PL5 were fixed in 95% ethanol for DNA extraction and analysis.

DNA Extraction. Individual pieces of fixed pleopods from the males and females were rinsed three times with distilled water, transferred to another microcentrifuge tube containing one ml of preservative solution (8 M urea, 1% n-lauryl-sarcosine, 20 mM sodium phosphate, 1 mM EDTA, pH 6.8), and incubated at 65 ºC for three days. The PL5 were individually transferred to a 96-well plate containing 50 µl of preservative solution, for a two-day incubation at 65 ºC. Total DNA was extracted from the pleopods and individual PL5 with the Sprintprep plasmid purification system according to the manufacturer's protocol (Agencourt, Beverly, MA).

Genotyping. The broodstock and progeny were genotyped at three microsatellite loci, CNM-MG398, CNM-MG487, and *Pvan*017; all of which were derived from sequencing of *L. vannamei* genomic libraries[12] (Table 1). Unlabeled primers were purchased from Bio-

Table 1. Microsatellite loci and primer sequences for the detection of broodstocks and offspring (from Perez, *et al.,* 2005[12]).

Microsatellite Loci	Primer sequences Forward & Reverse (5'→3')	Annealing temperature (ºC)
CNM-MG 398	F: GGG AAG AAT ATG TAA TG	45-25
	R: TAA CAA GTG CCT GAA A	
CNM-MG 487	F: GAC AGA CAG TGG TGG CG	45
	R: CGT TCT CCT TGC GTG ATG	
PVAN- 017	F: ATG GTG AAT ATA AGG AAG CT	42
	R: TGT GAT ATG GTT TTT GGA G	

Synthesis and Operon Technologies and labeled primers (labeled with Beckman fluorescent phosphoramidite dyes) were purchased from Proligo (Boulder, CO). The forward primers were labeled with one of the Beckman fluorescent phosphoramidite dyes: D3 (CNM-MG 398, *Pvan*362), D4 (CNM-MG487) and D2 (*Pvan*017). Loci were amplified from total genomic DNA in 20 µl reaction volumes containing 10 ng template DNA, 0.25 µM forward and reverse primers, 0.2 mM each dNTP, 2 mM $MgCl_2$ and 1 unit of *Taq* DNA polymerase (Promega, Madison, MI). Amplification parameters were 3 min at 94 ºC; followed by 35 cycles of 30 sec at 94 ºC, 30 sec at the optimal annealing temperature (42 ºC for *Pvan*017, 45 ºC for CNM-MG478, and a touchdown protocol for CNM-MG398 starting at 45 ºC and reduced 2 ºC every 3 cycles), 30 sec at 72 ºC; and a final extension of 60 min at 72 ºC. Amplified products were separated on a CEQ 8000 automated sequencer (Beckman Coulter, Fullerton, CA) along with a fluorescently labeled 400-bp size standard. Genotypes were scored using the Beckman Coulter CEQ 8000 fragment analysis software. Differences in paternity were

analyzed using a chi square test.

Results

The genotypes of the parents of three crosses and the predicted genotypes of their offspring at three loci were determined with Beckman Coulter CEQ 8000 fragment analysis software (Fig. 2). The genotypes of the males and females are shown in Table 2, with the predicted genotypes of the offspring at locus CNM-MG 487 as an example. The occurrence of null alleles at some loci was inferred from the microsatellite alleles observed for broodstock and related offspring. Using all three loci, offspring were unambiguously assigned to one of the two males for each cross (Table 3). The results from cross A indicated that 82% of the offspring were fathered by male M004 ($p < 0.01$). Similarly, the contributions of the two potential male broodstock were significantly uneven for crosses B ($p<0.01$), but not for cross C. Because of some null allele results, not all genotypes could be assigned for all individuals.

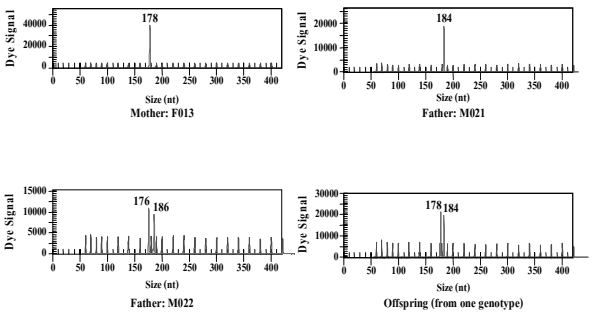

Fig 2. An example of electrograms of parental and offspring of cross C amplified by CNM-MG 398 primers and analyzed on a CEQ 8000 analyzer. Fragment size in base pairs shown on horizontal axis, arbitrary fluorescence on the vertical axis.

Table 2. Genotypes of broodstock and predicted genotypes of offspring. Alleles are given as observed size of the amplified products or as an inferred null allele. Genotypes from locus CNM-MG 487 are shown here as an example. F, female; M, male

Cross A: F002 x M003 x M004					
		M003: 297/null		M004: 291/294	
F002	288	288/297	288/null	288/291	288/294
	297	297/297	297/null	297/291	297/294

Cross B: F008 x M011 x M012					
		M011: 288/297		M012: 291/294	
F008	294	294/288	294/297	294/291	294/294
	297	297/288	297/297	297/291	297/294

Cross C: F013 x M021 x M022					
		M021: 294/297		M022: 291/294	
F013	288	288/294	288/297	288/291	288/294
	294	294/294	294/297	294/291	294/294

Table 3. Paternity analysis of three crosses of *L. vannamei* PL5.

	Paternity Assignment		Unassigned		Significance
Cross A	M003	M004	Mismatch	No Score	
	3 (6%)	41 (82%)	2 (4%)	4 (8%)	$P < 0.01$
Cross B	M011	M012	Mismatch	No Score	
	4 (8%)	46 (92%)	0	0	$P < 0.01$
Cross C	M021	M022	Mismatch	No Score	
	19 (38%)	31 (62%)	0	0	$P > 0.05$

Discussion

This study shows the potential for analyzing male broodstock performance by inseminating a single female with sperm from two *L. vannamei* males at the same time. Parameters of the progeny that could be compared without bias are the survival rate from nauplius to PL, metamorphosis rate (from nauplius to zoea, mysis and postlarva) and size of late PL. These parameters are very important for hatchery practice and could be improved as a result of proper selection of high-performance males by this method. The mismatch and no-score microsatellite marking observed in this study is probably due to degraded DNA that results in no PCR amplification; a similar result was observed in the microsatellite study of the European lobster *H. gammarus*[11].

The difference in the contribution to offspring of the two males could be due to differences in the number or quality of sperm in each spermatophore of individual males or in the survival rate from nauplius to PL5 of the two populations. In the selective breeding program, the males are selected based on their ability to produce sperm that have high potential to fertilize eggs and result in offspring with high survival rates. In *L. vannamei*, older and larger males produce better quality sperm than younger and smaller ones[13]; therefore, the difference in offspring contribution from the two males in this study could be due to differences in age and body weight as well.

Ideally, the microsatellite loci used to determine paternity should produce bands (scores) with are distinctly different, without null alleles and heterozygotes[14]. While it is true that the higher number of loci used the more accurate the determination of paternity would be, the use of too many loci also becomes cumbersome in practice. For comparison of two males, it is possible to use only one suitable locus,

but if more males are compared, it is likely that a higher number of loci would have to be employed. Selection of suitable microsatellite loci specific for each cross is, therefore, very important for this work.

Conclusion

By inserting two spermatophores from two different males into one female broodstock of *Litopenaeus vannamei*, paternity of the offspring at postlarval stage could be identified with microsatellite markers. This method can be used to compare the performance of two or more males regarding fertilization capability, survival rate and growth rate of the offspring, generated from the same female broodstock, at the same time and under the same environment.

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