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Pattern of Inheritance of Microsatellite Loci in the Squid *Loligo pealeii* (Mollusca: Cephalopoda)

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Abstract: Six microsatellite loci are described for the squid *Loligo pealeii*. All loci exhibit some degree of allelic diversity. The pattern of inheritance was tested for 3 loci through an analysis of the filial genotypes from a female-male mating. At all 3 loci, the ratios of the filial genotypes conformed to the ratios expected by Mendelian inheritance. The hypervariable loci will be useful in studies on sexual selection in this species, whereas the relatively less variable loci will be useful to address questions of population structure.

Key words: squid, microsatellites, inheritance, sexual selection, cephalopod.

INTRODUCTION

The commercially and biomedically important squid *Loligo pealeii* has a complex mating system (Hanlon and Messenger, 1996; Hanlon et al., 1997). During the summer inshore spawning season, females lay eggs in communal beds; in a day, individual females may lay tens of egg capsules, each with 100 to 300 eggs. Large "consort" males court females, copulate with them in the "parallel" position, place spermatophores in their mantle cavities, and guard them from other males. Other males attempt to copulate with guarded females in the "head-to-head" position, placing sperm in the females' seminal receptacles. These "sneaker" males are often successful in their attempts to copulate. Egg capsules, then, may contain the progeny of 2 or more males—consorts and sneakers.

Molecular methods are valuable tools to answer questions at the level of the individual and the population (e.g., Ferraris and Palumbi, 1996; Parker et al., 1998; Haig, 1998). One such approach is the use of microsatellite loci (e.g., O'Connell and Wright, 1997). Microsatellites have been developed for two congeners: an eastern Atlantic squid, *Loligo forbesi* (Shaw, 1996), and an eastern Pacific squid, *Loligo opalescens* (Reichow and Smith, 1999). In *L. forbesi* microsatellite loci have been used successfully to show multiple paternity within egg fingers laid by a single female in the wild (Shaw and Boyle, 1997), and to help resolve population structure (Shaw et al., 1999).

The advantages of microsatellite loci include codominant alleles, high allelic diversity at loci, and the ability to determine genotypes from small samples of tissue through the polymerase chain reaction (PCR) (Jarne and Lagoda, 1996; Schlotterer, 1998). Microsatellite loci are typically consistent with the expectations of Mendelian inheritance (e.g., O'Connell and Wright, 1997): independent segregation and random assortment of alleles. A direct test for Mendelian inheritance is to pair mates and analyze the genotypic frequencies of their offspring (e.g., Banks et al., 1999). We used this method to test the inheritance pattern

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of microsatellite loci developed recently for the squid *Loligo pealeii*.

Materials and Methods

Mating Trial: Pattern of Inheritance

Pattern of inheritance was examined through the genotypes of the offspring from a female (female A) and a male (male B). Female A was trawl-caught in Vineyard Sound on September 11, 1998, and placed in a large tank with many other squid. On September 14th, the female was removed from this tank, and sperm were evident in the seminal receptacle below her mouth. To remove sperm from this source, the female was placed into a large tub (2% ethanol, 98% seawater, by volume) and anesthetized within 1 minute. Her seminal receptacle was swiftly and completely excised with sterile scissors. She was then quickly transported to a 3-m-diameter recirculating tank, and she fully revived within 1 minute of being placed into the tank. Female A was kept in isolation in this tank for 48 hours. Female *L. pealeii* have no known organs for sperm storage within the mantle cavity (Drew, 1911), and Maxwell and Hanlon (2000) failed to find spermatophores within the mantle cavities of several females that had been kept in isolation for 1 or 2 days. Female A did not lay eggs during this isolation period.

A trawl-caught male (male B) was then added to the female's tank. On the following morning (September 17th), 25 newly laid egg capsules were found in the tank. Male B was then removed from the tank and dissected; gill tissue was taken and frozen at −20°C for DNA extraction. Female A died on September 20th; she did not lay another clutch of eggs. Upon dissection gill tissue was taken, and no spermatophores were found within her mantle cavity. The egg capsules were incubated and allowed to hatch in a large tub. Fifty-one free-swimming hatchlings were randomly sampled and immediately frozen at −20°C. We determined parental and filial genotypes at 3 polymorphic microsatellite loci (Table 1) at which female A and male B shared no more than 1 allele. Departure from the expected 1:1:1:1 ratio of filial genotypes was analyzed using a χ^2 goodness-of-fit test.

Microsatellite Loci

Genomic DNA was extracted from 20 to 40 mg of adult tissue or from whole individual embryos, using either a modification of the phenol–sodium dodecylsulfate (SDS) procedure (Maniatis et al., 1982) or Chelex reagent (Walsh et al., 1991).

Cloning, screening, sequencing, and primer development were performed by Genetic Identification Services (Chatsworth, Calif.). Briefly, digested genomic fragments were ligated into the *Hin*DIII-cut site of pUC19 plasmid and then cloned into *Escherichia coli* strain DH5α. Colonies were screened for several trinucleotide repeat motifs (AAT, AAG, CAA, and ATG). Positive clones were sized by PCR (using custom-made cloning size primers) prior to sequencing; plasmid inserts greater than 350 bp were sequenced. These methods yielded 20 microsatellite loci (GenBank accession numbers AF169206–AF169224 and AF165913). Five loci were selected to test for allelic diversity (see Table 1). A sixth locus, *Lfor3*, was adapted from *Loligo forbesi* (Shaw, 1996). All six loci were tested for allelic diversity using DNA extracted from female A and male B and the gills or armtips of 18 randomly selected adults trawled from Nantucket Sound in May 1997.

We estimated heterozygosity at each locus by randomly selecting 75 adults that were trawled during the 1999 inshore spawning season (May–October). We extracted DNA from their gills. At 3 loci (*Lp1*, *Lp4*, and *Lfor3*), we determined the genotypes of all 75 adults; we determined the genotypes of 20 of these 75 adults at the 3 remaining loci (*Lp2*, *Lp5*, and *Lp12*). At each locus, we reported the Nei (1987) unbiased estimate of observed heterozygosity.

For all loci, PCR was carried out in a thermocycler (GeneAmp, System 9700) (Perkin-Elmer). Standard reactions (10 μl) contained 10× PCR buffer (Perkin-Elmer), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.0 pmol of forward and reverse primers, one labeled with IRD 800, and *Taq* DNA polymerase. PCR cycles are given in Table 1 for each locus. PCR products were loaded onto a 6% denaturing gel (Long Ranger, 25 or 41 cm long and 0.25 mm thick) run in 1.2× Tris/boric acid/EDTA (TBE). The gel bands were separated and analyzed on a LI-COR 4000 DNA sequencer. Fragment sizes were determined by comparison to commercial size standards run in the same gel using GelPro Analyzer software (Media Cybernetics, Silver Spring, MD).

Results and Discussion

We originally attempted to adapt the 10 sets of primers designed for *L. forbesi* (Shaw, 1996) to amplify *L. pealeii* DNA, anticipating little divergence between these 2 north Atlantic congeners. The *Lfor3* primers used in PCR with *L.*

Table 1A. Primer Sequences, Repeat Motif, PCR Cycles, Observed Heterozygosity (H_o), and Number of Alleles Found Among 20 Individuals in *L. pealeii**

Locus	Primer sequence	Repeat motif	PCR program	No. of alleles	(*n*) H_o %	Fragment size, bp
Lp1	F: 5′-AAGCCTTAAAGAGAGATCGC-3′ R: 5′-GGAGCGTTTACTTCTAACTTCC-3′	$(AAG)_{20}$	300s at 94°C, 25–35 cycles of 30s at 94°C, 30s at 59°C, 30s at 72°C, and 120s at 72°C	25	95 (75)	223–350
Lp2	F: 5′-AGGGCAAAAAATGGACGT-3′ R: 5′-GCTGTTGGCTATTTATGTCG-3′	$(ATC)_{15}$	300s at 94°C, 25–35 cycles of 30s at 94°C, 30s at 57°C, 30s at 72°C, and 120s at 72°C	6	90 (20)	127–143
Lp4	F: 5′-CCAATTACTCTCTCTTGCACA-3′ R: 5′-AACGTAGATGAGGTGTGTGG-3′	$(AAG)_{22}$	300s at 94°C, 25–35 cycles of 30s at 94°C, 30s at 57°C, 30s at 72°C, and 120s at 72°C	14	93 (75)	156–236
Lp5	F: 5′-TGATAAAGGTGGTTGGTGAGA-3′ R: 5′-TCCGGGCACAAGTATTTAAG-3′	$(GAT)_{10}$	300s at 94°C, 25–35 cycles of 30s at 94°C, 30s at 57°C, 30s at 72°C, and 120s at 72°C	5	80 (20)	148–162
Lp12	F: 5′-TCGATCAGATCAATGCAGATT-3′ R: 5′-AACGATGCCTTAAACAGCAG-3′	$(GAT)_{17}$	300s at 94°C, 25–35 cycles of 30s at 94°C, 30s at 57°C, 30s at 72°C, and 120s at 72°C	3	60 (20)	169–177
Lfor3	F: 5′-GGTCATGTCATTCTCTGCAC-3′ R: 5′-ACATTTATCCATTAACAGAGTAGCA-3′	$(AAT)_{22}$	300s at 94°C, 20–25 cycles of 30s at 94°C, 120s at 55°C, 10s at 72°C, and 600s at 72°C	10	86 (75)	104–131

*Nucleotide sequences for cloned loci have GenBank accession numbers AF165913, AF169206, AF169208, AF169209, AF169216, and U66149.

Table 1B. Genotypes (in allele sizes, bp) of Female A, Male B, and 51 Offspring at Three Loci*

	Parental genotypes							
Locus	Male B	Female A	Offspring genotypes				χ^2	*P*
Lp2	129/135	126/129	126/129 (15)	126/135 (5)	129/129 (16)	129/135 (15)	6.3	>.05
Lp4	181/204	172/177	172/181 (8)	172/204 (20)	177/181 (11)	177/204 (12)	6.2	>.10
Lfor3	122/125	113/122	113/122 (11)	113/125 (12)	122/122 (15)	122/125 (13)	0.7	>.80

*Observed numbers of offspring for each genotype are given in parentheses. At each locus, expected genotypic ratios for the offspring are 1:1:1:1.

pealeii DNA amplified a DNA fragment of the expected size. Isolation and sequencing of the band revealed 99% identity between the species. In contrast, for example, the *Lfor7* primers given in Shaw (1996) produced an amplicon of the right size but revealed only 50% identity and lacked the short repeated sequences. This suggests a high mutation rate at this locus. The remaining 8 *L. forbesi* primers required lower annealing temperatures, showing base mismatches in these regions of the loci and consequently more random PCR products.

All 6 loci exhibited some degree of allelic diversity (see Table 1). As indicated by the number of alleles and heterozygosity values (see Table 1), variation exists between the loci. As an example, 14 alleles were found at locus *Lp4* for 18 randomly selected individuals; all individuals except squids 5 and 10 were heterozygous at this locus (Figure 1). For all 3 loci at which female A and male B shared no more than 1 allele, we failed to detect a significant departure from the expected 1:1:1:1 ratio of filial genotypes (see Table 1; $\alpha = 0.05$ for each χ^2 test). We also performed χ^2 tests on the

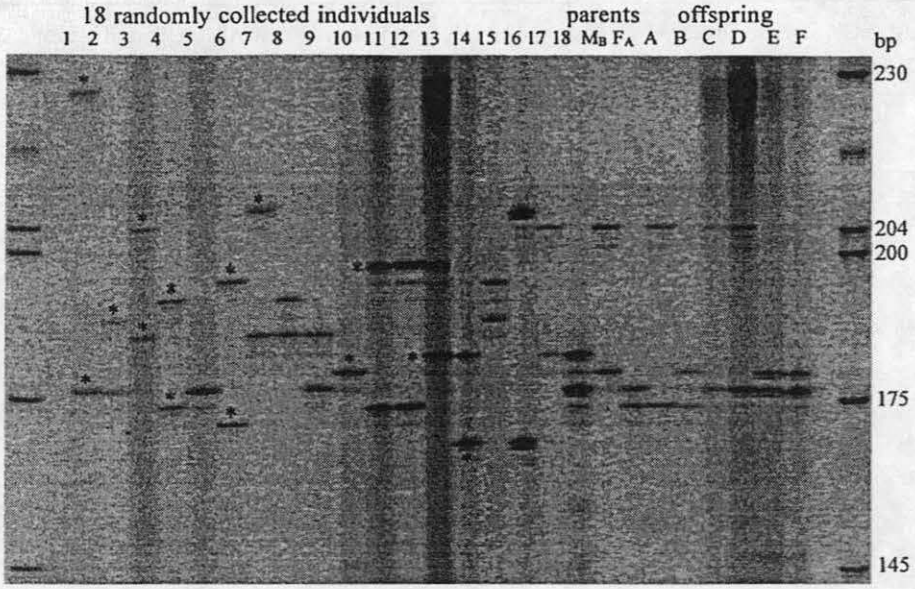

Figure 1. Genotypes at locus *Lp4*. On the left (lanes 1–18), the genotypes of 18 randomly collected adults from the field show 14 different alleles (*) in total. On the right, genotypes of the parents used in the mating experiment, male B (lane M_B) and female A (lane F_A), and 6 of their offspring *A–F* (lanes A–F); all offspring show alleles inherited from both male B and female A. The leftmost and rightmost lanes are 350-bp size standard markers.

frequencies of alleles found among the offspring and failed to detect a significant difference from the expected frequencies at any locus. These results provide strong evidence for Mendelian inheritance of these microsatellite alleles. Importantly, none of 51 progeny showed alleles that were not present in female A or male B. This observation indicates that female A was devoid of sperm and spermatophores when she was paired with male B. Figure 1 depicts the genotypes of female A and male B and 6 of their offspring at locus *Lp4*.

The hypervariable loci will be useful in studies of sexual selection on this species, such as the reproductive payoffs of different male mating tactics and the degree of multiple paternity within and between egg capsules in the field. Loci with relatively fewer alleles will be useful in determining population structure. *Loligo pealeii* ranges in the western Atlantic from Central America to Canada (Summers, 1983). Off the eastern United States, *L. pealeii* is a valuable and heavily exploited resource (McKiernan and Pierce, 1995), and determination of stock identity will aid in the management of this species. A previous study using allozymes could not resolve the question of subpopulation structure (Garthwaite et al., 1989). Shaw et al. (1999) have demonstrated subtle differences in population structure in *L. forbesi* using microsatellites where other methods (i.e., use of mitochondrial DNA and allozymes) had previously shown little subpopulation differentiation; thus microsatellites are likely to be useful in studying population structure of *L. pealeii*.

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