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Reproductive behaviour of captive *Fenneropenaeus merguiensis*: Evidence for monogamy and high between family variances for offspring number

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ABSTRACT

The purpose of this paper was to determine aspects of the reproductive biology of captively bred *Fenneropenaeus merguiensis* under commercial broodstock production conditions that relate to the design and implementation of genetic improvement programmes for this species.

First, we tested whether there is evidence for polygamy vs monogamy by genotyping females, the material found in their thelycums, and material that leaches out of the thelycum using DNA microsatellite loci. All genotypes in all animals and tissues tested could be accounted for using a monogamy model.

Second we compared the accuracy of pedigrees formed under assumptions of monogamy vs polygamy. Pedigrees were formed using microsatellite genotypes from 73 dams and 400 offspring. Sibship groups and dam–offspring groups from pedigrees developed assuming monogamy almost always had the same mtDNA haplotypes, suggesting a high accuracy of the pedigrees, but those formed under the assumption of polygamy were less accurate, and together these results also support the monogamy model.

Third, we assessed the between family variance in offspring family numbers from two sets of mass spawnings of about 40 inseminated females per spawn. About half of the offspring originated from just 20 percent of the dams, i.e. many dams contributed few offspring. These data can help predict optimal sample sizes required for accurate future estimates of genetic parameters.

1. Introduction

Across aquaculture, new species are being domesticated and new genetic selection programmes developed, including one for the banana shrimp, *Fenneropenaeus merguiensis*. A sound understanding of the reproductive biology of shrimp in captivity, whether the species mating behaviour is polygamous or monogamous and the between family variances in offspring number can assist with the planning and operation of genetic improvement programmes and the collection of data concerning genetic parameter estimates.

The relevance of monogamy vs polygamy under conditions of mass matings in captivity is that monogamy precludes the creation of half sib groups and can limit the estimation of genetic parameters since maternal effects cannot be partitioned from the additive effects (at least in shallow pedigrees). If so, dedicated artificial insemination (AI) experiments may be required for estimation of genetic parameters. Further, in some cases especially where few marker loci are available, or when only DNA from one parent (the mothers) is available, accurate pedigree assignment using DNA markers can rely of which assumption – monogamy or polygamy – is used.

In view of the utility of this information for genetics, there are surprisingly few data whether mating in shrimp species or indeed among the decapod crustaceans is monogamous or polygamous. The lack of data is further surprising given that mating systems are a fundamental aspect of reproductive biology in this group. Those few data available for wild crustacean suggest that mating behaviour is linked to life history traits, territoriality, and habitat type. For example, temporary monogamy was inferred by behavioural and morphometric observations on the symbiotic shrimp *Pinna carnea* (Baeza et al., 2011), and thought to be linked to the commensal behaviour of this shrimp species. The snapping shrimp (genus *Alpheus*) appear to be monogamous, a behaviour thought to the linked to the territorial nature of this species (Mathews, 2002). Both serial monogamy and serial polygamy were observed from behaviour of the American lobster, *Homarus americanus* (Atema, 1986; Cowan and Atema, 1990). Specifically for shrimp, overnight video recordings of *Penaeus monodon* did not reveal polygamous mating behaviour for females (Marsden et al., 2013). These reports have several things in common, namely, the use of behavioural observations during a given time interval as the primary data, that they leave open the possibility of serial polygamy and lastly, the conclusions were not confirmed using DNA markers.

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Another aspect of shrimp reproduction important for genetic planning is the relative contributions of different dams to the offspring in the next generation after mass matings since this variation can impact the efficiency and accuracy of the estimation of genetic parameters. Great variation will limit the numbers of parents and resulting families in the ensuing pedigrees and AI and or a period of separate rearing may be required for efficient experimental designs.

We have assessed using nine newly developed DNA microsatellite markers whether females carry DNA from one or more males in their thelycum. We have also compared the accuracy of pedigrees constructed using DNA markers under assumption of either monogamy or polygamy by checking the pedigrees using mtDNA haplotypes, assuming that real dam–offspring and full sib groups will have the same mtDNA haplotypes. Also, using DNA markers we have estimated the variable contributions to offspring numbers made from mass spawning dams in two different spawning events under commercial conditions.

2. Methods

2.1. Shrimp samples

All shrimp were from the Seafarm farm site at Cardwell, Australia (latitude 18° 16′ 0S, longitude 146° 1′ 60E, altitude 0 m). The daily average temperature in Cardwell is between 14 and 32 °C, with the minimum average of 19 °C and the maximum average of 29 °C over the last 103 years (Australian Bureau of Meteorology, 2012). The water temperature in cultured ponds varies between 25 °C and 32 °C. The annual rainfall is 2129 mm, occurring mainly from December to April with a peak in January, February and March.

For the tests of DNA from males in females, thirty-five thelycums were dissected from sexually mature and potentially inseminated female broodstock at the Seafarm site and shipped from Seafarm to the University of the Sunshine Coast. One eyestalk from each female was also dissected, placed in ethanol along with the dissected thelycum and shipped to USC for analysis. Upon arrival at USC, 16 samples were examined under a dissecting microscope with 8 being identified as containing a spermatophore inside or outside of the thelycum. For all 16 samples, DNA was extracted from the eyestalk plus a section of muscle tissue removed from the underside of the dissected thelycum. Additionally, each tube was centrifuged at 2000 rpm for 5 min and the resulting pellet was extracted for DNA. For the 8 samples visually assessed as having spermatophore present, DNA was extracted from the spermatophore following removal from the thelycum. To compare pedigrees formed assuming polygamy vs monogamy, two pedigrees, A and B, were considered. Pedigree A had 37 known dams and 200 sampled offspring, and pedigree B had 36 dams and 200 sampled offspring. For these pedigrees, whole animals were shipped to USC frozen below −60 °C. All DNA extractions were performed using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. DNA was PCR amplified and subsequently genotyped using nine microsatellite primer pairs developed from 454 next generation sequencing as follows.

2.2. Development of microsatellite loci

Approximately 5 μg of a pooled DNA sample from 20 *F. merguiensis* individuals was submitted to the Australian Genome Research Facility (AGRF; Brisbane, Australia; http://agrf.org.au/) and used to construct a random library that was sequenced on a 454 shot-gun GS-FLX platform (Roche Applied Science; Mannheim, Germany). Sequences were trimmed for length and quality using CLC Genomics Workbench v6 software (CLC Bio, Aarhus, Denmark). We obtained 65 129 reads with an average length of 367 bp and searched for microsatellite loci having a minimum of six repeats for di-nucleotides, and four repeats for tri- and tetra-nucleotides, using the QDDv2b pipeline (Meglécz et al., 2010) and PRIMER 3 (Rozen and Skaletsky, 1999). Primers were designed for 84 microsatellite-containing sequences with suitable flanking regions.

Microsatellite loci were initially screened for successful amplification and variation using eight wild and eight farmed animals and amplified individually in 12.5 μL reactions containing approximately 20 ng of genomic DNA, 1× reaction 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), 200 μM of each dNTP, 250 ng BSA (Roche), 2.0 mM $MgCl_2$, 0.4 μM of each primer, and 0.5 U of *Taq* F1 DNA polymerase (Fisher Biotec; Wembley, Australia). PCR was performed using a MaxyGene® thermocycler (Axygen; Tewksbury, USA) with the following cycling conditions: initial denaturation step at 95 °C for 3 min; 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 45 s; and with a final extension at 72 °C for 10 min. PCR products were visualised on 3.0% agarose 0.6× TBE gels (140 V; ~110 min) stained with EtBr to look for evidence of polymorphism, prior to labelling the forward primer of each of 16 potentially useful microsatellite loci with FAM, VIC, NED or PET fluorescent dyes (Applied Biosystems, Melbourne, Australia).

2.3. Genotyping

Loci with consistent PCR amplification, clear allelic variation, and clarity of electrophoretic signatures were selected for genotyping, nine were used for the thelycum and related samples and ten to determine the pedigrees. Primer pairs were analysed for complementarity and possible interactions using Multiplex Manager software (Holleley and Geerts, 2009) and subsequently grouped into 2 pools (pool 1 with 4 primers and pool 2 with 5 primers) for amplification using Qiagen Multiplex PCR Plus Kits (Qiagen, Germany). Final volumes were optimized (25 μL) to reduce costs. PCR reactions contained ultra-pure water, Multiplex PCR Master Mix (2×), Q-Solution (5×), primer premix (10×) and DNA (10 ng/μL). Cycling conditions were: an initial denaturation at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 90 s, and 72 °C for 30 s; with a final extension at 68 °C for 30 min. PCR products were separated by capillary electrophoresis on an AB 3500 Genetic Analyser (Applied Biosystems) and fragment sizes were determined relative to an internal lane standard (GS-600 LIZ; Applied Biosystems) using GENEMARKER v1.95 software (SoftGenetics; State College, USA) and double-checked manually. Individuals with low or missing peaks were amplified and genotyped a second time. MICRO-CHECKER v2.2.3 (van Oosterhout et al., 2004) was used to look for evidence of large allele dropout, extreme stuttering and null alleles, based on 1000 bootstraps and a 95% confidence interval. Tests for HWE at each locus and genotypic linkage equilibrium among pairs of loci were conducted in FSTAT v2.9.3.2 (Goudet, 2001). Numbers of alleles, observed and expected heterozygosities, and the fixation index (F_{IS}) as a measure of past inbreeding (Wright, 1965) were determined using GENALEX v6.41 (Peakall and Smouse, 2006). Polymorphic information content (*PIC*) was computed in CERVUS v3.0 (Kalinowski et al., 2007).

2.4. Mitochondrial DNA sequencing and analysis

The mtDNA control region (D-Loop, 310 bp) was amplified and sequenced using the primers DLoopF (5′-TCCTCTTGTTTTCCCCCTTT-3′) and DLoopR (5′-GGATTCAATATAGGCATTTAT-3′) (Wilson et al., 2000). Reaction volumes of 25 μL contained approximately 10 ng of genomic DNA, 1× reaction 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), 200 μM of each dNTP, 250 ng BSA (Roche), 1.5 mM $MgCl_2$, 0.4 μM of each primer, and 0.25 U of *Taq* F1 DNA polymerase (Fisher Biotec; Wembley, Australia). PCR was performed using a MaxyGene® Gradient Thermal Cycler (Axygen Scientific) with the following cycling conditions: initial denaturation step at 95 °C for 3 min; 35 cycles at 94 °C for 30 s, annealing temperature of 55 °C for 30 s, and 72 °C for 45 s; and final extension at 72 °C for 10 min. A sample of each PCR product was run on 1.5% agarose