

ORIGINAL ARTICLE

Multiple paternity in the common long-armed octopus *Octopus minor* (Sasaki, 1920) (Cephalopoda: Octopoda) as revealed by microsatellite DNA analysis

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Keywords

Microsatellite; multiple paternity; *Octopus minor*; progeny; sperm competition.

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Accepted: 18 February 2016

doi: 10.1111/maec.12364

Abstract

Multiple paternity was assessed in *Octopus minor* using microsatellite DNA markers. *Octopus minor* adults were captured in traps and kept in indoor cement ponds. The females spawned after several days. Ten broods (B1–B10), each containing 15 embryos and the maternal octopus, were maintained until the embryos reached the stage at which DNA could be extracted and used for genotyping. Multiple paternity was tested using five microsatellite DNA markers and the results proved the hypothesis that multiple paternity occurs in *O. minor* (observed in six out of the 10 broods). Analysis with GERUD software gave the minimum number of fathers and showed that four broods were sired by a single male, three were sired by two different males and three were sired by three different males. In brood B10, the number of fathers identified by the single-locus method was one fewer than that by the GERUD method. Of the six multiple paternity broods, four (66.7%) showed significant skew from equal paternal contributions, which indicated that sperm competition and/or cryptic female choice may be important for post-copulatory paternity bias in this species.

Introduction

Octopus minor (Sasaki, 1920) is a benthic, neritic cephalopod. It is widely distributed in the coastal waters of China, the Korean Peninsula, and from the south of Sakhalien Island to the whole of Japan (Fig. 1; Dong 1988). Sometimes referred to as *Octopus variabilis* in Japan and China (Dong 1988; Lu *et al.* 2012), *O. minor* is sexually dimorphic and the males can be identified by the third right arm, which is a specialized hectocotylus (Dong 1988; Qian *et al.* 2013).

The female *Octopus* lays and attaches fertilized eggs, which are relatively fewer in number than other octopus, to the underside of shelters, producing large, benthic hatchlings (Bo *et al.* 2014). The embryonic phase lasts 72–89 days at 21–25 °C (Qian *et al.* 2013).

Multiple paternity occurs widely in cephalopods and has been proven to occur in loliginid squids (Buresch *et al.* 2001; Shaw & Sauer 2004; Iwata *et al.* 2005, 2011), cuttlefishes (Naud *et al.* 2005) and octopuses (Cigliano 1995; Hanlon & Messenger 1996; Voight & Feldheim 2009; Quinteiro *et al.* 2011) and a number of prior studies has observed fertilization by multiple males in one egg capsule, or one egg-clutch (Buresch *et al.* 2001; Quinteiro *et al.* 2011). The majority of offspring within the distal end of the string were sired by the most successful male with few offspring sired by other males; by contrast, the proximal end of the string contained high frequencies of offspring sired by the other males (Shaw & Sauer 2004). The massive spermatozoa can be stored in oviducal glands for months, during which time the females copulate repeatedly (Wodinsky 1972; Mangold & Von-Boletzky 1973; Froesch & Marthy 1975; Cigliano 1995;

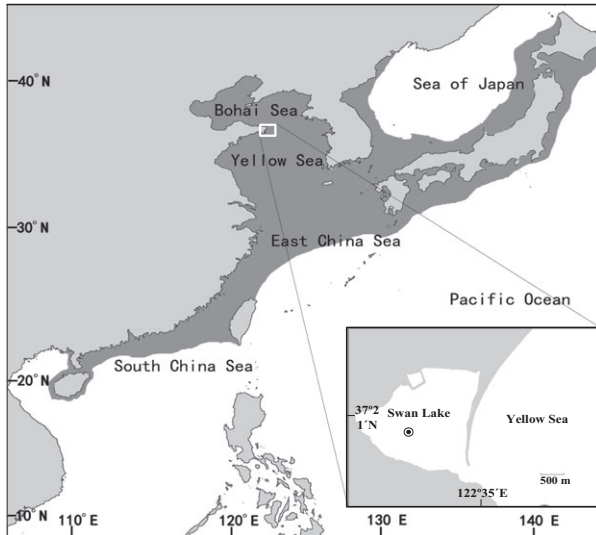


Fig. 1. The distribution of *Octopus minor* and an enlarged map of Swan Lake, showing the location of the sampling site (dark gray zone indicates the distribution of *Octopus minor* and the dot shows the sampling site).

Voight & Feldheim 2009). Hanlon & Messenger (1996) speculated that there was unified potential for long-term sperm storage because of the solitary lifestyle and the single spawning event. Multiple paternity in *Octopus minor* is a reasonable possibility as the spermatophore is stored in the *O. minor* female body for more than 15 days (Qian *et al.* 2013) and there is a delay between mating and fertilization.

Multiple paternity, as well as other mating schemes, can be a crucial determinant of rates of change in gene diversity and inbreeding in natural populations and these parameters can now be readily incorporated into predictive models of effective sizes for the study of evolutionary biology and conservation genetics (Sugg & Chesser 1994). Multiple paternity can affect the effective population size by decreasing the variance in male reproductive success and thereby increasing the effective size of a population (Balloux & Lehmann 2003; Pearse & Anderson 2009). Offspring get a genetic advantage by acquiring superior paternal genes as a defense against incompatibility (Zeh & Zeh 2001; Neff & Pitcher 2005). In consequence, multiple paternity is an issue that needs to be taken into account for population and conservation genetics and it would create benefits from the correct sex ratio of broodstock in culture design and management (Quinteiro *et al.* 2011). In addition, the appropriate use of multiple paternity strategies may assist fishery management in alleviating the sharp decline of *Octopus minor* populations from overfishing (Kim *et al.* 2008).

Observations of mating behaviors can help to verify multiple paternity (Cigliano 1995; Hanlon & Messenger 1996; Wodinsky 2008). Observations of the copulation and spermatophore transfer behaviors in various octopus species have previously been conducted (Mann *et al.* 1970; Hanlon & Messenger 1996; Wodinsky 2008). The male extends its hectocotylus into the female oviduct. One to three spermatophores are transferred simultaneously by a peristaltic ripple running down the groove from Needham's sac past the penis. The high dry weight fluid in the spermatophore draws in water, which leads the spermatophore to evaginate and release the sperm. The oviducal glands of the octopus are able to store sperm, which attach themselves to the wall of the spermathecae by their acrosome (Froesch & Marthy 1975). Cigliano (1995) suggested that the male octopus, when mating with a female that had mated within the previous 24 h, increases the time of copulation in order to remove the deposited sperm. However, it is, unlike among other octopus species, rare to observe copulation behavior of *Octopus minor* under light because of its lucifugous habits, which creates an obstacle to revealing multiple-mating event by typical experimental methods. Therefore, microsatellite DNA markers are an important tool for revealing multiple paternity in *O. minor*. Paternity testing using microsatellite DNA markers has made a significant contribution to the resolution of actual reproductive outcome and fertilization success in many taxa (Buresch *et al.* 2001; Shaw & Sauer 2004; Voight & Feldheim 2009; Quinteiro *et al.* 2011). Although *O. minor* has become a commercially important cephalopod in the north of China (Kim *et al.* 2008; Zheng *et al.* 2014) with the launch of artificial propagation and release, its paternity pattern remains unknown so far. Zuo *et al.* (2011) developed 12 *O. minor* microsatellites, which can be used to test for multiple paternity. The present study used these microsatellite DNA markers to ascertain whether multiple paternity exists in *O. minor*.

Material and Methods

Sample collection

A total of 120 *Octopus minor* adults was captured, using traps, from Swan Lake, Rongcheng, Shandong Province, China (Fig. 1). They were kept in five indoor cement ponds (5.7 × 2.7 × 1.5 m) that contained polyvinyl chloride polymer tubes as shelters and had a water depth of 0.5 m. Seawater was supplied into ponds directly or several days after filtering. Mean water temperature was 22.3 ± 2.8 °C, salinity 28.5 ± 1.5 and pH 8 ± 0.1. The ponds were equipped with aeration devices and about 50% seawater by volume was replaced daily. Crabs (*Hemigrapsus sanguineus*) were supplied daily as food. The

adult female *O. minor* hid in the shelters, defending themselves from others, and spawned in the shelters after several days. Females and their corresponding eggs were then separated artificially as soon as spawning occurred in order to eliminate disturbance from other adults.

A subset of muscle samples from 43 adult individuals was also used for population genotyping. Ten broods (B1–B10), each containing 15 embryos and the maternal female, were maintained until the embryos reached the stage at which DNA could be extracted and used for genotyping. DNA was extracted from muscle tissue that was excised from adult individuals and embryos, and was stored immediately in 95% ethanol.

DNA extraction and microsatellite genotyping

Total genomic DNA was isolated from muscle tissue by a modified version of the phenol-chloroform purification procedure described by Li *et al.* (2002). Embryos, females and population samples were genotyped at six microsatellite loci: *OM02*, *OM03*, *OM04*, *OM05*, *OM07* and *OM08* (Zuo *et al.* 2011). The PCR amplifications were performed in a final volume of 10 μ l. The reactions contained 1 \times PCR buffer, 0.25 U *Taq* DNA polymerase (Takara), 0.2 mM deoxy-ribonucleotide triphosphate (dNTP) mix, 1.5 mM MgCl₂, 1 μ M of each primer pair and approximately 100 ng of template DNA. The PCR reactions were performed under the following conditions: 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at the annealing temperature as indicated by Zuo *et al.* (2011), and 45 s at 72 °C. A final extension of 5 min at 72 °C was used. The PCR products were resolved by a 6% denaturing polyacrylamide gel using silver staining. A 10-bp DNA ladder (Invitrogen) was used as a reference marker to identify allele size.

Data analysis

Parsimony approaches have been established to determine the number of possible fathers that contribute gametes to a progeny array from a single mother. The simplest method is the single-locus minimum method (Fiumera *et al.* 2001), in which the paternal alleles are identified by removing the maternal alleles from each offspring at each locus. The minimum number of fathers for a given brood is the maximum number of paternal alleles at any one locus, divided by two and rounded up to an integer. However, this method uses data from only the most informative genetic locus, neglecting other loci. Consequently, the actual number of fathers can never be lower than that given by this method.

The GERUD software applies a parsimony approach to obtain the minimum possible number of paternal multi-

locus genotypes by means of reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents (Jones & Ardren 2003). The parental reconstruction method reintegrates parental genotypes based on the correlation of parental alleles across multiple loci within the offspring. It then assigns individual offspring to males (DeWoody *et al.* 2000). GERUD takes two steps to obtain the minimum number of fathers necessary to explain the progeny array. Firstly, the paternal alleles are identified by removing the maternal alleles. Secondly, GERUD determines the combinations of paternal genotypes, making full use of the multilocus data, to explain the offspring array using the minimum number of fathers. GERUD can rank the likelihood of paternal genotype combinations in accordance with segregation patterns and genotypic frequencies in the population (Jones & Ardren 2003).

Allele frequencies, heterozygosity, homozygote frequencies and null allele frequencies were estimated in the females, sampled embryos and the population samples, using CERVUS v. 3.0 (Kalinowski *et al.* 2007), where the frequency of null alleles was calculated as Summers & Amos (1997). To assume sampling independence, the test for Hardy–Weinberg equilibrium (HWE) was performed on the population sample using GENEPOP v. 4.3 (Raymond & Rousset 1995). Genotype linkage disequilibrium was also assessed using ARLEQUIN v. 3.5 (Excoffier & Lischer 2010). Paternal alleles were identified as being either non-maternal, homozygous or heterozygous alleles that were identical to those of the mother. Multiple paternity was suspected when the number of paternal alleles in a single half-sib progeny array was greater than two. The expected exclusion probabilities, the minimum number of possible fathers and their most probable inferred genotypes were obtained using GERUD v. 2.0 (Jones 2005). The minimum number of possible fathers was also obtained using the single-locus minimum method.

In order to measure the degree of reproductive skew, the binomial skew index B was calculated by SKEW CALCULATOR 2003 (<http://www.eeb.ucla.edu/Faculty/Nonacs/PI.htm>). Significant levels of B were estimated by simulation with 10,000 permutations. Zero, positive or significant negative values implied a random distribution of offspring among fathers, skew or an overly equal distribution of offspring, respectively (Nonacs 2000; Liu & Avise 2011; Xue *et al.* 2014).

Results

All individuals were successfully genotyped at the six microsatellite loci. Table 1 shows the observed and expected heterozygosities, HWE test results and the null allele estimated frequencies. Table S1 shows the allele fre-

Table 1. Number of alleles (k), number of individuals genotyped at each microsatellite loci (n) observed (Hobs) and expected (Hexp) heterozygosities, and null allele frequencies (fn) estimated by CERVUS v. 3.0 and Hardy–Weinberg equilibrium (HWE) test conducted by GENEPOP v. 4.3 for six microsatellite loci from samples of *Octopus minor*.

locus	k	n	Hobs	Hexp	HWE	fn
population sample						
OM02	3	41	0.463	0.607	0.021	0.1139
OM03	7	39	1.000	0.787	0.057	−0.1303
OM04	4	43	0.651	0.701	0.344	0.0234
OM05	5	41	0.585	0.531	0.820	−0.0705
OM07	5	43	0.791	0.716	0.345	−0.0733
OM08	10	37	0.730	0.835	0.008	0.063
females/progeny sample						
OM02	3	160	0.344	0.630	ND	0.289
OM03	7	158	0.804	0.778	ND	−0.0196
OM04	5	159	0.811	0.715	ND	−0.0703
OM05	4	160	0.513	0.523	ND	0.0171
OM07	5	160	0.719	0.734	ND	0.0024
OM08	10	158	0.816	0.815	ND	−0.0048

ND = not determined.

quencies, heterozygosity frequencies and homozygote frequencies in the females, embryos and the population sample. The OM02 locus had the least number of alleles. The presence of null alleles was suspected at the OM02 locus (fn, null allele estimated frequencies, >0.05) in females/progeny samples and was observed in B2 and B10. Significant linkage disequilibrium was detected between OM02 and OM05 ($P < 0.01$). Therefore, the data for OM02 were discarded and we used the other five microsatellite DNA markers in the following paternity analysis.

brood	number of genotyped progeny	GERUD method					number of fathers by S-LM method	
		minimum number of fathers	F1	F2	F3	freq + seg ^a		
B1	15	2	12	3		1.33E−23	0.175	2
B2	15	1	15				NA	1
B3	15	1	15				NA	1
B4	15	1	15				NA	1
B5	15	3	5	5	5	3.44E−34	−0.067	3
B6	15	2	13	2		1.53E−28	0.269	2
B7	15	3	6	5	4	7.14E−36	0.002	3
B8	15	2	12	3		5.27E−25	0.175	2
B9	15	1	15				NA	1
B10	15	3	7	4	4	6.25E−33	0.214	2

^aLikelihood based on frequencies and Mendelian segregation (freq + seg). NA, Not available. F1–3 representing different fathers. Significant levels of B-value was used to measure the degree of reproductive skew (zero, positive or significant negative values implied a random distribution of offspring among fathers, skew or an overly equal distribution of offspring, respectively).

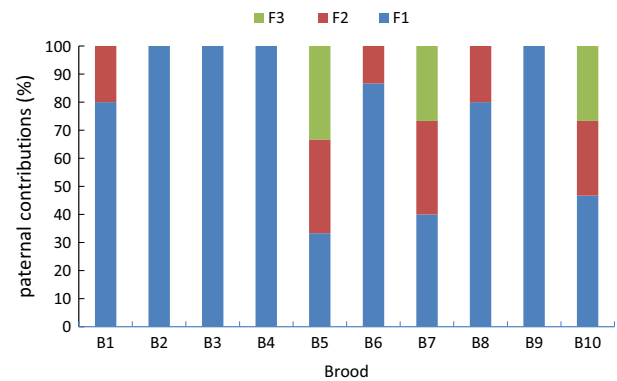


Fig. 2. Relative contributions of fathers within each brood of *Octopus minor*. The x-axis shows the number of broods and the y-axis shows the percentage of offspring sired by different fathers. Each column represents a brood with the different colored boxes showing different fathers and the percentage of their offspring in the corresponding brood. F1, F2 and F3 represent different fathers in each brood.

Multiple paternity was detected in six (60%) out of the 10 broods (see Table 2 and Fig. 2). The GERUD results showed that the minimum number of fathers ranged from one to three, with no two identical genotype combinations of fathers. One male sired all of the offspring of B2, B3, B4 and B9, with multiple paternity occurring in the remaining six broods (see Table 2 and Table S2, showing the inference of the minimum number of fathers and their most probable genotype combinations). Of these, four (B1, B6, B8 and B10; 66.7%) showed significant skew from equal paternal contributions ($P < 0.05$) and a single male was observed to have fathered more than 50% of the assayed progeny.

Table 2. Minimum number of fathers in 10 broods as determined by the GERUD and S-LM methods using five microsatellite loci for *Octopus minor*. (S-LM methods, the paternal alleles are identified by taking the maternal alleles out in each offspring at each locus and therefore the minimum number of fathers is the maximum number of paternal alleles at any one locus divided by 2 and rounded up).

Estimates of the minimum number of fathers produced by the single-locus method and those estimated by the GERUD method were identical for nine of the broods, and only differed in B10. In that case, the minimum number method estimated two fathers, while the GERUD method estimated three.

Discussion

This study has shown that multiple paternity occurs in *Octopus minor*. There was a relatively short time period between capture and spawning, which indicated that copulation had taken place in the wild. This implies that the different number and relative contributions of fathers of different broods is a naturally occurring phenomenon.

The minimum number of fathers in this study ranged from one to three. Four (B1, B6, B8 and B10) out of six (66.7%) broods exhibited a significantly uneven distribution of fertilization success of fathers (Table 2, Fig 2) and all four broods showed a principal contribution from a single male, who fathered more than 50% of progeny. Similar reproductive skews have also been observed in *Octopus vulgaris* (Quinteiro *et al.* 2011), *Graneledone boreopacifica* (Voight & Feldheim 2009), *Loligo pealeii* (Buresch *et al.* 2001), *Loligo vulgaris* (Shaw & Sauer 2004) and *Loligo bleekeri* (Iwata *et al.* 2005, 2011). However, the frequency of multiple paternity in *Octopus minor* (60%) in this study is lower than that recorded previously in *Octopus vulgaris* (the multiple paternity frequency was 100%: three sires in three broods and four sires in one brood; Quinteiro *et al.* 2011). The different paternity rates indicate that sperm competition and/or cryptic female choice may be important for post-copulatory paternity bias in these species (Shaw & Sauer 2004; Quinteiro *et al.* 2011). Iwata *et al.* (2011) demonstrated that sperm precedence in the seminal receptacle was not biased toward longer sperm. In octopods, both sexes have multiple mates and sperm competition is likely to exist, although it has not been demonstrated (Cigliano 1995; Hanlon & Messenger 1996; Quinteiro *et al.* 2011). More work is needed to understand the mechanisms of sperm precedence and cryptic female choice, which will be made clear by more observations of their mating and spawning behaviors through more elaborate experiments, such as those involving the use of infrared monitors. This study will lead to other investigations into the mechanism and procedure of sperm competition and/or cryptic female choice in octopods.

Acknowledgements

We are indebted to Mr Pei-Liang Wang of Mashan Group Co. Ltd (Shan-Dong Province, China) for his kind assistance in specimen collection. The study was sup-

ported by National Science Funding of China (no. 31172058) and the National Marine Public Welfare Research Project of China (no. 201305043).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. The allele frequencies at five microsatellite loci for the *Octopus minor* females, embryos and population samples.

Table S2. The inference of the minimum number of fathers and their most probable genotype combinations at five microsatellite loci.