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SHORT REPORT

Genetic evidence for multiple paternity in the mangrove land crab *Ucides cordatus* (Decapoda: Ocypodidae)

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Abstract

Studies on mating patterns of marine crustaceans are rare, but the few studies on brachyuran crabs to date suggest that polyandrous mating is uncommon. In the present study, we used six microsatellite loci to genotype 8–16 larvae obtained from each of 10 gravid females of the crab *Ucides cordatus* (Brachyura: Ocypodidae). Based on the number of non-maternal alleles detected among the offspring, at least 40% of the females were inseminated by more than one male. We suggest forced copulation and maintenance of sperm in spermathecae throughout the year as possible causes of polyandry in *U. cordatus*.

Key words: Mating behavior, polyandry, sexual selection

Introduction

While the male benefits of multiple mating are well known (Bateman 1948), the female advantage of polyandry is more controversial (DiBattista et al. 2008). Female monogamy should be favoured due to the high costs of multiple mating, such as increased risk of predation (Koga et al. 1998), reduced vigilance and mobility during mating, energy and time loss for re-mating, physical injuries, and diseases from copulation (Jennions & Petrie 2000), in addition to the observation that a single mating often ensures fertilization (DiBattista et al. 2008). However, with the advent of new molecular techniques, moderate to high levels of multiple mating in several animals groups have been revealed (Birkhead & Moller 1998).

There are two kinds of explanation for this phenomenon: material and genetic benefits. The former includes parental care (Nakamura 1998), adequate sperm supply (Drnevich et al. 2001), chemical defence (González et al. 1999), transfer of nutrients

(Arnqvist & Andrés 2008) and stimulants (Wagner et al. 2001), and assurance of fertilization by non-sterile males. On the other hand, avoiding genetic incompatibility and inbreeding (Jennions & Petrie 2000), bet-hedging (Fox & Rauter 2003), gaining 'good genes' (Keller & Reeve 1995) and increasing genetic diversity among offspring (Moore & Ball 2002) are examples of potential advantages of polyandry. Knowing the ecological and evolutionary determinants of mating strategies is important for the management and conservation of threatened species (Reynolds 1996; Kichler et al. 1999; Moore & Ball 2002; Neff & Pitcher 2002; Jensen et al. 2006) given that they influence the intensity of sexual selection (Fleming & Gross 1994; Evans & Magurran 1999), the effective population size (Sugg & Chesser 1994) and the genetic variability and introgression within a population (Baer & Schmid-Hempel 1999).

The mangrove land crab *Ucides cordatus* (Linnaeus, 1763) is one of the commonest species in the mangrove ecosystems of the Western Atlantic (Costa 1979). It is present from Florida (USA) to

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Santa Catarina (Brazil) and accounts for a large proportion of animal biomass in many estuaries (e.g. Koch 1999). It is considered to be a keystone species in Brazilian mangroves (Nordhaus et al. 2006) because of its role in processing decaying plant material, in nutrient and energy cycling (Cardoso 2004), soil aeration, and sediment drainage (Blankensteyn et al. 1997). Moreover, *U. cordatus* is among the most important fishery species in northeastern Brazil (Diele et al. 2005). However, a combination of overexploitation (Ministério do Meio Ambiente 2004; Amaral & Jablonski 2005), habitat loss, and Lethargic Crab Disease (Boeger et al. 2005) have greatly affected their stocks, reducing mean body size and numbers (Amaral & Jablonski 2005), leading to an urgent need to develop efficient management strategies to maintain the genetic integrity of local populations (Oliveira-Neto et al. 2007b). The goal of the present study was to use molecular methods to test for the existence of polyandry in *U. cordatus*.

Material and methods

Samples

Ten gravid females of *Ucides cordatus* were collected in Guaratuba Bay, state of Paraná, Brazil, as part of a large-scale initiative for restocking overexploited populations (Silva et al. 2009). Each female was housed separately until she released her larvae into the rearing tanks. Ten larvae at the stage of zoea 1 were collected from each female. Samples of muscle tissue from one of the pereopods of each female were then preserved in EDTA–DMSO buffer (Seutin et al. 1991) at -20°C until their DNA was extracted using the ChargeSwitch kit (Invitrogen®). The larvae had their eyes removed before DNA extraction using the DNeasy kit (QIAGEN®). DNA concentration and purity of the samples were measured with GeneQuant Pro spectrophotometer (Amersham Bioscience®).

Development of microsatellites primers

Polymorphic microsatellite markers were commercially developed by Genetic Identification Services (Chatsworth, CA, USA). Methods for DNA library construction, enrichment and screening were as described previously (Jones et al. 2002). Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes (Rsa I, Hae III, Bsr B1, Pvu II, Stu I, Sca I, Eco RV). Fragments in the size range of 300–750 bp were adapted and subjected to

magnetic bead capture (CPG, Inc., Lincoln Park, NJ, USA), using biotinylated capture molecules.

Libraries were prepared in parallel using Biotin-CA(15), Biotin-GA(15), Biotin-ATG(12) and Biotin-TAGA(8) as capture molecules in a protocol provided by the manufacturer. Captured molecules were amplified and restricted with HindIII to remove the adapters. The resulting fragments were ligated into the HindIII site of pUC19. Recombinant molecules were electroporated into *Escherichia coli* DH5alpha. Recombinant clones were selected at random for sequencing, and enrichment levels were expressed as the fraction of sequences that contained a microsatellite. Sequences were obtained on an ABI 377, using ABI Prism Taq dye terminator cycle sequencing methodology.

The optimal amplification reaction mix for all primer pairs consisted of $1 \times$ BiolaseC Buffer (from $10 \times$ stock solution supplied by manufacturer), 2 mM MgCl_2 , 0.2 mM each dNTP, 6 μM each primer (forward primer fluorescent-labelled), 0.025 U/ μl BiolaseC Taq polymerase, and 0.2 ng/ μl template DNA in 50 μl final reaction volume. Samples were amplified in a Perkin–Elmer–Cetus thermal cycler by an initial 3 min of denaturation at 94°C , followed by 35 cycles of denaturation (94°C , 40 s), annealing (55°C , 40 s), and extension (72°C , 30 s), with final extension time of 4 min at 72°C .

DNA was extracted using the PureGene DNA Extraction KitR kit (Gentra Systems, Minneapolis, MN, USA) following the manufacturers instructions. Microsatellite loci were amplified in 10 μl reactions in the following reaction mix: MgCl_2 , 2 mM; dNTPs (premixed), 0.2 mM each; primers, 0.3 μM each; BioTaq DNA PolymeraseR (Bioline USA, Canton, MA, USA), 0.025 U/ μl ; template DNA, 0.2 ng/ μl . Polymerase chain reaction (PCR) was conducted in a RoboCycler Gradient 96R thermocycler (Stratagene, Inc., La Jolla, CA, USA) by an initial denaturation (94°C , 3 min), followed by 35 cycles of denaturation (94°C , 40 s), annealing (55°C , 40 s), and extension (72°C , 30 s), and a final extension at 72°C for 4 min. PCR products were labelled using one of the conventional sequencing dyes NED, HEX or FAM (Applied Biosystems, Inc.). Amplification products were separated on polyacrylamide gels in an ABA 377 DNA sequencer and sized using Genotyper 2.5 software and Rox 400 HD size markers (Applied Biosystems, Inc., Foster City, CA, USA). All six loci were highly variable, with 10–61 alleles, varying from 0.51 to 0.97 in observed heterozygosity (Table I). The most variable locus was B3, with 61 alleles, and the less variable was C109, with 10 alleles.

Table I. Microsatellite loci details for *Ucides cordatus*, including PCR primer sequence (F, forward; R, reverse), the core repeat motif and its allele size (bp), optimal annealing temperature ($T^{\circ}\text{C}$), number of alleles per population, and observed (H_{O}) and expected (H_{E}) heterozygosity, in population analysis.

Locus	Sequence	Repeat	Size (bp)	T ($^{\circ}\text{C}$)	No. alleles	H_{O}	H_{E}
A5	F: TTG-CTC-GTT-CGT-ATC-CAG-TAG R: ACG-CCT-TAG-AAA-AGA-AGA-AAC-C	(CGAG) ₅ (CA) ₁₄	270	58	14	0.8684	0.8862
A11	F: TGG-TGA-ATG-AGT-CAA-ACT-GAG R: TGC-TTC-CTT-GCA-TAC-ATA-CTG	(CA) ₁₄	182	52	14	0.5781	0.6529
A120	F: AAC-TCC-CGC-TAC-CTC-TCA-CTC R: TGG-TCT-TTG-CAC-ACA-TGA-TTC	(CA) ₁₆	181	55	55	0.5516	0.5577
B3	F: CGA-GGC-ATC-GTC-TTT-AGA-G R: CAA-TCC-GCT-TCC-TAT-TCA-C	(GT) ₁₇	175	55	61	0.9167	0.9722
B124	F: TTT-CCT-CGA-ATG-TAT-AAA-CAG-G R: TCC-ACA-AAG-CGA-CTC-TCT-AG	(GA) ₁₉ (GT) ₁₁	122	55	28	0.8051	0.8360
C109	F: GCC-ATC-ATC-ACC-ATC-ACC R: TCC-CCT-TCT-TCC-TAT-TGT-CC	(CAT) ₆	285	62	10	0.4583	0.5113

Microsatellites analyses

Primer sets for six *Ucides cordatus* microsatellite loci (Table I) were amplified with fluorescence-labelled primers in 10 μl PCR reactions with the following final concentrations: 0.6 μM forward labelled primer (fluorescent), 0.6 μM reverse unlabelled primer, 0.2 mM dNTP, 0.025 U Taq Platinum, 1 \times Buffer, 1.5 mM MgCl and around 0.5 ng/ μl of DNA template. A 3-min denaturing step at 94 $^{\circ}\text{C}$ was followed by 35 cycles of 30 s denaturation at 95 $^{\circ}\text{C}$, 60s annealing at 52 $^{\circ}\text{C}$ (A11), 58 $^{\circ}\text{C}$ (A5) or 62 $^{\circ}\text{C}$ (C109) and 60 s extension at 70 $^{\circ}\text{C}$ followed by a final extension of 1 h at 72 $^{\circ}\text{C}$. Another programme was used for primers A120, B3 and B124: 94 $^{\circ}\text{C}$ for 3 min, followed by 35 cycles 40 s at 94 $^{\circ}\text{C}$, 40 s at 55 $^{\circ}\text{C}$ and 30 s at 72 $^{\circ}\text{C}$, and a final extension of 72 $^{\circ}\text{C}$ for 40 min. Genotyping was performed on an ABI 3130 sequencer (Applied Biosystems) and fragment analysis was conducted using the software GenMapper 3.7v (Applied Biosystems). Multiple paternity analysis was based on Mendelian rules of inheritance, in which the minimum number of mates estimated to generate the allelic distributions in each family is half the number of non-maternal alleles for each locus (Toonen 2004).

Results

Ten females and 8–16 of their larvae were genotyped with (or using) each primer set (Table I). Multiple paternity was found for four of the 10 females examined (40%) (Table II). There are at least three non-maternal alleles observed among the offspring of these females. This estimate of the frequency of polyandry is highly conservative, given that several factors might have contributed to underestimating the number of matings, such as the presence of homozygous males, the presence of the same alleles in males and females, and the fact that not all of the

offspring was sampled (Neff et al. 2002; Toonen 2004).

Discussion

The existence of at least three non-maternal alleles in the offspring of 40% of the tested *Ucides cordatus* females is unequivocal evidence for the occurrence of polyandry in the species, at least in the studied population. The existence of polyandry in this species might reflect its peculiar mating behaviour. During their mating season, males and females leave their nests and engage in a period of intense above-ground walking and fighting, a phenomenon traditionally known as ‘andada’ (Góes et al. 2000). In particular, males use their pereopods to subdue females and to drag them to burrows (Costa 1979). This phenomenon suggests that forced mating could be the cause of polyandry in *U. cordatus*. In addition, there is evidence for lower larval release rate in larger females (Hattori & Pinheiro 2003), which could result from larger females being able to resist males more effectively.

Another mechanism that can result in polyandry in *U. cordatus* is the ability to maintain viable sperm in spermathecae throughout the year. Despite the seasonal mating period, the transfer of spermatophores result in a large supply in spermathecae throughout the year (Sant’Anna et al. 2007), with the sperm remaining viable for several months (Castilho 2006; Castilho-Westphal et al. 2008). Therefore, the contribution of more than one male to the offspring of a given season might be a consequence of the sperm remaining from mating in the previous year. Furthermore, the absence of packed spermatophores prevents the stratification of sperm from different males (Sant’Anna et al. 2007), which could give an advantage to the last copulating male (van der Brink & McLay 2008). With

Table II. Male alleles from a sample of 8–16 brooded offspring from each of the 10 females of *Ucides cordatus* and the minimum number of males with which each female must have mated to generate the observed allelic distribution among the offspring.

Family	B3	A120	C109	A11	B124	A5	Males
1	10 and 21	1 and 2	3 and 4	4 and 5	8 and 11	2 and 13	1
2	18	1 and 5	3 and 4	3 and 4	8 and 9	4 and 6	1
3	29 and 34	1 and 4	3 and 4	1 and 6	9 and 11	7, 9 and 12	2
4	12, 18, 28 and 36	1 and 5	3 and 4	3 and 4	1, 9 and 10	2, 8 and 10	2
5	26 and 30	1 and 2	3 and 4	3	9 and 16	8	1
6	16 and 34	1	3 and 4	3 and 5	9 and 10	7 and 10	1
7	17 and 28	1 and 2	3 and 4	3 and 4	9 and 13	8 and 9	1
8	1 and 23	1, 3 and 4 or 2	3 and 4	2 and 4	2, 3 and 9	6 and 17	2
9	13 and 15	1 and 2	3 and 4	3 and 4	2 and 9	2 and 8	1
10	16, 18 and 17 or 26	1	3 and 4	5 and 6	9 and 2	6 and 10	2

unstratified spermathecae, the first or the last male of *U. cordatus* to copulate would seem to have equivalent chances to fertilize eggs (Sant'Anna et al. 2007). The presence of polyandry has important consequences for the understanding of the biology of *U. cordatus* and might help explain the considerable genetic diversity observed in the species (Oliveira-Neto 2007a,b).

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