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Isolation and characterization of 24 polymorphic microsatellite loci in *Sepioteuthis lessoniana*

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ABSTRACT. Owing to their codominant, multiallelic, and highly polymorphic nature, microsatellite markers have been used widely in population genetics and biological resource conservation studies. To investigate the genetic structure of *Sepioteuthis lessoniana*, we developed 24 microsatellite DNA markers and assessed the polymorphism of each locus in a wild *S. lessoniana* population. The number of alleles per locus ranged from 4 to 26, and the observed and expected heterozygosities varied from 0.188 to 1.000 and 0.392 to 0.959 with an average of 0.675 and 0.852, respectively. These microsatellite loci will be useful tools in future studies of population genetic structure in this species.

Key words: Microsatellite; Cephalopoda; *Sepioteuthis lessoniana*; Enriched genomic library

INTRODUCTION

Bigfin reef squid, *Sepioteuthis lessoniana* (Lesson, 1830), are marine animals found from the shores from the Hawaiian Islands to the tropical Pacific Ocean and the Red Sea. They are commonly mistaken as cuttlefish, but they are indeed a completely different species. *S. lessoniana* has been placed within the *Sepioteuthis* branch in the cephalopod phylogenetic tree based on mitochondrial gene arrangement (Akasaki et al., 2006). Allozyme electrophoresis (Triantafillos and Adams, 2005) has been used to investigate the taxonomic status of northern calamari, and results indicate that *S. lessoniana* most likely comprises two "cryptic" biological species in Shark Bay, western Australia.

The only previous genetic investigations undertaken on *S. lessoniana* have demonstrated three taxa in the waters around Japan based on allozyme profiles (Izuka et al., 1994, 1996). Cryptic or sibling species are those not recognized *a priori* on morphological grounds and are a relatively common phenomenon among marine invertebrate groups (Knowlton, 1993; Thorpe et al., 2000). Otherwise cryptic species do exist in loliginid squid in the region (Yeatman and Benzie, 1994); therefore, a need has arisen to resolve the species status of *S. lessoniana*, preferably with molecular genetic techniques.

Owing to their codominant, multiallelic, and highly polymorphic nature, microsatellite markers have been used widely in population genetics and biological resource conservation research. Herein we present the first polymorphic microsatellite markers for *S. lessoniana*. These markers will be useful in population genetic studies and the conservation of this species.

MATERIAL AND METHODS

One live *S. lessoniana* was sampled from Lingshui, Hainan Province, China (18.48°N, 110.02°E). Genomic DNA was isolated from mantle muscle and subsequently digested with *Mbo*I. DNA fragments were ligated to oligonucleotide adapters (Gardner et al., 1999). We used electrophoresis on a 2% NuSieve GTG (Cambrex, USA) agarose gel to isolate small fragments (350-1000 bp). Biotin-labeled dinucleotide repeat sequences [$(CA)_{15}$] were hybridized to these DNA fragments, and the hybrid mixture was incubated with streptavidin-coated magnetic spheres (Promega, USA). After washing to remove the non-simple sequence repeat (SSR) fragments, the eluted single-stranded DNA contained the selected microsatellite DNA. As the primer, adapter A was used to amplify the selected DNA fragments using hot start polymerase chain reaction (PCR).

The amplified DNA fragments were ligated with pMD19-T plasmid vectors (TaKaRa, Japan) and the complexes were transformed into *Escherichia coli* DH5α competent cells (Toyobo, Japan). White clones were PCR screened with two vector primers and a non-biotin-labeled $(CA)_{12}$ primer. Screening amplifications were performed as follows: 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s, and a final extension of 5 min at 72°C.

We selected 360 clones for amplification, 97 of which were isolated for sequencing using a BigDye Terminator Cycle sequencing kit and an ABI PRISM 3730 Genetic Analyser (Applied Biosystems). Screening by the SSR Hunter 1.3 software (Li and Wan, 2005) showed that 80 sequences contained microsatellites with at least four uninterrupted repeats. With the exception of the biotin-labeled AC SSR probes, TC, AG, AT, AAG, AGT, ACAG, AGAT, ATAG, AAAG, and AGAA occurred in the microsatellites, which suggested that this probe made a positive contribution to the creation of the SSR-enrichment library from the genomes. All PCR primers were designed with PRIMER 5 (http://www.premierbiosoft.com/). We discarded the hybrid clones, duplicates and those sequences with short unique regions flanking the microsatellite array. In total, 52 PCR primer pairs were designed.

We used polyacrylamide gel electrophoresis to test the primer pairs in 30 *S. lessoniana* individuals sampled from Lingshui, Hainan Province. PCR amplifications were carried out in 10-μL volumes containing 0.25 U *Taq* DNA polymerase (TaKaRa), 1X PCR buffer, 0.2 mM deoxyribonucleotide triphosphate mix, 1 μM each primer set, 1.5 mM $MgCl_2$, and approximately 100 ng template DNA. The PCR thermal conditions were as follows: 3 min at 94°C, followed by

Table 1. Levels of variability at 24 polymorphic microsatellite loci in the *Sepioteuthis lessoniana*.

Locus	Accession No.	Repeat motif	Primer sequence (5'-3')	Ta (°C)	No. of alleles	Size range (bp)	H_O	H_E	P
Sle02	JQ696966	$(CT)_5(CT)_7(CT)_7(TTTC)_6$	TCTACGGCTACATTTACCTTT GGGGTGAGCATTTGAGTAAG	56	16	311-379	0.656	0.921	0.0000*
Sle03	JQ696967	$(TG)_{25}(AG)_5(AG)_{12}(AG)_5$	AAGGCGAAGTAATACAGA CACTTCTTAGTCCCCTCT	56	15	179-221	0.807	0.813	0.5101
Sle04	JQ696968	$(CT)_9(CA)_{14}(CA)_7$	CTCCCTCACATCTATTACCA GAGAGCAAGATGAAAGTGAAG	56	13	204-232	1.000	0.893	0.0350
Sle06	JQ696969	$(GA)_4(AG)_5(GT)_{20}(GA)_{14}$	TGTACCAAGTGTTATCTCCG GTTGCTATCAGTTTCTGTTAGTC	56	16	273-329	0.688	0.931	0.0000*
Sle07	JQ696970	$(TG)_6(GA)_4(GA)_4(TG)_4(GA)_5(GA)_4$	AGCGTGCATGAGAAGGAA CTGCAATGGGCTTGTTTAC	56	8	235-265	0.625	0.741	0.0000*
Sle09	JQ696971	$(AG)_8(GT)_{12}(AG)_{20}(AG)_4$	GTGTTAAAAGGTCCACTAAAG GCCCACCCATAAATAAATAC	62	13	276-334	0.633	0.906	0.0004
Sle10	JQ696972	$(AC)_7(AT)_4$	TATGCTACAAAACCAAAC ATATTCCACCTTGAAAAC	56	4	275-281	0.188	0.392	0.0006
Sle11	JQ696973	$(TG)_5(TG)_9(TG)_8(GC)_4(CT)_{11}(TG)_{31}$	GCTATAATACGCTCAAGTACCGA CAGCTATCTCAGCCACCAAA	62	16	348-408	0.625	0.923	0.0000*
Sle12	JQ696974	$(AAAG)_6(AGAA)_{14}$	GCCTATTTATTACATTGGTCG TTTTACAACTTCACTCTTCCTTT	58	26	168-284	0.968	0.959	0.1816
Sle15	JQ696975	$(AC)_{31}$	ACGAGAAGTTGAGTTATCTGAAA AACTGAAGTAAATTGGTAAATCAG	60	14	193-233	0.724	0.893	0.0000*
Sle16	JQ696976	$(AGT)_9$	TCGCACAACACCAGCATAGAG CGTCCGATTCACCGCTTACT	60	8	120-144	0.903	0.800	0.0293
Sle17	JQ696977	$(GC)_5(GT)_{16}$	CAGTAAGCGGTGAATCGGAC AGTGCGTATGCGTACCCTTT	60	21	126-192	0.967	0.945	0.0626
Sle22	JQ696978	$(TC)_9(CA)_{27}(TC)_{12}$	CACAGTACAGCAAGGATTTCT CTGTCAGTCGGTGAGTTTGT	60	11	229-281	0.875	0.875	0.0262
Sle24	JQ696979	$(CT)_8(TC)_4(CT)_4$	TCTGTTTCTTTATTCCTACCTCT GGGATTGTTCCAGTTGAGC	60	4	275-287	0.344	0.597	0.0000*
Sle25	JQ696980	$(AGAT)_{16}$	GATCGAAACATTTGAGCACT ATTTCCCATTCTGTCTTTGT	56	13	130-180	0.406	0.904	0.0000*
Sle29	JQ696981	$(ACAG)_5(GT)_{27}$	GAAAGACGCCAAGAGCATC ATTTCGTTTCGTTACCGTTG	62	19	270-314	1.000	0.942	0.4457
Sle31	JQ696982	$(TG)_{14}(GT)_{37}$	GCCGTTGACTGATATGCTAA GTGGGACGTTTGTCGTTT	62	12	342-378	0.548	0.915	0.0000*
Sle32	JQ696983	$(GA)_6(ATAG)_4(AG)_{16}(GT)_{26}(AG)_5$	GCCGAAATGAAGATGAGA CGTCTCCTCCTCACTTACTC	56	17	258-314	0.833	0.934	0.0024
Sle33	JQ696984	$(CA)_{25}$	CATCGTTGCCGTGTAGTCT TGATGTCCTTTCCATATCACTA	56	15	292-330	0.828	0.921	0.2520
Sle36	JQ696985	$(GA)_{20}(GA)_4(TG)_6(TG)_6(TG)_5(TG)_5$	CATCCGATTATCACTGCCT TTCCCGATATTTCTATTACTCA	60	17	305-347	0.367	0.928	0.0000*
Sle37	JQ696986	$(GA)_{12}(GT)_{11}(GT)_{11}(GA)_8(AG)_4$	AGTCAGTCTGTCAGTCGGTG CAGTACAGCAAGGATTTCTCA	60	8	267-287	0.519	0.806	0.0067
Sle41	JQ696987	$(AAG)_{15}$	ACCCGAGGAGACTTACTATCACTC TGTTGTTGCTGGCTGCTCT	56	12	222-270	0.308	0.910	0.0000*
Sle43	JQ696988	$(GT)_7(GA)_9(AG)_5(GA)_4(GA)_8(GT)_5(GA)_6(AG)_7(GA)_6(AG)_5(GA)_8(GA)_{10}(GA)_8$	AGGGACAGATGAAGAAGGT CGTCTTGATAAAACAGCGT	60	9	295-365	0.581	0.760	0.0091
Sle48	JQ696989	$(GT)_{10}(GA)_{12}(AG)_{10}(GA)_4(GA)_4(TG)_{11}(TG)_7(GA)_5(AG)_4(AG)_4(GA)_{10}(TAGA)_4$	AGAAGCAAGGAGAAATATAGACAG AACATTCCAACCAAAAGAGG	60	11	352-394	0.813	0.842	0.0965

Ta = anneling temperature; H_O = observed heterozygoty; H_E = expected heterozygoty. *Significant departure from Hardy-Weinberg equilibrium (P = 0.000).

35 cycles of 45 s at 94°C, 45 s at the annealing temperature listed in Table 1, and 45 s at 72°C, with a final extension of 5 min at 72°C. As the reference marker, a 10-bp DNA ladder (Invitrogen) was used to identify allele size.

The PCR products were resolved on 6% denaturing polyacrylamide gel using silver staining. The number of alleles and observed and expected heterozygosities were estimated with the Microsatellite Analyser program (Dieringer and Schlötterer, 2003), and tests for linkage disequilibrium and deviations from Hardy-Weinberg equilibrium were performed using Genepop 4.0 (Rousset, 2008).

RESULTS AND DISCUSSION

Of the 52 primer pairs developed, 24 microsatellite loci showed obvious polymorphism in the *S. lessoniana* population (see Table 1). The number of alleles at each locus ranged from 4 to 26 with an average of 13.25, and the observed and expected heterozygosities varied from 0.188 to 1.000 and 0.392 to 0.959 with an average of 0.675 and 0.852, respectively. No linkage disequilibrium existed between pairs of loci. Significant departure from Hardy-Weinberg equilibrium was found at 10 loci (Sle02, Sle06, Sle07, Sle11, Sle15, Sle24, Sle25, Sle31, Sle36, and Sle41). These deviations may have been caused by the presence of null alleles at these loci (Pemberton et al., 1995). Genetic variability of these loci indicated their practicability as appropriate population genetic markers for the exploration and investigation of population genetic diversity in this species.

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REFERENCES

Akasaki T, Nikaido M, Tsuchiya K, Segawa S, et al. (2006). Extensive mitochondrial gene arrangements in coleoid Cephalopoda and their phylogenetic implications. *Mol. Phylogenet. Evol.* 38: 648-658.

Dieringer D and Schlötterer C (2003). Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Mol. Ecol. Notes* 3: 167-169.

Gardner MG, Cooper SJB, Bull CM and Grant WN (1999). Isolation of microsatellite loci from a social lizard, *Egernia stokesii*, using a modified enrichment procedure. *J. Hered.* 90: 301-304.

Izuka T, Segawa S, Okutani T and Numachi K (1994). Evidence on the existence of three species in the oval squid *Sepioteuthis lessoniana* complex in Ishigaki island, Okinawa, southwestern Japan, by isozyme analyses. *Jpn. J. Malacol.* 53: 217-228.

Izuka T, Segawa S and Okutani T (1996). Biochemical study of the population heterogeneity and distribution of the oval squid *Sepioteuthis lessoniana* complex in southwestern Japan. *Am. Malacol. Bull.* 12: 129-135.

Knowlton N (1993). Sibling species in the sea. *Ann. Rev. Ecol. Syst.* 124: 189-216.

Li Q and Wan JM (2005). SSRHunter: development of a local searching software for SSR sites. *Yi. Chuan* 27: 808-810.

Pemberton JM, Slate J, Bancroft DR and Barrett JA (1995). Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Mol. Ecol.* 4: 249-252.

Rousset F (2008). Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol. Ecol. Resour.* 8: 103-106.

Thorpe JP, Solé-Cava AM and Watts PC (2000). Exploited marine invertebrates: genetics and fisheries. *Hydrobiol* 420: 165-184.

Triantafillos L and Adams M (2005). Genetic evidence that the northern calamary, *Sepioteuthis lessoniana*, is a species complex in Australian waters. *ICES J. Mar. Sci.* 62: 1665-1670.

Yeatman J and Benzie JAH (1994). Genetic structure and distribution of *Photololigo* spp. in Australia. *Mar. Biol.* 118: 79-87.

