PRIMER NOTE

Characterization of microsatellite markers in the squid, *Loligo bleekeri* (Cephalopoda: Loliginidae)

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Running title: Microsatellite markers in *Loligo bleekeri*

Abstract

Loligo bleekeri has a long spawning season, the size of mature males changes during the season: dimorphic (large / small) early in the spawning season, and monomorphic (small) later in the spawning season. To understand how copulatory behaviors relate to the dimorphism, we developed five polymorphic microsatellite loci in *L. bleekeri*. The level of polymorphism ranged from 10 to 22 alleles with expected heterozygosities ranged from 0.79 to 0.93, suggesting that the novel polymorphic loci should be useful for parentage analysis of *L. bleekeri*.

Loligo bleekeri occurs widely off the coast from southern Korea to northern Japan, where commercial fisheries exploit the spawning aggregations. The spawning season of *L. bleekeri* extends over six months, and the size of mature males changes during the season: dimorphic (large / small) early in the spawning season, and monomorphic (small) later in the spawning season (Natsukari & Tashiro 1991). Before maturation, female Loliginidae store sperm in their seminal receptacles after "head to head" copulation. Just before spawning, they receive spermatophores during "male parallel" (Drew 1911) and "sneaking" copulation (Hanlon *et al*. 1994; Hanlon 1996). Loliginidae have three copulatory behaviors relating closely to male body size, and a female has sperm of several males available during spawning. Parentage analysis is a valuable tool to understand complex reproductive relations. Microsatellite markers have been isolated in other loliginid squid (Shaw 1997; Emery *et al*. 2000; Reichow & Smith 1999; Maxwell *et al*. 2000), demonstrating multiple paternities both within

clutches and within egg capsules (Shaw & Boyle 1997; Emery *et al*. 2001; Buresch *et al*. 2001). But the relation to copulatory behavior is unclear. Furthermore no microsatellite loci have been isolated in loliginid species living in the western Pacific Ocean. Here, we report the characterization of five microsatellite markers in *L. bleekeri* to understand the copulatory behaviors relating to the dimorphism of body size.

Adult squid were caught in the coastal water off southern Hokkaido, and stored at –20 °C. Gill tissue from each specimen was homogenized by overnight incubation at 50 °C with a solution of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 7.5), 1.4 M NaCl, 2% CTAB, 0.2% 2-mercaptoethanol and 150 μg/ml proteinase K, and total DNA was extracted with phenol-chloroform, ethanol-precipitated (Munehara & Takenaka 2000). For PCR primer design, 150-300 bp fragments digested with *Hae*III were selected by agarose gel electrophoresis and purified using a Gel Band Purification Kit (Pharmacia Biotech Ltd). The DNA fragments were ligated into the plasmid vector Blue-script and transformed into *Escherichia coli* XL1B, recombinant colonies were screened with $(GT)_{15}$ and $(GA)_{15}$ oligonucleotide probes that had a 3' terminal labeled with digoxigenin (Boehringer Manheim Ltd). Recombinant DNA fragments were sequenced on an automated sequencer (Gene Rapid, Pharmacia Biotech Ltd), following the manufacturer's recommendations. PCR primer pairs were designed for five loci.

PCR reaction mixes contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.19 mM of each dNTP, 3.3 μM of each primer, 1.5 U *Taq* polymerase (Takara Ltd), and 30 μg template DNA, and water to a final volume of 6.8 μL. PCR reactions were performed for 60 s at 94 °C, then 28~30 cycles of 30 s at 94 °C, 50 s at the annealing temperature (Table 1), and 80 s at 72 °C, with a GeneAmp PCR system 2400 thermocycler (Perkin-Elmer). The PCR products were electrophoresed in 7% polyacrylamide gel, and visualized by the silver staining method (Tegelström 1986). The size of the PCR products were estimated with 10 bp ladder markers and PCR products from the individual used for primer design.

The results of polymorphism and heterozygosity at each locus within 32 randomly selected individuals are presented in Table 1. The number of alleles ranged from 10 to 22, and expected heterozygosities ranged from 0.79 to 0.93. These microsatellite loci should provide to be helpful for the study of the reproductive system of *L. bleekeri*.

Table 1 Primer sequences and characterization of five microsatellite loci isolated from *Loligo bleekeri*. T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity

Locus	Repeat unit	Primer sequence (5'-3')	T_a (°C)	Size range (bp)	No. of individuals	No. of alleles	H_0	H_E	DDBJ Accession no.
Lb1	$(AC)_{16}CC(ACACAG)_3(AC)_7$	TATGCGTTACACTACCACCT ACGATAACCATTACACGACG	57	144-172	32	14	0.94	0.82	AB100368
Lb2	$(GT)_5(GA)_{15}$	TCTTAATTGAACGCCAGATT CTCGAGGAAACTATTTAAACT	51	138-172	32	17	0.88	0.88	AB100369

Lb3	$(TC)_{14}$	GCCATCCGAACAAACTTTAT GTTGCTATCAGCGTCCATTT	51	127-175	32	22	0.94	0.93	AB100370
Lb4	$(GA)_7TA(GA)_6$	CCACGTTGTTCCATGTGTTA CCGAGGGCTTGGTAAATATA	52	158-180	32	11	0.88	0.79	AB100371
Lb5	$(TC)_{13}(TA)_{12}(CA)_4$	TTTTGACATGGTGCCGCGAT ATATGCCCCTCTTTGCTTGC	60	104-128	32	10	0.84	0.81	AB100372

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