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Parentage determination of Kuruma shrimp *Penaeus* (*Marsupenaeus*) *japonicus* using microsatellite markers (Bate)

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Abstract

The application of microsatellite markers for parentage determination is gaining both acceptance and popularity in aquaculture. In this study we used simulations and controlled matings to examine the potential of microsatellite markers in assigning parentage to Kuruma shrimp (*Penaeus japonicus*) progeny. Simulations based on allele frequency data from a captive population of *P. japonicus* demonstrated that at least five loci would be required to assign progeny to their correct maternal parent (with 95% confidence) when drawn from a breeding population of 30 dams and 150 putative sires. Based on this information, nauplii from 22 matings where maternal parents were known were typed at six microsatellite loci and subjected to parentage analysis. Assignment success of progeny to their "true" mother was lower than predicted by the simulations, with only 47% of progeny assigned correctly. Null alleles and allelic dropout resulting from poor quality DNA contributed to this disparity. The benefits of DNA parentage analysis as a tool to retain pedigree information in shrimp selective breeding programs are discussed.

Keywords: Microsatellites; DNA parentage; Maternity; *Penaeus japonicus*; Shrimp

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1. Introduction

Progress in the domestication and genetic improvement of farmed shrimp has varied considerably among species and geographic regions (Preston and Clifford, 2002). In general, the uptake of genetic technologies to improve the productivity of farmed shrimp has been relatively slow compared to that of most agrifoods. Reasons for the slow progress include ready availability of wild broodstock and postlarvae, a lack of understanding of shrimp reproductive biology, as well as misguided perceptions of a low potential for genetic gain. Lack of progress is most acute in the Eastern Hemisphere where the predominant *Penaeus monodon* industry is almost totally reliant on offspring from wild broodstock (Preston et al., 2001). The recognition that reliance on wild broodstock for production is non-sustainable has led to increased efforts to domesticate various shrimp species worldwide in order to improve the sustainability of postlarval supplies, reduce risks of disease and gain benefits from selective breeding. Along with domestication comes the opportunity to increase the productivity of shrimp farming through genetic improvement.

One shrimp species farmed in Australia where the technology of production is sufficiently advanced to allow implementation of a genetic improvement program is the Kuruma shrimp, *Penaeus* (*Marsupenaeus*) *japonicus*. This species occurs naturally throughout the coastal waters of the Indo-West Pacific. The profitability of this relatively small industry is strongly dependent on the cost of production and as a consequence the species is well suited to a genetic improvement program as a method to boost production efficiency (Preston et al., 2001). Preliminary studies on the response to selection for growth rate in the Kuruma shrimp have indicated responses as high as 10.7% per generation, even with modest intensities of selection (i.e. 29%) (Hetzel et al., 2000).

Like all crustaceans, Kuruma shrimp grow by shedding their exoskeleton. This precludes the use of external identification tags such as those routinely used to identify fish and molluscs. Several internal markers are available (e.g. elastomer dyes, passive transponders: Godin et al., 1996; Caceci et al., 1999; Jerry et al., 2001). However, these cannot be utilised if the breeding program is run in conjunction with commercial production, as insertion of tags renders the end product unsaleable. Additionally, farmed shrimp are usually stocked into ponds as postlarvae (~2 mm in size) making them impractical to tag at this small size. Maintenance of reliable pedigree information is one of the most significant barriers to the development of a breeding program for this species.

Parentage inference using highly polymorphic codominant genetic markers is becoming increasingly common in aquaculture genetic improvement programs as a way to establish pedigrees (e.g. Garcia de Leon et al., 1998; Norris et al., 2000). The use of DNA markers such as microsatellites allows progeny from different families to be communally stocked together and retrospectively assigned to their family of origin, provided parental genotypes are known. There is no requirement to mark or segregate the progeny before stocking into the ponds. This means that microsatellite markers can potentially be used to determine family groups in commercial shrimp breeding programs.

Our study was conducted in order to assess microsatellite DNA markers previously developed for *P. japonicus* (Moore et al., 1999) in their ability to assign correct parentage to shrimp progeny. In this study we used computer simulations to predict the number of

loci required to give a specific level of allocation success for a predetermined number of candidate dams and sires. We then used known parentprogeny relationships to test the power of six microsatellite markers to correctly assign parentage under field conditions.

2. Materials and methods

2.1. Computer simulation analyses

The simulation study was designed to estimate the number of loci that would be required to confidently assign parentage to a theoretical breeding population of shrimp. Simulations were based on hypothetical parent and offspring genotypes that were created using allele frequency information from eight previously isolated microsatellite loci (Moore et al., 1999). Additionally, two hypothetical loci were simulated based on an average polymorphic information content (PIC) of 0.8. This brought the total numbers of markers available for the parentage assignment simulations to 10 (Table 1). Parental genotypes were generated assuming Hardy–Weinberg equilibrium and no linkage between loci. Once parental genotypes had been generated, one potential male and female parent were randomly chosen from the pool of potential breeding animals and were designated as the parents of a family group. Progeny genotypes were created based on these sire and dam genotypes assuming no mutation or transmission error between parents and their progeny.

The assumptions made in the simulations were that the breeding program consisted of the mating of 30 dams to any one of 150 candidate sires. This assumption was based on a proposed breeding program where females are each mated in tanks containing up to five males. To mimic an actual selection program 300 progeny (10 per family) from the matings were selected to be genotyped at the 10 microsatellite loci. These 300 progeny were taken to represent the fastest growing individuals in the pond population and would constitute the broodstock for the next round of selection. Parentage assignments were conducted similarly to that for the known maternal parent–offspring analyses as outlined

Table 1
Numbers of alleles (k), expected heterozygosities $H(E)$, polymorphic information content (PIC), and probabilities of exclusion based either on the genotype of one parent known (Excl 1) or no parents known (Excl 2) for the simulation study of 10 microsatellite loci on 480 Kuruma prawns

Locus	k	$H(E)$	PIC	Excl 1	Excl 2
CSPJ002	12	0.868	0.853	0.575	0.732
CSPJ003	22	0.801	0.783	0.466	0.642
CSPJ005	24	0.905	0.899	0.689	0.815
CSPJ008	14	0.743	0.714	0.368	0.548
CSPJ010	14	0.871	0.857	0.587	0.741
CSPJ012	15	0.797	0.770	0.437	0.613
CSPJ014	6	0.562	0.479	0.163	0.285
CSPJ015	6	0.510	0.448	0.135	0.267
Simloci1	9	0.834	0.812	0.500	0.670
Simloci2	10	0.844	0.826	0.525	0.691
Average	13	0.774	0.744	0.445	0.600

below, except it was assumed in the simulations that there was no a priori information on the identity of the true parent. Assignment to parents was performed in a two-step analysis, with the maternal parent determined from a pool of 30 candidate mothers and then the paternal parent drawn from the pool of 150 candidate fathers.

2.2. Parentage identification with known maternal parental information

In order to validate the theoretical expectations predicted by the simulations, as well as to evaluate how reliable the microsatellite loci were in assigning parentage in a real-life commercial breeding program, a breeding experiment was conducted where shrimp nauplii from a known maternal parent could be sampled. Broodstock used in this experiment were derived from three primary sources: mature animals collected from the wild (W), those bred in captivity and selected for fast growth for one generation (G1), and those that had been bred in captivity and selected for fast growth for five generations (H5). Broodstock animals were collected from the ponds in March 2000 and conditioned in 12 tonne sand-based round fibreglass maturation tanks until late September. Filtered (1 μm) 28 °C seawater was supplied constantly to achieve 100% exchange per day. Shrimp were fed to satiation daily with commercially produced shrimp pellets (Higashi maru™) and chopped fresh squid.

Mating groups were established in early August. At mating broodstock were tagged and a pleopod removed for subsequent genetic analyses. The pleopod was frozen and stored at −20 °C. Eyestalk ablation was performed in late September where all broodstock were transferred into groups within non-substrate spawner tanks (i.e. 12 tonne tanks without sand substrate). Ready to spawn females, with stage 4 ovaries (as described by Crocos and Kerr, 1983) were selected from spawner selection tanks and transferred to individual 100 l round plastic tubs supplied constantly with 29 °C, 1 μm filtered and ozone treated seawater. Eggs were collected on 140 μm screens, washed and allowed to hatch. Upon hatching, a subsample of nauplii from each family were sampled and frozen at −20 °C for genetic analyses. In total, 30 families were produced (e.g. 13 W, 9 H5×G1, 8 G1×H5). However, due to the mortality of several females between spawning and the collection of tissues for genotyping only 22 families were used in the genotyping experiment. In total 22 maternal and 168 paternal parents were pleopod sampled and 110 nauplii were collected.

2.3. DNA extractions and marker amplification

To obtain genomic DNA, individual nauplii were pipetted along with 30 μl of PCR buffer (67 mM Tris−HCl (pH 8.8); 16.6 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 0.2 mg/ml gelatin) into the depressions of a 96-well PCR plate. This plate was heated to 99 °C for 15 min, before spinning at 4000 rpm for a further 10 min. Proteinase K (100 μg/ml) was then added to the wells for 2 h, before the plate was heated and spun as before. Following the final spinning, the 96-well plate was stored at −20 °C until required.

Genomic DNA from adult shrimp was extracted from 40 mg of pleopod tissue, (shattered after freezing in liquid nitrogen) using a DNAeasy™ 96 Well Tissue Kit (QIAGEN®) with a 2 h incubation for proteinase K digestion.

Six microsatellite loci were selected for parentage assignment (i.e. CSPJ003, CSPJ010, CSPJ011, CSPJ012, CSPJ014, and CSPJ015; Moore et al., 1999). All reactions were made up to a final volume of 20 μl and contained 0.5 μM of the forward and reverse primers, approximately 50 ng of DNA template, 0.4 units of *Taq* Plus DNA polymerase (Fisher Biotech), 30 μM of each dNTP (Pharmacia Biotech), 3 mM $MgCl_2$, and PCR buffer.

Microsatellite amplifications were performed in 96-well plates using an MJ Research PTC-200 thermocycler and the following protocol: 3 min initial denaturation at 94 °C, 30 cycles of 1 min denaturation at 94 °C, 2 min annealing at 55 °C and 1 min elongation at 72 °C. A final extension at 72 °C for 30 min was used to ensure complete addition of adenine to the PCR product (Smith et al., 1995).

Following amplification the PCR products were diluted, dried down and combined with load dye and Genescan-500 Tamra™ size standard (Applied Biosystems), before being denatured and visualised on a 4.8% denaturing polyacrylamide gel. Results were collected using an ABI 377 Prism DNA autosequencer and analysed using GeneScan® 3.1 and Genotyper® 2.5 software.

2.4. Parentage analysis

Exact tests for conformance to Hardy–Weinberg equilibrium at each locus were performed using the program GENEPOP (Markov chain method; Raymond and Rousset, 1995). Utility of the microsatellite loci to determine parentage in Kuruma shrimp, as well as to the possible presence of null alleles, was assessed using the likelihood-based approach in CERVUS Version 2.0 (Marshall et al., 1998). Null allele frequency estimates of greater than 5% were considered significant. The simulation module within CERVUS was used in a stepwise manner to estimate the required critical differences in the likelihood ratio between the first and second most probable candidate parents. To determine the number of mismatches between known mothers and their offspring, an initial simulation was done with the rate of typing error set at 1%. Because all possible maternal genotypes were known it was assumed that a genetic mismatch between the known parent and offspring were the result of either miss scoring or mutation. Analysis parameters for this simulation run were as follows: 10,000 replication cycles, a pool of 22 candidate maternal parents, 100% of the candidate parents sampled and genotyped, 90% of loci typed. Output from the simulation run was then used in the parentage assignment module of CERVUS to evaluate assignment success to the correct maternal parent when there was no a priori information on the correct parent (i.e. what would occur in a commercial breeding program).

3. Results

3.1. Computer simulations

Summary statistics for the 10 markers used in the simulations of the hypothetical shrimp breeding population are given in Table 1. The microsatellite loci used in the simulations ranged from being low in polymorphism ($k=6$) to highly polymorphic

(k = 24). On average there were 13 alleles per locus, with mean polymorphic information content (PIC) of 0.744. Theoretical total parentage exclusion probabilities based on the genotypes of the first and second parent were 0.998 and 0.999 for the 10 loci, respectively.

Simulations demonstrated that nine loci were required to assign 95% of progeny to both parents, with allocation success highly dependent on the number of loci used (Fig. 1). For example, allocation success for the maternal parent ranged from 11% with one locus to 99.7% if 10 loci were used. Likewise, correct assignment to the paternal parent ranged from 0% to 96.7% with 10 loci. Correct assignment to the maternal parent required fewer microsatellites than did assignment to the paternal parent, obviously due to fewer potential candidates to chose from in the maternal parent.

3.2. Known parent–offspring analyses

In total 302 individuals were genotyped. This included 110 offspring, 22 candidate mothers and 168 candidate fathers. However, due to difficulties in obtaining quality template DNA from some nauplii, there was a moderate proportion of allelic dropout for some of the progeny at one or more loci. Because whole nauplii had originally been used in the DNA extraction phase, re-extraction of DNA was not an option. Consequently, it was not possible to re-run samples to recover this information. To limit the potential loss of resolving power due to incomplete genotypes (i.e. 6 loci), only those progeny typed at three or more loci were included in the parental allocation analyses ($n = 98$).

Allele sizes ranged from 137 to 176 base pairs (bp) at *CSPJ003**, 90 to 116 bp at *CSPJ010**, 189 to 211 at CSPJ011 bp, 103 to 148 bp at *CSPJ012**, 146 to 155 bp at *CSPJ014**, and 110 to 122 bp at *CSPJ015**. Expected heterozygosities ranged from 0.47 at the *CSPJ015** locus, to 0.89 at *CSPJ011**. The six microsatellite loci (Table 2) used in the known parent–offspring analyses exhibited an average PIC of 0.654. This was lower than that predicted by the simulation study. Likewise, exclusionary power when one or both parents are unknown was lower for all loci except *CSPJ015**.

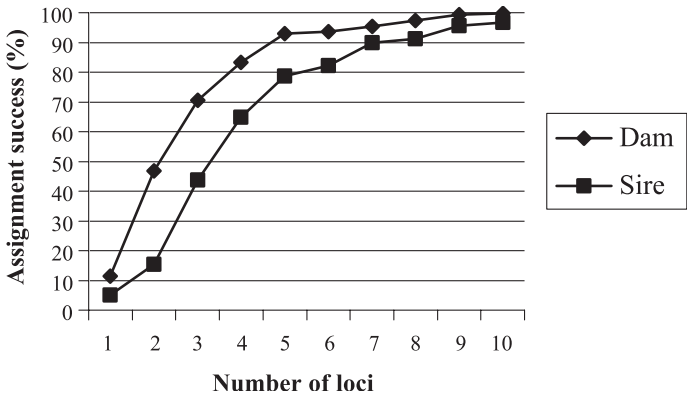

Fig. 1. Cumulative assignment success of Kuruma prawn progeny to correct dam and sire based on hypothetical parent and offspring genotypes.

Table 2
Number of individuals (n) genotyped, number of alleles (k), expected heterozygosities $H(E)$, polymorphic information content (PIC), and probabilities of exclusion based either on the genotype of one parent known (Excl 1) or no parents known (Excl 2) for 302 Kuruma prawns genotyped at six microsatellite loci

Locus	n	k	$H(E)$	PIC	Excl 1	Excl 2	HW	Null frequency
CSPJ003	286	14	0.625	0.600	0.240	0.427	***	+0.023
CSPJ010	280	10	0.809	0.786	0.460	0.637	***	−0.014
CSPJ011	276	18	0.886	0.873	0.624	0.769	***	+0.086
CSPJ012	284	11	0.754	0.722	0.374	0.553	***	−0.024
CSPJ014	265	5	0.564	0.468	0.162	0.271	*	+0.038
CSPJ015	232	5	0.474	0.531	0.149	0.293	NS	+0.062
Average		11	0.695	0.654	0.334	0.590		

HW = Conformance to Hardy-Weinberg expectations, NS = $P>0.05$.
* $P<0.05$.
*** $P<0.001$.

From a total of 489 comparisons, there were 27 cases of mismatching alleles at a particular locus between the known maternal parent and their offspring. Further scrutiny revealed that two-thirds of these mismatches occurred when either the progeny or their parent exhibited a homozygous genotype. The HW test results (Table 2) indicated five out of six markers did not follow HW equilibrium. This strongly suggests the presence of null alleles at up to five of the loci. Expected probabilities of null alleles at each of the loci are given in Table 2. Of the remaining progeny with mismatches not explained by the possibility of null alleles, all but two were different from their parent by a single microsatellite repeat. This suggested either that these individuals had been mis-scored, or that a repeat shift had occurred due to a de novo mutation from the maternal lineage. Although we believe that the most likely cause of the mismatch was due to scoring error, it must be remembered that an individual female can produce up to 150,000+ offspring in a single spawning and the opportunity for mutations in the parental germ cell line to be detected in offspring will be higher than for terrestrial animals. The remaining two progeny mistyped had vastly different alleles from their known parent and it was concluded that this was likely due to mislabelling during the collection stage.

Allocation success was lower than that predicted by the simulation analysis, with only 46 out of the total 98 (47%) progeny subjected to parentage analyses correctly assigned to their true mother. This is compared to 92% predicted by the simulations. Because the earlier simulations demonstrated that nine loci would be required to confidently assign progeny to sire, coupled with the fact that the true sire was not known for each family group, the analysis was restricted to only assigning progeny to the maternal parent.

4. Discussion

When conducting selective breeding programs with shrimp or other highly fecund species, it is important to retain information on genealogical relationships among

individuals over multiple generations. This permits the design of breeding programs that maximize the genetic response for traits such as growth, while minimizing the potential detrimental effects of accumulated inbreeding. Conventionally, physical tags such as coloured elastomer implants have been used to maintain family identity in several important aquaculture crustacean species (Uglem et al., 1996; Linnane and Mercer, 1998; Caceci et al., 1999; Godin et al., 1996; Jerry et al., 2001; Arce et al., 2003). However, their application to breeding programs involving shrimp has several drawbacks; namely that tagging is size limited, the tagged animal becomes commercially unsaleable and that tagging is a labour-intensive process that dramatically limits the number of animals from each family that can realistically be tagged. In highly fecund species maximum selection intensity is achieved via assessment of the total population. Consequently, breeding programs that are restricted to a few individuals per family—as is the case with using tags—may fail to achieve maximum selection intensity thereby limiting potential genetic gain (Doyle and Herbinger, 1994). The use of DNA parentage analyses as a biological tag has the potential to overcome many of the limitations imposed by conventional tagging as it can be non-invasively, retrospectively, applied after the growth phase of animals have been evaluated. A further benefit is that at the same time as progeny are being assigned to parents they can simultaneously be used to estimate genetic relatedness among individuals.

Despite the obvious potential of DNA pedigreeing when applied to aquaculture breeding programs, the present study highlights the importance of experimentally evaluating the performance of markers before they are routinely employed. As highlighted by Wilson and Ferguson (2002), simulation approaches are useful in assessing the probable performance of a marker set of loci with specified allelic distributions; however, they are of limited utility when deciding how many loci are actually required and how these loci may perform under real situations. We evaluated the performance of six microsatellite markers previously isolated by Moore et al. (1999) and found that although we were able to confidently resolve maternal parentage in around 50% of cases, the power of these markers to resolve parentage was much lower than that predicted by our simulations. Simulations based on allele frequencies of parents suggested that six loci would allow assignment of progeny to their correct dam in 92% of cases. In reality, however, assignment success was half this. Discrepancies between the simulations and real data sets were considered to be largely due to the presence of null alleles at four of the six loci that were not accounted for in the simulations, and/or substantial allelic dropout caused by poor quality genomic DNA. For some individuals we had great difficulty in extracting amplifiable DNA from nauplii, possibly due to the presence of polysaccharides or other inhibitors. Consequently, we were only able to genotype around 60% of the larvae at all six loci and this obviously was a significant contributor to the reduced resolution of our marker set. In a real selection program, however, allelic dropout would not be as great a problem as genotyping would be performed on juveniles and/or adult individuals.

The likelihood of finding null alleles using the markers developed by Moore et al. (1999) is not restricted to our experimental population. Sugaya et al. (2002a) also assumed the presence of null alleles at the *CSPJ010**, *CSPJ014** and *CSPJ015** markers when examining their inheritance mode in progeny of seven wild caught *P. japonicus* females

with spermatophores implanted prior to capture. Null alleles appeared to be present in five of the seven families at the various loci genotyped. Indeed, null alleles were found in a subsequent genetic diversity analysis at the same three loci in four Japanese Kuruma shrimp populations (Sugaya et al., 2002b). It appears, therefore, that at least four of the markers of Moore et al. (1999) do not amplify all alleles in *P. japonicus*.

We aimed in this parentage study to utilize the markers of Moore et al. (1999). However, difficulties in amplifying, subsequent scoring, and likelihood of null alleles for many of the loci immediately made it apparent that not every microsatellite is suitable for inclusion in a parentage determination marker suite. Based on the number of loci that we could reject as being suitable from the suite developed by Moore et al. (1999), it is apparent that upwards of twice as many loci as are actually utilized may need to be evaluated before inclusion. Although likelihood-based parentage determination tests similar to that performed here can take account to some degree of mismatches between progeny and parents, the inheritance mode of all markers should still be tested against known offspring–parent relationships. Any markers that exhibit suspect inheritance, difficulties in scoring and so on should be excluded. Indeed, Pemberton et al. (1995) suggested that only individuals heterozygous at a particular locus should be used in parentage tests so as to minimize errors in assignment due to null alleles. We do not take such an extreme view, as due to breeding practices many aquaculture animals have reduced diversity and commonly are homozygous at one or more loci. Instead of discarding this data, once the inheritance mode of alleles has been validated, homozygous individuals can provide valuable information for parentage tests.

4.1. Utility in shrimp breeding programs

One of the major benefits of applying DNA parentage analyses to shrimp breeding programs is that families can be communally reared from birth minimizing potential confounding genetic and environmental influences. Bagley et al. (1994) and Herbinger et al. (1999) demonstrated for salmonids that single tank rearing of full-sib families induces differences in growth performance compared to when reared as mixed family tanks, while Coman et al. (2002) demonstrated significant interactions between family performance of *P. japonicus* when reared at different temperatures. These results indicate that confounding environmental factors can dramatically influence the growth performance of aquaculture species. Because large numbers of *P. japonicus* families can be spawned within a few days of each other, an ability to stock nauplii as mixed families would allow more efficient genetic parameter estimation and selection practices based on both within- and between-family variances to be conducted with a minimal confounding effect of environment. This combined selection approach would maximise the potential for genetic progress over existing within-family approaches by allowing the total genetic variance in the breeding population to be accessed. Equally as important, communal rearing negates the requirement of expensive specialised facilities and labour-intensive tagging schemes. Indeed, once the marker sets are established, DNA parentage analyses may compete favourably in respect to operating costs to those of conventional tagging based breeding programs. Before DNA parentage analysis is routinely applied to breeding programs in *P. japonicus*,

however, additional markers will need to be developed and evaluated to increase the success of allocation.

Although our simulation and validation was restricted to *P. japonicus* it is likely that similar principles apply to other species of farmed shrimp.

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