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Genetic parentage in the squat lobsters *Munida rugosa* and *M. sarsi* (Crustacea, Anomura, Galatheidae)

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ABSTRACT: *Munida* is the most diverse and cosmopolitan genus of the galatheid squat lobsters. The group has attracted much attention in recent years from both systematic and evolutionary perspectives, yet information on the biology, ecology and evolution of this genus is very limited. We investigated the genetic parentage of 2 North Atlantic species, *M. rugosa* and *M. sarsi*, sampled from the Clyde Sea on the west coast of Scotland. Microsatellite markers were used to establish the parental contribution from embryos of berried females (*M. rugosa*, n = 25 and *M. sarsi*, n = 5). The frequency of multiple paternity observed in both species (86% for *M. rugosa* and 100% for *M. sarsi*) is the highest ever reported for any marine crustaceans. Invariably more than 2 sires were involved in each case (minimum of 2 to 3 for *M. rugosa* and 4 for *M. sarsi*). Our findings indicate that multiple paternity is likely to be the norm in both species. Within most multiply sired broods, sire contribution was highly skewed towards a single male (66% of broods for *M. rugosa* and 100% for *M. sarsi*). Furthermore, embryos from different sires were randomly distributed across the female's brood patch. This is the first report of multiple paternity in galatheids. While a number of theories can account for the high incidence of multiple paternity in these species (e.g. convenience polyandry as a result of cryptic female choice, forced copulations, the influence of fishing pressures), at present it is not possible to disentangle their individual and/or combined effects.

KEY WORDS: Galatheids · Crustacean mating system · Polyandry · *Munida* spp.

INTRODUCTION

The mating behaviour of decapod crustaceans has received considerable attention in recent years (reviewed by Correa & Thiel 2003, Duffy & Thiel 2007). Many studies have elaborated on male mating behaviours such as mate guarding and aggression (Wada et al. 1997, Jivoff & Hines 1998, Rondeau & Sainte-Marie 2001), or sperm competition. The latter has focused on sperm stratification (Urbani et al. 1998), removal (Beninger et al. 1991) and/or limitation (MacDiarmid & Butler 1999, Rondeau & Sainte-Marie 2001, Rubolini et al. 2005). More recently, attention has shifted towards the examination of female behaviour. These studies have addressed questions related to mate selection (Sainte-Marie et al. 1997), convenience polyandry (Thiel & Hinojosa 2003) and cryptic female choice (Walker et al. 2002, Thiel & Hinojosa 2003).

A major difficulty in examining the mating behaviour of marine crustaceans in general is associated with the obvious logistical difficulties of observing mating interactions in their natural environment. The elusive burrowing behaviour and/or nocturnal activities of many species (De Grave & Turner 1997) further complicate studies on mating. While laboratory experiments have contributed to a better understanding of the mating behaviour of some species (e.g. Urbani et al. 1998, Thiel & Hinojosa 2003), the mating systems of the majority of crustaceans are still poorly understood. Molecular studies are now providing a powerful alter-

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native for investigating the mating strategies of otherwise elusive and/or difficult target species (e.g. Bilodeau et al. 2005)

Molecular investigations of paternity among marine crustaceans are still rare in comparison with other taxa. With a few exceptions, focusing on brachyuran crabs (Urbani et al. 1998, McKeown & Shaw 2008), the majority of other studies on marine crustaceans have reported on the incidence of multiple paternity, e.g. porcelain crab *Petrolisthes cinctipes* (Toonen 2004), crayfish *Orconectes placidus* (Walker et al. 2002), American lobster *Homarus americanus* (Gosselin et al. 2005) and ghost shrimp *Callichirus islagrande* (Bilodeau et al. 2005). Knowledge of mating strategies is particularly relevant for species subject to intense fishing exploitation. For instance, in the American lobster, Gosselin et al. (2005) showed that mating strategies can vary geographically in correlation with different levels of exploitation. The authors suggested that selective fishing pressure for large males could be responsible for such a pattern.

Munida is the most diverse and cosmopolitan genus of the galatheid squat lobsters and, as such, has attracted much attention in recent years from both a systematic and evolutionary perspective. However, information regarding the biology, ecology and evolution of this genus is still limited. *Munida rugosa* and *M. sarsi* are endemic to the north-eastern Atlantic. They inhabit rocky or soft mud substrata in shelf waters ranging from the shallow to the deeper continental slope. Similar to other *Munida* species, *M. rugosa* and *M. sarsi* are gonochoric, but little is known about the mating behaviour of these species. They are benthic as adults with a planktotrophic larval phase and a developmental period that lasts 3 to 4 mo (Schmidt 1965, Gore 1979, Van Dover & Williams 1991). Clarification of the mating system of *Munida* species is now of particular relevance not only because of the increasing target fisheries, but also due to the impact from indirect fishing pressures. In western Scotland, for instance, *M. rugosa* comprises a large component of bycatch from the large-scale commercial *Nephrops norvegicus* fisheries (Bergmann & Moore 2001).

Pothanikat (2005) produced the only report detailing the mating behaviour of *Munida sarsi*, which was based upon laboratory observations. The report stated that coerced mating occurs in the hard-shelled state, with a male holding a female in place with his chelipeds, while trying to insert a spermatophore into the abdominal area of the female with the help of his fifth pereiopods. Females possess no internal organs for sperm storage, and there is no information as to how the female handles the spermatophore or how egg insemination takes place. Following insemination, however, the female's abdomen is cupped under the cephalothorax, forming a spawning chamber where eggs are brooded until they hatch. It is not known whether a female can mate with more than 1 male during a breeding season. While the timing of reproduction is unclear for *M. sarsi*, in western Scotland *M. rugosa* produce broods from November, with hatching occurring from March to May (Lebour 1930, Zainal 1990, Coombes 2002). Extruded broods contain up to ~32 000 eggs which are approximately 0.5 mm in diameter (Wenner 1982, Tapella et al. 2002).

Other crustacean species displaying mating behaviour similar to squat lobsters, including forced copulation (e.g. crayfish) and lack of sperm storage structures (e.g. rock shrimp), are characterised by multiple mating. While it is still unknown as to whether multiple mating behaviour translates into multiple genetic paternity in rock shrimp, this has been confirmed for crayfish (Walker et al. 2002). Thus, we hypothesised that genetic multiple paternity is the mating strategy of the galatheid species investigated. In order to test this hypothesis in *Munida rugosa* and *M. sarsi*, we generated and compared microsatellite multilocus genotypes of females and their egg broods in order to deduce paternal contribution.

MATERIALS AND METHODS

Study sites and collections. Given their elusive nature and habitat preferences (individuals often bury themselves in muddy substrata), it is notoriously difficult to study the mating behaviour of these species in their natural environment. This is particularly the case for egg-carrying females, making them very hard to obtain. Indeed, at least for *Munida rugosa*, fisheries have been reported to be biased towards males (Coombes 2002). Furthermore, in comparison to *M. rugosa*, *M. sarsi*, as the deeper water species, is considerably more difficult to sample. Thus, despite considerable effort, sampling success for ovigerous females was limited. *M. rugosa* (n = 25) and *M. sarsi* (n = 5) ovigerous females were collected by a 2 m beam trawl using a 50 mm mesh in the Clyde Sea (western Scotland) in March 2005 and February 2006. The abdomen and associated eggs from each female were stored in 99% molecular grade ethanol for subsequent analysis. Species identification was confirmed by genetic screening using diagnostic mtDNA markers (Bailie 2008).

DNA extraction and amplification of microsatellites. Genomic DNA was extracted from the muscle tissue of the ovigerous females as described by Taggart et al. (1992). For *Munida rugosa* samples, 11 eggs were randomly collected from each of the 4 pleopodal regions (totalling 44 eggs female^{-1}). A similar sampling

strategy was carried out for *M. sarsi*, except that 23 eggs were randomly removed from each of the 3 pleopodal regions (totalling 69 eggs female^{-1}). This sequential egg sampling strategy (i.e. obtaining samples from all pleopodal regions) was carried out in order to account for any potential variation in the timing or order of egg extrusion by females. DNA egg extraction was carried out using a standard Chelex extraction protocol using a 96-well format microtitre plate (information available from the authors upon request).

Microsatellite primer development for both *Munida rugosa* and *M. sarsi* followed the protocol described by Kijas et al. (1994) based on enriched partial genomic libraries, with modifications as reported by Boston et al. (2009) and McInerney et al. (2008, 2009). Microsatellite-containing regions in the genome of both *M. rugosa* and *M. sarsi* are particularly complex, being characterised by the presence of cryptic repeated elements including transposable elements (Bailie et al. 2010). This makes the development of microsatellites for these species exceptionally difficult. Nevertheless, following the approach outlined by Bailie et al. (2010), 3 informative microsatellite markers were successfully developed for *M. rugosa* (see Table 1 for details). While no microsatellite markers were successfully developed for *M. sarsi*, all 3 of the *M. rugosa* markers were found to cross-hybridise and were used to screen all samples.

Single locus polymerase chain reaction (PCR) amplifications for microsatellite genotyping in a Li-Cor system were carried out in 12 µl reaction volumes containing 1× Promega *Taq* polymerase buffer, 1.5 to 2.5 mM $MgCl_2$ 100 µM dNTP, 1 to 2 pM of each microsatellite primer, 50 ng template DNA and 0.5 to 1 U of Promega *Taq* DNA polymerase. PCR cycling conditions consisted of 1 cycle at 95°C for 5 min, followed by 24 to 28 cycles at 95°C for 1 min, 42 to 56°C for 1 min, 72°C for 1 min, followed by 1 cycle of 72°C for 5 min (Table 1). Amplified products were denatured at 80°C for 3 to 5 min, and 1 µl was loaded into a 25 cm 6% 1× TBE polyacrylamide gel using a commercially available size-standard ladder for the Li-Cor system (Microstep-13a/b and 20a, Microzone) to accurately estimate the size of allelic fragments. Gels were run on the Li-Cor system at a constant power of 40 W at a temperature of ~50°C for 1 to 2 h. Genotypic scoring was carried out using the computer software Gene Profiler (Scanalytics).

Statistical analysis. The power of the 3 microsatellite markers to detect multiple paternity was assessed through simulation analysis as implemented in the Probability to Detect Multiple Matings (PRDM) program (Neff & Pitcher 2002). This simulation takes into consideration the potential number of sires, degree of paternal skew and brood size analysed under a range of possible mating scenarios across both species. Mating scenarios, which take into consideration equal, moderately skewed and highly skewed male breeding success, were chosen to reflect both information available from crustacean mating system studies (Walker et al. 2002, Bilodeau et al. 2005, Yue et al. 2010) and recommendations by Neff & Pitcher (2002). Allelic frequency population data for *Munida rugosa* and *M. sarsi* samples from the same area were available from a parallel study into the population structure of both species, which was carried out within our research group.

The occurrence of multiple paternity of a brood was established by the occurrence of more than 2 paternal alleles across at least 2 loci (to allow for the possibility of mutation at 1 locus). On confirmation of multiple paternity, GERUD (Jones 2001) was used to estimate the minimum number of possible males, to reconstruct all possible male genotypes and to rank the likelihood of each male's contribution to the brood in correlation with the known maternal genotypes. When more than 2 paternal alleles were identified at a single locus only, χ^2 statistics were used to test whether the remaining 1 or 2 loci displayed evidence for significant deviations from expected Mendelian genotypic ratios.

Table 1. Primer details of microsatellite loci developed in this study. Product size represents the size in base pairs (bp) of the cloned allele from which primers were designed, annealing temperature (Ta), $MgCl_2$ concentration and number of PCR cycles used in amplification reactions with this Ta (denaturation and extension temperatures were 95 and 72°C, respectively, for all primer sets). F: forward; R: reverse. •: specific primer of the pair labelled with fluorescent dye

Primer	Sequence 5′ to 3′	Product size (bp)	Ta (°C)	$MgCl_2$ (mM)	No. cycles
MR62F	TAAACGACCAATCCCATTAGAC	154	52	1.5	28
MR62R•	TATATTTGGAGTAAAGTGG				
MR63F•	TCTTGAGAAAGATAGAAATAT	132	42	2.5	25
MR63R	CTTGCGCAAGCGGGAATAA				
MR778F	GGAAACCAACTCATTATTACTTAC	135	56	2.5	24
MR778R•	CCGTGGCCACCCCCTTAG				

RESULTS

Although very difficult to develop, the resulting 3 microsatellite marker loci (*MR62*, *MR63* and *MR778*) were found to be sufficiently informative for parentage analysis in both *Munida rugosa* and *M. sarsi*. Summary genetic statistics for the population samples screened for both species (*M. rugosa:* no. of individuals, n = 80 and *M. sarsi:* n = 18 from an ongoing population study) are reported in Table 2. *MR778* was found to be monomorphic in *M. sarsi*; thus, the screening of *M. sarsi* broods was conducted with only 2 microsatellite loci (*MR62* and *MR63*). No significant departure from Hardy-Weinberg equilibrium was observed globally in either species. No genotypic disequilibrium was observed between loci, and no null alleles were detected in the present study.

While only 2 (*Munida sarsi*) or 3 (*M. rugosa*) microsatellite markers were used for parentage analysis, these invariably displayed adequate power (27 to 100%) for detecting multiple paternity in both species depending on the mating scenario considered (Table 3). Not surprisingly, the power to detect multiple paternity increases with brood size. The logistical difficulties in characterising genotypes of the high number produced by some crustacean species has resulted in studies (e.g. Urbani et al. 1998, Walker et al. 2002, Gosselin et al. 2005) implementing a pooling approach. The drawback of such an approach is that the true number of males can be significantly underestimated, particularly if the male contribution is highly skewed; therefore, we elected to screen individual eggs.

Table 2. *Munida rugosa* and *M. sarsi*. Summary statistics for population samples of *M. rugosa* (no. of individuals, n = 80) and *M. sarsi* (n = 18). Allelic diversity (K) observed and expected heterozygosities (H_{obs} and H_{exp}, respectively) for 3 microsatellite loci

Species	Locus	K	H_{obs}	H_{exp}
M. rugosa	*MR62*	14	0.845	0.825
	MR63	6	0.436	0.445
	MR778	7	0.493	0.496
M. sarsi	*MR62*	14	0.96	0.92
	MR63	4	0.21	0.27
	MR778		Monomorphic	

Table 3. *Munida rugosa* and *M. sarsi*. Probability of detecting multiple paternity (PRDM) for 2 or 3 microsatellite loci (MR: *M. rugosa*, no. of individuals, n = 3; MS: *M. sarsi*, n = 2) used assuming 3 mating scenarios: (1) even contribution, (2) moderately skewed towards 1 male and (3) largely skewed towards 1 male (or 2 males when 4 were taken into consideration). This was conducted against 2 to 4 males and with ranging brood sizes (N). Average numbers of *M. rugosa* and *M. sarsi* eggs screened were 32 and 40, respectively. **Bold** type refers to values discussed in text. No. males = mating scenario, Ratio MC = Ratio of male contributions

No. males	Ratio MC	Species	Brood sizes (N) 10	20	32	40	60	86
2	50:50	MR	0.831	0.886	0.893	0.895	0.894	0.894
		MS	0.863	0.918	0.925	0.924	0.923	0.925
	67:33	MR	0.794	**0.876**	0.893	0.894	0.892	**0.896**
		MS	0.829	0.908	0.923	0.924	0.925	0.925
	95:5	MR	**0.272**	0.454	**0.607**	0.668	0.770	0.834
		MS	0.289	0.486	0.634	0.698	0.801	0.866
3	33:33:33	MR	0.946	0.983	0.988	0.988	0.988	0.988
		MS	0.963	0.992	0.995	0.994	0.995	0.995
	57:28.5:14.5	MR	0.905	0.971	0.983	0.985	0.987	0.988
		MS	0.927	0.982	0.991	0.993	0.994	0.995
	86:9:5	MR	0.593	0.808	0.905	0.938	0.967	0.980
		MS	0.620	0.837	0.928	0.955	0.980	0.990
4	25:25:25:25	MR	0.976	0.996	0.998	0.998	0.999	0.999
		MS	0.985	0.999	1.000	**1.000**	1.000	1.000
	40:20:20:20	MR	0.966	0.994	0.998	0.998	0.998	0.999
		MS	0.979	0.998	0.999	1.000	1.000	1.000
	40:40:10:10	MR	0.945	0.987	0.995	0.996	0.998	0.998
		MS	0.962	0.994	0.998	0.999	0.999	1.000

The average number of eggs (32 for *Munida rugosa* and 40 for *M. sarsi*) for which consistent genotypic data were obtained per brood was adequate for detecting multiple paternity with a PRDM between 60 and 100% across both species (Table 3). Dependent on the skew detected, the average number of eggs (N) screened does not greatly improve the likelihood of detecting multiple paternity. For example, for *M. rugosa* with a mating scenario of slight paternal skew (e.g. 67:33), the power to detect paternity when screening 20 eggs is 87%, with increased screening of 86 eggs only improving the power to 89%. This indicates that the power of detecting multiple paternity is surprisingly high despite the low number of markers available. The number of additional paternal alleles observed among the offspring of *M. rugosa* broods provided evidence of multiple paternity by, firstly, displaying more than 2 paternal alleles in more than 1 locus, or when more than 2 paternal alleles were identified at a single locus deviating from the Mendelian inheritance of a 1:1 ratio (Table 4).

Multiple paternity was evident in 21 (84%) of the *Munida rugosa* families. Thirteen females (52%) mated with a minimum of 2 sires while the remaining 8 (32%) mated with a minimum of 3 (Table 4). Given

Table 4. *Munida rugosa*. (A) Allelic inheritance microsatellite DNA profiles for 25 berried females. In each case, allelic profiles, identified by the length of the amplification product (in bp), result from the screening of an average of 32 fertilised eggs. Female alleles and putative sire alleles (in *italics*) are reported per locus. (B) Average minimum number of males per family with which each female must have mated in order to generate the observed allelic distribution among the offspring is also reported. FG: female genotype

(A)

M. rugosa female ID	Microsatellite loci *MR62* FG	*MR62* Paternal alleles	*MR63* FG	*MR63* Paternal alleles	*MR778* FG	*MR778* Paternal alleles	Min. no. males
1[a,b]	204, 232	*204, 212, 240*	133, 133	*133*	135, 135	*127, 131,135, 139*	3
2[b]	212, 212	*204, 212, 220*	133, 133	*133, 137*	123, 131	*135*	2
3	204, 204	*204, 212*	133, 133	*133*	127, 135	*135*	1
4[a,b]	204, 204	*204, 208, 212, 240*	133, 137	*133, 137*	131, 135	*127, 131, 135, 139*	3
5[a,b]	212, 220	*212, 220, 208, 216, 232*	133, 137	*133, 137*	135, 135	*135*	3
6[b]	204, 240	*204, 240, 212*	133, 133	*133, 137*	131, 135	*127, 135*	2
7[a,b]	208, 220	*200, 204, 208, 216*	133, 133	*133, 137, 141*	135, 135	*131, 135*	3
8[b]	196, 208	*196, 212, 240*	133, 137	*133, 137*	135, 151	*135*	2
9[a,b]	212, 224	*196, 204, 212, 228*	133, 137	*133, 137*	131, 139	*107,131, 135, 139*	3
10	212, 212	*206, 208*	133, 137	*133, 141*	135, 139	*131, 135*	1
11	204, 204	*204, 212, 240*	133, 137	*133, 137*	135, 135	*131, 135, 139*	2
12[b]	196, 204	*204, 212*	133, 133	*133, 125, 141*	135, 135	*131, 135*	2
13[b]	204, 212	*204, 208*	133, 137	*141*	135, 135	*127, 135, 139*	2
14[a]	204, 208	*204, 208, 212, 236*	133, 133	*133*	135, 135	*127, 135, 139*	2
15[b]	212, 216	*204, 212, 220*	133, 133	*133, 141*	131, 139	*135, 139*	2
16[b]	204, 212	*204, 212*	133, 137	*133, 137*	135, 135	*131, 135, 139*	2
17[b]	204, 236	*208, 212, 216,*	133, 133	*133, 137*	135, 135	*135, 139*	2
18[b]	204, 216	*204*	137. 137	*133*	131, 135	*127, 135, 139*	2
19[b]	212, 240	*208, 212, 220, 228, 232*	125, 129	*133*	135, 135	*135, 139*	3
20[a,b]	212, 236	*200, 204, 208, 212, 220, 236*	133, 137	*133, 137*	135, 139	*127, 135, 139*	3
21[a,b]	204, 208	*204, 212, 216, 220, 232*	133, 133	*133*	135, 135	*127, 135, 139*	3
22	212, 240	*212*	133, 133	*133, 145*	135, 135	*135*	1
23[a]	204, 204	*204, 208, 212*	133, 133	*133, 137*	135, 135	*127, 131, 135*	2
24[a]	204, 204	*204, 208, 212*	133, 137	*133, 125, 145*	135, 135	*131, 135, 139*	2
25	204, 208	*204, 240*	133, 137	*133, 137*	135, 135	*127, 135*	1

(B)

Min. no. sires	Percent of total broods	No. broods (= mating females)
3	32	8
2	52	13
1	16	4

[a]Families with multiple paternity detected at more than 1 locus or with more than 3 paternal alleles at a single locus; the likelihood of a false result due to mutation is decreased in these cases

[b]Families with multiple paternity detected by significant deviations from expected Mendelian genotypic ratios

that evidence for multiple paternity in 44% of the cases (11 females) is provided by more than 1 locus or a single locus with more than 2 paternal alleles, it is unlikely that this is an artefact of high levels of mutation.

In 66.7% of the detected cases of multiple paternity, paternal contribution was often significantly skewed (*t*-test, $p < 0.001$) towards 1 particular male (i.e. sum of 38.2 and 28.5% from mating scenarios 95:5 and 86:8:6, respectively; Table 5). The estimated PDRM probability of detecting more than 1 male within a given family using these markers ranged from 46 to 93% (Table 5). The numbers observed in the present study are within the upper extreme of this range (i.e. 84% resulting from the sum of 32 and 52% for 8 and 13 families associated with 3 and 2 sires, respectively, see Table 4).

Table 5. *Munida rugosa*. Summary of the estimated frequency of multiple paternity. Six scenarios were considered for the number of fathers and their reproductive skew. The probability of detecting this mating scenario in the current *M. rugosa* dataset was simulated by the Probability to Detect Multiple Matings (PRDM). Values discussed in the text are in **bold**

No. fathers	Paternal skew	No. families	Frequency of observed pattern (%)	Probability of detecting pattern
2	50:50	1	4.7	**0.754**
	67:33	3	14.4	0.748
	95:5	8	**38.2**	**0.466**
3	33:33:33	1	4.7	0.931
	57:28.5:14.5	2	9.5	0.922
	86:8:6	6	**28.5**	**0.783**

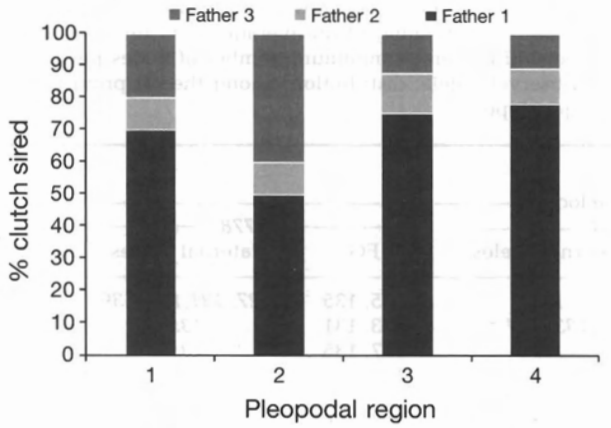

Fig. 1. *Munida rugosa*. Paternal contributions made to Family 7 female's clutch based upon the highest likelihood ranked combination of paternal males through GERUD analysis. In this case, contribution of presumed males (represented by varying shades of grey) is given by pleopodal region. Three sires were identified and contributed to each of the 4 pleopodal segments

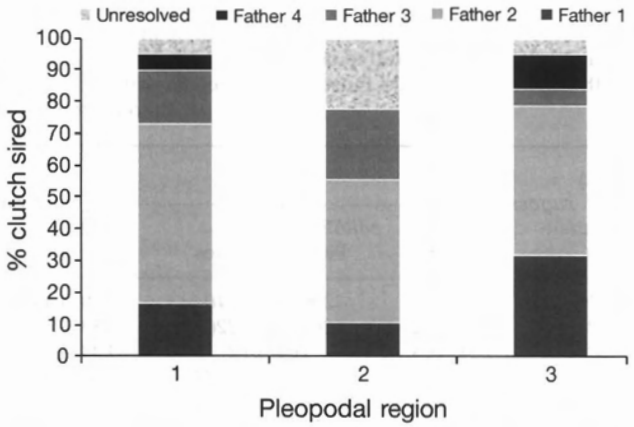

Fig. 2. *Munida sarsi*. Paternal contributions made to Family 5 female's clutch based upon the highest likelihood ranked combination of paternal males through GERUD analysis. In this case, contribution of presumed males (represented by varying shades of grey) is given by pleopodal region. Four sires were identified in this family, and eggs unable to be assigned to an individual male due to the allelic makeup of the offspring and other putative males were classified as 'unresolved'

Conversely, for the remaining 25% of cases, the markers provide little power to detect multiple paternity, hence potentially explaining the instances of single paternity observed (identified in 16% of cases, see Table 4). The probability of detecting multiple paternity decreases (e.g. 46 to 78% when taking into account 2 to 3 males, respectively) with increasing paternal skew (Table 5). However, despite this and extreme paternal skew (i.e. >95%), multiple paternity was detected in two-thirds (66.7%) of *Munida rugosa* families in this investigation.

The microspatial distribution of contributing males in *Munida rugosa* broods exhibiting multiple paternity (86%) was evident in each of the 4 pleopodal segments. It was also clear that second and third males (unless very underrepresented) also contributed to 2 to 3 of the 4 pleopodal segments in 52% of broods (e.g. Fig. 1), thereby suggesting the possibility of egg mixing or simultaneous egg extrusion.

In congruence with *Munida rugosa*, the allelic distribution in all 5 *M. sarsi* broods (100%) provided unequivocal evidence of multiple paternity. A minimum of 4 sires were required to explain each brood's allelic make up (Table 6). This contrasts with the results observed for *M. rugosa*, where a maximum of 3 sires was detected. Similar to what was observed in *M. rugosa*, there appears to be considerable variation in the relative contribution of individual males siring

Table 6. *Munida sarsi*. Summary of allelic inheritance from a sample of an average of 40 brooded offspring from each of 5 berried females. Female alleles and male alleles (in *italics*) are reported (excluding fertilised eggs for which parentage could not be unambiguously resolved among putative males). Each female must have mated with a minimum of 4 males in order to generate the observed allelic distribution among the offspring (as estimated using GERUD). All families had multiple paternity detected at more than 1 locus or with more than 3 paternal alleles at a single locus, and the likelihood of a false result due to mutation is decreased in these cases. In addition, all families had multiple paternity detected by significant deviations from expected Mendelian genotypic ratios

M. sarsi female ID	Microsatellite loci			
	MR62		*MR63*	
	Female genotype	Paternal alleles	Female genotype	Paternal alleles
1	192, 212	*188, 192, 200, 212, 216, 224, 232*	129, 133	*129, 145*
2	188, 224	*148, 168, 172, 188, 196,200, 224, 284*	129, 129	*117, 129, 141*
3	212, 220	*188, 192, 200, 212, 216, 220, 236*	129, 129	*129, 133, 141*
4	220, 224	*188, 212, 220, 224, 244, 308*	129, 133	*117, 129, 133, 145*
5	200, 328	*200, 236, 316, 324*	129, 129	*129, 137, 141*

the offspring of particular females. A single male was highly favoured in all mating scenarios, but the varying proportions of contributions made by subsequent males differed. As for *M. rugosa*, when more than 1 male was present (in all 5 families) each male's contribution was evident in each of the 3 pleopodal segments (Fig. 2), with few exceptions.

DISCUSSION

Given the difficulties in obtaining microsatellites for these and possibly other galatheid species (Bailie et al. 2010), those identified in the present study are not only extremely valuable but are currently the only markers available for these galatheids. In the present study, these 3 markers were found to be sufficiently polymorphic to detect genetic contribution from multiple males. Invariably, while more than 1 marker is needed to discard mutation bias, the detection of multiple paternity does not require a large number of marker loci. Thus, the number of markers used in this case is comparative to the majority of other crustacean molecular paternity studies published to date (e.g. Urbani et al. 1998, Walker et al. 2002, Streiff et al. 2004, Toonen 2004, Bilodeau et al. 2005, Gosselin et al. 2005). The secretive and nocturnal nature of galatheids (De Grave & Turner 1997) makes behavioural observations difficult. Molecular work has proven essential to provide novel information on the mating strategy of these elusive galatheids. Indeed, the present study provides the first genetic evidence for the occurrence of multiple paternity in any Galatheidae species.

In addition to identifying multiple paternity, this is the first investigation to demonstrate that polyandry is the predominant mating strategy in *Munida rugosa* and *M. sarsi* from the Clyde Sea area. We argue that the few instances of monogamy identified in *M. rugosa* (14%) are likely to be an artefact of the comparatively low power of markers employed in this particular case. Thus, the frequency of multiple paternity observed is likely to be an underestimation of the true extent for this species in the wild. The identification of a higher number of contributing males to broods in *M. sarsi* in comparison with that seen in *M. rugosa* broods is interesting. While we only examined 5 *M. sarsi* broods, this species occurs at higher densities than *M. rugosa* (Hartnoll et al. 1992), making it feasible for *M. sarsi* to have more opportunity for male encounters.

The findings of this investigation (i.e. polyandry) are comparable to what has been observed in the majority of other crustacean species including the thalassinidean ghost shrimp *Callichirus islagrande* (Bilodeau et al. 2005), American lobster *Homarus americanus* (Gosselin et al. 2005), rock shrimp *Rhynchocinetes typus* (Thiel & Hinojosa 2003), porcelain crab *Petrolisthes cinctipes* (Toonen 2004), snow crab *Chionoecetes opilio* (Urbani et al. 1998) and crayfish *Orconectes placidus* (Walker et al. 2002). Contrary to these studies, however, the incidence of polyandry in both *Munida rugosa* (84%), but in particular *M. sarsi* (100%), is higher than that observed in other crustacean species. Prior to the present study, the highest levels of multiple paternity have been observed in species such as *O. placidus* (60%, Walker et al. 2002), porcelain crab (80%, Toonen 2004) and Norway lobster *Nephrops norvegicus* (54%, Streiff et al. 2004).

Previous reports of multiple paternity in crustaceans have elaborated on a number of explanations for this behaviour, including convenience polyandry, cryptic female choice (by re-mating, discarding sperm from selected males or delaying ovulation) or forced copulations (Thiel & Hinojosa 2003, Walker et al. 2002). Alternatively, the reduced number of males resulting from sex-biased fisheries may result in a female mating with more than 1 male in order to obtain sufficient sperm to fertilise her brood (Gosselin et al. 2005). The multiple mating identified in *Munida rugosa* and *M. sarsi* females is compatible with 1 or more of these hypotheses. Limited laboratory observations have shown that forced copulations occur in *M. sarsi* (Pothanikat 2005), while sex-biased fisheries have also been reported for *M. rugosa* (Coombes 2002).

Ra'anan & Sagi (1985) suggested that forced copulations, where males transfer sperm externally to the females, are common in crustacean species. This argument is corroborated by a number of observations of this behaviour in species such as the crayfish *Austropotamobius italicus* (Rubolini et al. 2006), rock shrimp (Thiel & Hinojosa 2003) and *Munida sarsi* (Pothanikat 2005). It is interesting to note that for species where forced copulations—involving external sperm transfer and external fertilisation—occur, multiple mating has often been observed (Walker et al. 2002, Thiel & Hinojosa 2003). It is possible that forced copulations may be triggered by a number of factors. For instance, males might have to force copulation because hard-shelled females do not require protection, and hence can potentially afford to be choosy. Alternatively, following Bateman's principle, males might want to increase their biological fitness by mating with a large number of females.

While the incidence of forced copulations by males of *Munida* species implies strong male influence on mating behaviour, female choice cannot be underestimated. For the rock shrimp, based on behavioural observations, Thiel & Hinojosa (2003) suggested that cryptic female choice plays an important part in this species' mating behaviour. The authors reported that *Rhynchocinetes typus* actively chooses male sper-

matophores favouring larger individuals. Thus, at least for this species, females can influence the breeding success of particular males even though copulations were forced. Therefore, for rock shrimp species, direct genetic benefits might be the main factor favouring this particular mating strategy (reviewed by Jennions & Petrie 2000).

In order to counteract male bias through forced copulations and to reduce the risk of injury, it is possible that females are able to make postcopulatory choices by selecting spermatophores from large males. This mechanism aids to increase genetic diversity and viability of the females' offspring, while also ensuring the provision of an adequate amount of sperm for the fertilisation of their broods. While sperm competition and/or sperm pack removal by male competitors cannot be excluded, it is also possible that the paternal skew in the parentage assignment observed in *Munida* species is due to female cryptic choice. Therefore, it is feasible that females could potentially favour 'better' males.

The already established squat lobster fisheries in western Scotland and reported squat lobster discards through *Nephrops* fisheries (Bergmann & Moore 2001) have the potential to perturb the mating system of the species. Direct or indirect fishing efforts may affect the operational sex ratio (OSR) of the *Munida* species, which can influence behavioural aspects of each sex. This is particularly true when one sex is gathered in higher numbers than the other, causing a skew in the sex ratio, thus increasing competition for each respective sex.

Studies of population biology conducted by Coombes (2002) suggested that male *Munida rugosa* were dominant in particular months of the year (i.e. February and June), and another study found comparatively reduced numbers of females in May and August (Hartnoll et al. 1992). Although it is unlikely that this reflects a true difference in sex ratio, the skew may represent cryptic behavioural responses related to the reproductive and moulting cycle, resulting in males being more vulnerable to fishing pressures in particular months. The incidence of multiple paternity may be influenced by geographical location or fisheries' exploitation levels as suggested for the American lobster (Gosselin et al. 2005). The removal of large males by fisheries forces females to multiply mate with smaller males in order to get enough sperm to fertilise their broods. Males have also been reported to economise their sperm (MacDiarmid & Butler 1999, Rondeau & Sainte-Marie 2001, Rubolini et al. 2005). This behaviour also forces females to mate with more than 1 male to ensure fertilisation of their broods. Consequently, the Clyde Sea fisheries may affect the mating system observed in *Munida* species in that area as compared to that seen in unexploited areas.

While external fertilisation may be followed by either simultaneous or consecutive egg extrusion in correspondence to each spermatophore, it is currently not known whether galatheid females can handle more than 1 spermatophore at any given time. Consecutive spermatophore handling and egg extrusion has been reported in the rock shrimp (Thiel & Hinojosa 2003), whereas a simultaneous mode of handling has been recorded in the mole crab *Emeritus asiaticus* (Subramoniam 1977, 1979). If females are capable of handling more than 1 spermatophore as eggs are being extruded, then males may compete by removing other males' sperm (Beninger et al. 1991). Thus, despite theories of cryptic female choice, the observed paternal skew could alternatively be the result of last male precedence, as has previously been reported in the snow crab (Sévigny & Sainte-Marie 1996). Therefore, the identification of significantly highly skewed broods (1 male sired >86% of the brood; 66% of *Munida rugosa* families and 100% of *M. sarsi* families) could be the result of cryptic female choice and/or last male precedence and/or sperm competition.

For both *Munida* species reported in the present study, the offspring from different sires were randomly distributed among the females' brood-patch. While such an observation is compatible with a simultaneous fertilisation mode, the alternative hypothesis of sequential egg extrusion cannot be ruled out if egg mixing occurs in the brood. Considering that egg mixing may result in egg loss, it is reasonable to assume that simultaneous fertilisation is the most likely scenario for these species.

CONCLUSION

The genetic parentage analyses conducted in this study have demonstrated that (1) both *Munida rugosa* and *M. sarsi* mate multiply with 2 to 4 sires, as respectively 86 and 100% of broods were multiply sired; (2) the offspring of different males were distributed throughout the females' pleopods; and (3) in most broods analysed (66 and 100%, respectively), it was evident that they were significantly highly skewed towards a single male. In this particular study, we cannot differentiate between all the theories discussed (convenience polyandry as a result of cryptic female choice, forced copulations and/or the influence of fishing pressures) or a combination thereof. Future work would benefit from comparisons of mating scenarios from fisheries exploited and unexploited areas and more detailed behavioural observations to disentangle the possible theories discussed.

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