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Multiple paternity in the common octopus *Octopus vulgaris* (Cuvier, 1797), as revealed by microsatellite DNA analysis

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Abstract

Two microsatellite DNA markers developed for the common octopus, *Octopus vulgaris*, were used to determine genotypes of four brooding female octopuses and 11 embryos sampled from their respective clutches, in order to confirm the multiple paternity hypothesis within this species. Two well-known reproductive behavioural patterns are apparently conflicting in mating outcome: the often-observed multiple mating and the role of the male's hectocotylus in removing stored spermatophores left by other males inside the female's oviducal gland. Genotyping data suggest that at least two males have been successful in fertilizing eggs sampled from each clutch, an evidence of multiple paternity in the common octopus.

Introduction

The Common Octopus, *Octopus vulgaris* (Cuvier, 1797), is a benthic, neritic cephalopod species whose Atlantic and Mediterranean distribution extends from the coastline to the outer edge of the continental shelf. This species is found at depths ranging from 0 to 200 m, and occupies a variety of habitats, such as rock reefs, coral reefs, sand substrates and seagrass beds. It can be described as an exploratory and opportunistic species but is inactive during part of the daytime (Mather and O'Dor 1991). *O. vulgaris* is a gonochoric species, where sexual dimorphism can be identified by the presence of some enlarged suckers on the second and third pair of arms in mature males (a potential tool for sex diagnostics) and the specialized third right arm of males, called the hectocotylus, that is used for spermatophore transfer (Hanlon and Messenger 1996; Mangold 1987; Packard 1961).

Octopuses are solitary animals that show little agonistic or courtship behaviour before mating (Hanlon and Messenger 1996). Mating in *O. vulgaris* is characterised by the copulatory activity of the males, who insert the hectocotylus into the mantle cavity of the females to transfer spermatophores into one of the two oviducts. The spermatozoa may be stored by females for up to ten months (Mangold 1987). Internal fertilization in octopods takes place in the oviducal gland as eggs pass down the oviduct (Hanlon and Messenger 1996). There is no evidence of pairing or cohabitation. Both sexes have multiple mates, and sperm competition is thought likely to exist (Cigliano 1995; Hanlon and Messenger 1996) but has not been demonstrated. The tip of the male's hectocotylised arm, the ligula, is spoon-shaped and may function in removing stored sperm from the spermatheca that lies inside the female's oviducal gland (Hanlon and Messenger 1996).

Octopus vulgaris shows two spawning peaks per year related to inshore seasonal migrations (Roper *et al*. 1984). The number of eggs laid by a female octopus is directly proportional to the size, with an estimated range of 100,000 to 500,000 observed for *O. vulgaris*. Females stop feeding just after laying their eggs and brood them for 25 to 125 days. The female dies soon after the paralarvae have hatched (Mangold 1987).

Octopus vulgaris is one of the most commercially valuable mollusc species of inshore fisheries in the Atlantic and Mediterranean. O*ctopus spp.* landings worldwide have increased slightly from the 1990s to the decade of 2000 and have tended to stabilize. Moreover, several research experiments have been carried out and are still ongoing to culture this valuable species (Chapela *et al.* 2006; Iglesias *et al.* 2007). Stocks must be managed judiciously due to recent high levels of exploitation. Basic reproductive information is needed and its genetic consequences must be considered to first assess how the fishery (which often targets spawning aggregations in shallow waters) impacts octopus populations and to enable appropriate management in aquaculture. Multiple paternity affects the effective population size (Karl 2008; Sugg and Chesser 1994), genetic diversity (McLeod and Marshall 2009), the offspring fitness and promotes population reproductive isolation (Jennions and Petrie 2000).

Microsatellite DNA loci have been extensively used in population genetic studies including characterization of cephalopod paternity patterns (Iwata *et al*. 2005; Shaw and Boyle 1997; Shaw and Sauer 2004). Several microsatellite markers have been isolated and characterized for *O. vulgaris* (Greatorex *et al*. 2000) and used to evaluate the genetic structure of its populations along the northwest coast of Africa (Murphy *et al*. 2002), the Mediterranean Sea (Casu *et al*. 2002) and around the Iberian Peninsula and the Canary Islands (Cabranes *et al*. 2008).

The present study uses previously described microsatellite markers to ascertain whether the observed multiple mating and copulatory behaviour in *Octopus vulgaris* produces multiple paternities.

Materials and Methods

A total sample of 124 unsexed wild adults of *Octopus vulgaris* were captured using traps and kept mixed and equally distributed into three 7.75 m^3 cages with individual PVC dens. The cages were suspended from an aquaculture raft in the Ría de Vigo (Galicia, NW Spain).

From this sample, four egg clutches (A–D) each containing 10 to 15 egg-strings, and muscle tissue from the corresponding four females, were obtained one month after sampling. A subset of muscle tissues from 41 individuals was also sampled for population genotyping. Egg-strings from each clutch were fixed in separate bottles of 90% ethanol. Eleven developing embryos were dissected out from each clutch and placed separately in 1.5 mL of 96% ethanol. Total DNA was isolated from each embryo and also from the muscular tissue of respective females and other unsexed sampled specimens using the NucPrep® DNA chemistry in an ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems). Individual embryos, females and population samples were genotyped at Oct3 (di-nucleotide repeat) and Ov12 microsatellite loci (tetra-nucleotide repeat) using µOct3F/µOct3R and µOct12F/ µOct12R primers (Greatorex *et al*. 2000) end-labelled with 6-FAM and TET fluorochrome dyes, respectively. PCR conditions in a GeneAmp 9700 thermocycler (Applied Biosystems) included an initial denaturation step at 96ºC for 5 min, followed by 35 cycles of denaturation at 96ºC for 25 s, annealing at 54ºC for 25 s, and extension at 72ºC for 25 s. Resulting PCR products, together with a GeneScan-350 ROX size marker (Applied Biosystems) were separated and detected in an automated DNA sequencer ABI Prism 377 (Applied Biosystems). Allele detection and size estimation were carried out with GenScan and Genotyper software (Applied Biosystems).

Allele frequencies and heterozygosity in the mothers, their sampled offspring and the population sample were estimated with CERVUS v3.0 (Kalinowski *et al*. 2007). The test for Hardy-Weinberg equilibrium (HWE) was performed only on the population sample to assume sampling independence. Maternal and paternal alleles at both loci were inferred using the exclusion criterion (Adam and Ardren 2008) by comparing the mother's genotype with the genotype of each offspring. Multiple paternity was suspected when the number of paternal alleles in a single clutch was greater than two. Thus, paternal alleles are identified as being non-maternal alleles, homozygous alleles or as heterozygous alleles that are identical to those of the mother.

The expected exclusion probabilities, the minimum number of possible fathers and their most probable inferred genotypes were obtained using GERUD v2.0 (Jones 2005). Paternal genotype probability was obtained through segregation patterns and genotypic frequencies in the population. Relationships between pairs of eggs from the same clutch were estimated by means of pairwise relatedness (r) (Queller and Goodnight 1989) with Kingroup (Konovalov *et al*. 2004) as implemented in the KINSHIP program (Goodnight and Queller 1999). Multiple paternity produces maternal half-siblings with an expected value of r=0.25, while the value for full siblings is around r=0.5.

Results

The two assayed microsatellite loci, Oct3 and Ov12, revealed considerable genetic variation which permitted the detection of multiple paternity in maternally identified clutches of *O. vulgaris*. No significant deviations from Hardy–Weinberg equilibrium were detected. The expected exclusion probability, for the combined two loci with one known parent, was 0.98. However, both the population and clutch genotyping samples showed low heterozygosity at the Oct3 locus, probably related to the presence of null alleles (Table 1). The presence of null alleles was suspected in the case of clutch A with Oct3 primers. Previous population studies suggested the same genotyping problems with these primers (Cabranes *et al.* 2008), however, this partial data was taken into account here because of its usefulness for strict exclusion analysis.

TABLE 1. Number of alleles (k), allele size range, heterozygotes (Het), homozygotes (Hom), observed (Hobs) and expected (Hexp) heterozygosities, HWE test and null allele estimated frequencies for two loci from samples of *Octopus vulgaris*.

Locus	k	N	Allele size	Het	Hom	Hobs	Hexp	HWE	Null Allele
Female/progeny sample									
Oct3	16	40		22	18	0.5500	0.8759	ND[1]	0.2295
Ov12	19	48		47	1	0.9792	0.9123	ND	0.0420
Population sample									
Oct3	29	29	108–180	17	12	0.5862	0.9468	NS[2]	0.2297
Ov12	34	39	160–425	32	7	0.8205	0.9437	NS	0.0673

[1] Not done, [2] Not significant

Four females (A–D) and their respective eggs sample (N=11) were genotyped for the two loci (Table 2). Within clutch A, the female showed a single allele (165), but the homozygous status can be discarded due to the presence of a different single allele in one of the embryos (no.5). Consequently, Oct3 shows five different alleles, a maternal null allele and, perhaps one or more paternal null alleles. In this clutch A, genotyping with Ov12 resulted in two maternal alleles and 7 paternal alleles. In clutch B, Oct3 shows five alleles, three of which are different from the maternal ones. Five different alleles were detected for the Ov12 marker. Clutch C showed three additional alleles for Oct3 and four for the Ov12 besides the two alleles detected in the female. Finally, clutch D showed five and six different alleles for Oc3 and Ov12 markers, respectively.

TABLE 2. Genotyping data for four *O. vulgaris* females (A–D) at two microsatellite loci and for a sample of their offspring.

Code	**Maternal Genotype**		**Offspring genotypes**	
	Oct3	Ov12	Oct3	Ov12
Mother A	165/0[1]	372/386		
Embryo				
no.1			165/0[1]	182/372
no.2			165/143	200/372
no.3			165/0[1]	182/372
no.4			165/0[1]	308/372
no.5			0/147	194/386
no.6			165/0[1]	308/372
no.7			165/0[1]	174/386
no.8			165/0[1]	194/386
no.9			165/163	194/372
no.10			165/163	178/372
no.11			165/167	160/372
Mother B	139/163	194/315		
Embryo				
no.1			139/163	186/315
no.2			139/139	186/315
no.3			139/139	186/315
no.4			139/139	186/315
no.5			139/148	304/194
no.6			139/139	194/194
no.7			163/163	198/194
no.8			139/163	186/194
no.9			139/153	186/194
no.10			139/139	186/194
no.11			163/141	186/315
Mother C	150/174	227/319		
Embryo				
no.1			174/174	186/319
no.2			174/174	182/319
no.3			174/174	186/319
no.4			174/174	186/227
no.5			150/150	182/319
no.6			150/140	300/227
no.7			150/144	182/319
no.8			174/174	182/227
no.9			174/174	182/319
no.10			150/150	182/319
no.11			150/120	339/319
Mother D	141/146	186/198		
Embryo				
no.1			139/141	190/198
no.2			139/141	319/198
no.3			139/141	319/198
no.4			146/146	213/186
no.5			141/146	178/186
no.6			146/146	190/186
no.7			141/146	319/198
no.8			130/146	319/198
no.9			180/146	319/198
no.10			146/146	190/186
no.11			146/146	178/198

[1] Allele 165 or null maternal/paternal allele.

Results show a principal contribution from a single male in three of the four egg-clutches (B, C, and D), who sired more than 50% of the assayed offspring. Moreover, male genotype and string specificity relationship was not observed.

The pairwise relatedness (*r*) values estimated from comparisons within each clutch were scattered, with a mean value ≤ 0.4 () suggesting the presence of half-siblings in each one progeny. The lowest mean *r* values were obtained for clutch 1 (r=0.23), 3 and 4 (r=0.30). For the analyzed offspring arrays, the minimum number of fathers ranged from 3 to 4 and their genotype was inferred from the higher likelihood estimated value (TABLE 2. Genotyping data for four *O. vulgaris* females (A–D) at two microsatellite loci and for a sample of their offspring.). The progeny sired by each male ranged from 2 to 7.

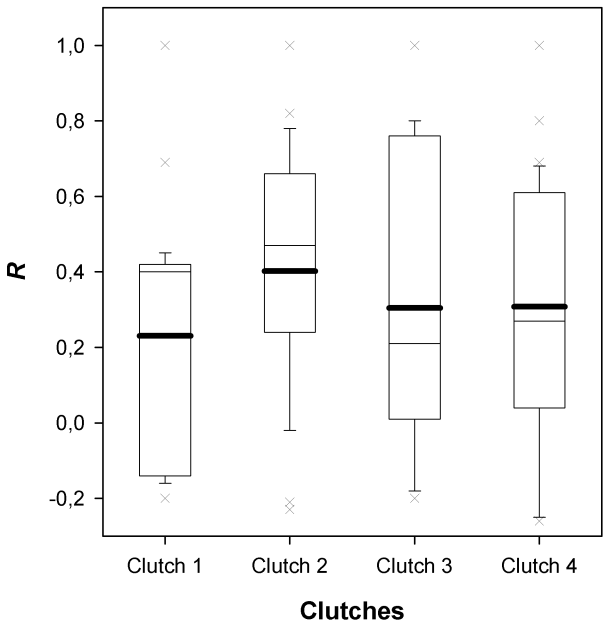

FIGURE 1. Plot of R values estimated for each of the four analyzed clutches of *O. vulgaris*. Box contains the 25th–75th percentiles whereas the x points are data outliers, the thin box line represents the median, the thick box line is at the mean and the error bars indicate the 90th and 10th percentiles. The gray dotted line indicates the value R=0.5, expected from full siblings.

TABLE 3. Genotype at two microsatellite loci for four *O. vulgaris* females (A–D) and the inference of the minimum number of fathers and their most probable genotype combinations.

	Maternal Genotype			Paternal Genotype			
Clutch Code	Oct3	Ov12	Minimum No. of fathers	Oct3	Ov12	No. of progeny	ML
A	165/0	372/386	4[1]	165/165	182/308	4	1.68E-19
				165/165	194/174	2	1.68E-19
				143/167	200/160	2	1.68E-19
				147/163	194/178	3	1.68E-19
B	139/163	194/315	3	139/163	194/198	2	1.09E-14
				139/153	186/186	7	1.09E-14
				148/141	186/304	2	1.09E-14
C	150/174	227/319	3	174/174	186/182	6	7.98E-16
				150/144	182/182	3	7.98E-16
				140/120	300/339	2	7.98E-16
D	141/146	186/198	3	139/146	190/319	6	7.32E-15
				146/146	213/178	3	7.32E-15
				130/180	319/319	2	7.32E-15

[1] Estimated without considering the presence of null allele/s

Discussion

The analysis of two microsatellite loci in four females and their respective progeny revealed that more than two paternal alleles were found in all assayed egg masses, thus supporting the hypothesis of multiple paternity in *Octopus vulgaris*. This reproductive pattern seems to be a general rule because more than two male contributors were inferred for all assayed offspring.

Half-sibling zygotes can originate in the female reproductive tract through random sampling of well-blended stored sperm from different males, by the differential use of sperm depositions (Shaw and Sauer 2004; Walker *et al.* 2006), after a process of sperm competition (Cigliano 1995; Hanlon and Messenger 1996) or mediated by a cryptic female choice. Eggs analyzed in this study were sampled from different places on each egg-mass from the same and different egg-strings. Although the detailed mechanism of storage and use of sperm in the oviduct of *O. vulgaris* is poorly understood, the observed principal contribution from a single male is congruent with the competitive copulation mechanism involving the use of the male's hectocotylus, to remove previously deposited spermatophores from the female spermatheca with limited efficiency. Consequently, as suggested for *Loligo bleekeri* Keferstein, 1866 (Iwata *et al*. 2005), the most frequent paternal alleles would likely be provided by the last copulation event.

Clutch samples were obtained from females maintained in aquaculture sea cages. However, the relatively short time that elapsed between capture and egg sampling suggests that copulation was likely to have taken place in the wild. Although further mating could have taken place inside the rearing cage, multiple mating has often been observed in this species (Hanlon and Messenger 1996). Thus, multiple mating cannot be attributed to stress arising from confinement within a high density space and a possibly altered reproductive behaviour. This suggestion can be supported by observations of identical reproductive behaviour in captive and wild octopus (Hanlon and Messenger 1996) and by the genotyping results from southern reef squid, *Sepioteuthis australis* Quoy & Gaimard, 1832, where egg masses obtained in captivity and those collected from the field (van Camp *et al.* 2004) were simultaneously analysed. Thus, the observed paternity pattern probably reflects the results of the mating behaviour in the wild and the related physiological and morphological adaptations.

Our results for multiple paternity in *Octopus vulgaris* suggests that this is an evolutionary conserved mating outcome (as a consequence of male competition and multiple mating) in cephalopods as previous studies have shown that it is widespread. Such studies include *Loligo forbesi* Steenstrup, 1856 (Shaw and Boyle 1997), *Loligo pealeii* LeSueur, 1821 (Buresch *et al*. 2001), *Loligo bleekeri* Keferstein, 1866 (Iwata *et al*. 2005), *Sepia apama* Gray, 1849 (Naud *et al*. 2004), *Sepioteuthis australis* (van Camp *et al*. 2004) and *Graneledone boreopacifica* Nesis, 1982 (Voight and Feldheim 2009).

Multiple paternity in *O. vulgaris* is an issue that needs to be taken into account for population and conservation genetics, since it affects the effective population size (Ne) (Karl 2008; Sugg and Chesser 1994). In consequence, genetically based estimates of exploited population size must be carefully considered. In addition, multiple paternity is a relevant issue in culture design and management to get an adequate male: female ratio in culture and broodstock selection, Also, for repopulation strategies for depleted octopus fisheries, considering a male-biased contribution will increase effective size (Sugg and Chesser 1994).

The number of eggs laid by a female *O. vulgaris* is high (100,000 to 500,000) and the females die of starvation after a single period of spawning and brooding. Multiple paternity can therefore maximise genetic recombination with multiple males in a single reproductive event. The exhaustive egg care during brooding furthermore guarantees a successful survival rate of genetically diverse offspring.

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