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NOTE

Multiple paternity within the brood of single females of *Loligo forbesi* (Cephalopoda: Loliginidae), demonstrated with microsatellite DNA markers

P. W. Shaw*, P. R. Boyle

Department of Zoology, University of Aberdeen, AB9 2TN, Scotland, UK

ABSTRACT: Microsatellite DNA markers developed for the squid *Loligo forbesi* were used to determine the genotype of a series of embryos obtained from egg strings of individual females. The results demonstrate that at least 2 males had been successful in fertilizing the eggs of a single female. The findings are compatible with observations of male competition at mating in other species of loliginid squid. They are discussed in relation to interpretation of specific questions of cephalopod ecology.

KEY WORDS: Cephalopoda Squid · *Loligo* · Microsatellite DNA · Genotype · Mating

Squids of the family Loliginidae display a semelparous, approximately annual life-history strategy, with oviparity, high individual fecundity, no parental care of offspring and non-overlapping generations (Boyle 1990, Boyle & Pierce 1994, Boyle & Boletzky 1996). High inter-annual variability in population sizes are also commonly observed (Boyle & Boletzky 1996). The common squid species of British coastal waters, *Loligo forbesi*, has typically complex population structures (Pierce et al. 1994) and recruitment patterns (Collins et al. 1997). From an individual perspective, such a semelparous lifestyle with uncertain recruitment can be predicted to result in the evolution of promiscuous mating strategies and the development of sperm competition (Parker 1970), or behaviours effecting the same result (Birkhead 1994).

Fertilization in cephalopods is invariably a process of paired matings in which packages of sperm (spermatophores) are transferred by a specially modified male arm to a female, where they are retained by various species-specific means (Arnold 1984, Mangold 1987). In some loliginid species, underwater observations of behaviour at breeding have revealed complex mating strategies in which males of 2 distinct size classes compete to fertilize the spawning female. The female is paired with a large dominant male which guards her as she approaches the substratum to attach her finger-like egg strings. At this point in the process, a small 'sneaker' male (Archer 1988) may attempt to mate with the female. Such behaviour has been described for *Loligo plei* (F. P. DiMarco & R. T. Hanlon unpubl. data in Hanlon & Messenger 1996), *Loligo vulgaris reynaudii* (R. T. Hanlon, M. J. Smale & W. H. H. Sauer unpubl. data in Hanlon & Messenger 1996), and *Loligo pealei* (Hanlon 1996). To what extent, if any, the 'sneaker' male is successful in fertilising any eggs was unknown, this being difficult to assess under field conditions and given the sensitivity of squid to captivity. Molecular genetic markers have proven to be extremely informative in such situations in other animals, initially using multi-locus 'DNA fingerprinting' (e.g. Packer et al. 1991, Pemberton et al. 1992) and, more recently, single-locus microsatellite DNA markers (e.g. Morin et al. 1994, Estoup et al. 1995) to genetically determine paternities.

The present study uses genetic fingerprinting to indicate that multiple matings in a loliginid squid may result in multiple paternity of the offspring of a single female.

Methods. Two egg masses (A and B) of *Loligo forbesi*, each containing 10 to 15 strings of eggs, were obtained from shallow water (<30 m) off the west of Scotland near Kinlochbervie. Two single strings from each mass were fixed in separate bottles of 90% ethanol. Ten developing embryos from each string

*Present address: Department of Biological Sciences, University of Hull, Hull HU6 7RX, United Kingdom.
E-mail p.w.shaw@biosci.hull.ac.uk

were dissected out and placed separately in 1.5 ml of ethanol. Duplicate samples of the surface covering of each string were also fixed.

Total DNA was extracted from each embryo using a modified CTAB (2% N-cetyl N,N,N-trimethylammonium bromide) extraction protocol optimised for squid tissue. Individual embryos were genotyped at each of 6 microsatellite loci (4 tri- and 2 di-nucleotide loci) using primers developed specifically for this species and known to give dependable banding patterns (Shaw 1997). Specific loci were polymerase chain reaction (PCR)-amplified using dedicated primers, one of which was end-labelled with ^{32}P, and the resulting products run out on a 6% denaturing polyacrylamide sequencing gel. Gels were exposed to autoradiographic film for 12 to 48 h, and the film was then developed to visualise the genotype banding patterns for each individual. Band (allele) sizes were determined against M13 sequences run on the same gel (details in Shaw 1997).

Results and discussion. High yields of good quality total DNA were obtained from all embryos, and the DNA amplified successfully for all loci in most samples. Banding patterns were clear and unambiguous (Fig. 1). The number of different alleles detected within single egg strings ranged from 3 to 6 per locus (Table 1). The samples of egg string surface yielded only very small amounts of DNA which gave confusing multi-band patterns after amplification (lanes 'ss' in Fig 1).

The results show that good quality fingerprints/multi-locus genotypes can be obtained from pre-hatching embryos in squid using dedicated primers. Unfortunately, the possibility of determining the genotype of the female depositing each string from the DNA extracted from the string surface could not be realised due to the multi-band patterns arising from amplification of this tissue. It is, however, possible with 6 such variable loci to reconstruct a putative female genotype that is not inconsistent with the observed genotype distributions amongst the offspring, making the usual Mendelian assumptions of 1 allele per genotype coming from the female and only 2 alleles in total present in the female. The proposed putative female and consequently the possible male genotypes contributing to each brood are indicated in Table 1. Although it is not possible to verify statistically the exact number and genotypic identities of the males contributing to the fertilisation of the embryos in each string, it is possible to make several important deductions about possible reproductive strategies giving rise to these brood samples:

First, strings 1 and 2 from egg mass A (A1 and A2 in Table 1) were almost certainly spawned by the same female. In contrast, strings 1 and 2 from egg mass B (B1 and B2 in Table 1) were definitely spawned by different females (no common allele at locus Lfor3).

Second, since single egg strings are extruded complete by an individual female, the finding of 6 different alleles at 2 single loci (Lfor2 and Lfor3 in strings A1 and A2—see Fig. 1) within the embryos of a single string confirms the occurrence of multiple paternity in this species of squid: 2 alleles must come from the female, the remaining 4 alleles coming from between 2 (heterozygous) and 4 (homozygous) males.

Third, this evidence indicates that both strings 1 and 2 from egg mass A resulted from the mating of 1 female with the same 2 males; 2 embryos out of the 10 tested in each string being the progeny of 1 of the males (indicated in Table 1). String 1 in mass B results from the mating of a different female with possibly 1 (there are no more than 4 alleles at any locus) but probably also more than 1 male (skewed distribution of alleles in the postulated paternal contribution: the postulated maternal genotype is based on the expectation of more even allele distributions—Table 1). Finally, string 2 in mass B results from the mating of a third female with at least 2 males: although there are no more than 4 alleles present within the brood at any 1 locus, the only possible consistent maternal genotype indicates at least 3 different paternal alleles to be present at 5 out of the 6 loci (although consistent genotypes of 2 males cannot be deduced, so only the combined paternal alleles are indicated in Table 1).

The most important conclusion from these results is that, unquestionably in at least one brood, multiple

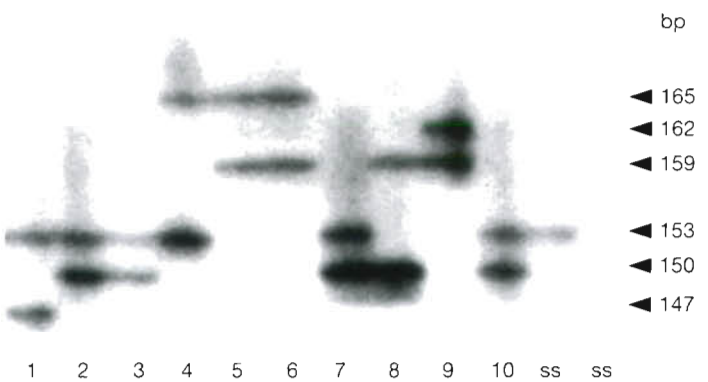

Fig. 1 *Loligo forbesi*. Autoradiograph showing banding patterns for brood A1 at locus Lfor2. Embryos 1 to 10, and samples of egg string skin (ss) are marked; the brood clearly exhibits 6 different size alleles ranging from 147 to 165 base pairs (bp)

Table 1. *Loligo forbesi*. Genotypes of embryos from 4 egg strings, across 6 microsatellite loci. Allele designations are size in base pairs (–/– = not scored). Possible maternal/paternal multi-locus genotypes deduced from allele complements are indicated for each brood (males for A1 and A2 are the same, +: offspring of male 2; paternal alleles, not genotypes are shown for B2; see text). [n]: alleles that are known to be masked by a 'null allele' when using a different set of primers (Shaw 1997) to amplify Lfor1*, so are scored as different alleles. No other loci exhibited null alleles

		Locus					
		Lfor1*	Lfor2	Lfor3	Lfor4	Lfor5	Lfor6
String A1							
Embryo	1+	139[n]/175	147/153	114/171	203/215	110/110	113/117
	2	139/148	150/153	162/171	203/203	108/110	93/119
	3	139/148	150/153	162/171	203/209	108/126	93/113
	4	139/175	153/165	120/171	203/209	110/114	107/113
	5	139/148	159/165	120/165	203/209	110/114	107/113
	6	139/175	159/165	120/165	203/209	108/126	93/113
	7	139/148	150/153	162/171	203/209	110/114	107/113
	8	139[n]/175	150/159	162/165	209/215	110/114	107/113
	9+	139[n]/148	159/162	165/174	203/203	110/126	107/119
	10	139/148	150/153	162/171	203/215	114/126	107/119
Female?		148/175	153/159	165/171	203/215	110/126	113/119
Male 1?		139[n]/139	150/165	120/162	203/209	108/114	93/107
Male 2?+		139[n]/139[n]	147/162	114/174	203/203	110/110	107/117
String A2							
Embryo	1	139/148	150/153	162/165	203/209	110/114	93/119
	2	139[n]/148	159/165	120/165	203/203	114/126	107/113
	3+	139[n]/175	147/153	114/171	203/215	110/110	113/117
	4	139/175	153/162	120/171	203/215	114/126	–/–
	5	139/175	159/165	120/165	209/215	110/114	107/119
	6	139/175	150/159	162/165	209/215	110/114	107/119
	7+	139[n]/175	153/162	171/174	203/215	110/126	113/117
	8	139[n]/148	153/165	120/171	203/203	108/126	93/113
	9	139/148	150/153	162/171	203/203	114/126	107/119
	10	139[n]/175	159/165	120/165	203/215	114/126	93/119
Female?		148/175	153/159	165/171	203/215	110/126	113/119
String B1							
Embryo	1	142/145	147/150	144/183	212/215	110/116	109/109
	2	142/145	147/150	153/183	212/215	110/116	113/113
	3	142/169	147/150	144/183	212/215	110/116	109/109
	4	151/169	147/150	153/183	203/212	110/116	109/109
	5	145/151	147/150	144/183	203/212	110/116	109/109
	6	142/169	147/150	144/183	212/215	110/116	109/109
	7	142/169	150/156	–/–	–/–	116/116	109/109
	8	142/169	147/150	153/183	212/215	110/116	109/109
	9	142/169	147/150	144/183	212/215	110/116	113/113
	10	151/169	150/156	153/171	203/215	110/116	109/113
Female?		145/169	150/150	144/153	203/212	116/116	109/113
Male?		142/151	147/156	171/180	212/215	110/116	109/113
String B2							
Embryo	1	166/166	147/153	150/174	215/218	104/120	93/111
	2	145/166[n]	147/153	150/174	218/221	112/120	93/93
	3	145/145	147/153	150/168	203/203	112/120	109/111
	4	145/145	150/150	–/–	–/–	104/104	93/101
	5	166/166	150/162	156/174	203/215	112/120	93/109
	6	145/145	150/153	–/–	–/–	112/120	109/109
	7	166/166	150/162	156/168	215/218	112/120	93/111
	8	145/145	150/162	156/168	203/221	112/120	93/111
	9	145/145	147/153	150/174	203/221	–/–	93/111
	10	145/166	147/150	156/168	218/221	–/–	93/109
Female?		145/166	147/150	168/174	203/218	104/112	93/109
Males?		145, 166, 166[n]	150, 153, 162	150, 156	203, 215, 221	104, 109, 120	93, 101, 109, 111

paternity occurs during mating of the squid *Loligo forbesi*. This result strongly implies that mating behaviour in this species, although not observed directly, involves the sort of competitive behaviour between males described for other loliginids (studies cited in Hanlon & Messenger 1996). In addition, the recognition of 2 different female genotypes from adjacent strings within egg masses, genetically confirms observations that females may lay their egg strings within existing egg masses deposited by other females, as described for *Loligo vulgaris reynaudii* (Sauer & Smale 1993) and *Loligo opalescens* (Fields 1965, Cousteau & Diole 1973).

The application of DNA fingerprinting to cephalopod reproductive ecology has reached the demonstration stage. Many more independent loci (>20) would be needed to confirm statistically the full- or half-sib relationships of embryos within single strings and, therefore, the absolute number of males contributing to the fertilization of the eggs from a single female. However, molecular genetic methods provide clear evidence for the first time that male competition at mating in cephalopods does potentially result in multiple paternity of the offspring of a single female. Future application of these techniques in conjunction with facilities for controlled captive manipulation of matings being developed (R. Hanlon pers. comm.) will allow testing of any link between alternative male mating strategies (see Hanlon & Messenger 1996) and the bimodal size-at-maturity distributions observed in male loliginids (Boyle et al. 1995).

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