Isolation and characterization of highly polymorphic microsatellites in the mitre squid, *Uroteuthis* (*Photololigo*) *chinensis*

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Abstract

The mitre squid, *Uroteuthis* (*Photololigo*) *chinensis*, is a commercially important species in many coastal regions of Asia. Little information is available on their population structure and mating system. We developed 12 polymorphic microsatellite markers using enrichment protocol. The number of alleles per locus ranged from 5 to 27, and levels of expected heterozygosity ranged between 0.70 and 0.97. These loci should prove useful for population and parentage studies. Cross-species amplifications of 21 microsatellite loci developed for *Loligo vulgaris* and *Loligo forbesi* in *U.* (*P.*) *chinensis*, and of all 33 microsatellite loci in two other commercially important *Uroteuthis* species were not successful except for one locus.

Body of the manuscript

Microsatellite DNA markers were firstly applied in population genetic studies of cephalopods in 1999 (Shaw *et al.*, 1999), and their use has increased in recent years (Shaw, 2002). For example, they were used to determine whether seasonal and/or geographical groups represent distinct populations in a number of loliginid squids and cuttlefishes (Garoia *et al.*, 2004; Reichow and Smith, 2001; Shaw *et al.*, 2004). *Uroteuthis* (*Photololigo*) *chinensis* supports a very important commercial fishery in mainland China and Taiwan on the continental shelf off Guangdong, southern Fujian and around the Pescadore Islands in the Taiwan Strait (Voss and Williamson, 1971). It accounts for up to 90% of the loliginid catch in several parts of China. It also accounts for 15-40% of the trawl catch in the Gulf of Thailand. *Uroteuthis* (*Photololigo*) *chinensis* is also caught in north Australian waters and it is believed to occur in small quantities in Indonesian, Malaysian and Philippine catches (Roper *et al.*, 1984). However, little is known about the population structure and mating system of this species. Thus we isolated and characterized 12 polymorphic micorsatellites from *U.* (*P.*) *chinensis* in order to examine its population biology.

Total DNA was extracted from ethanol-preserved mantle of an *U.* (*P.*) *chinensis* individual using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Microsatellites were isolated following the protocol of Glenn & Schable (2005). Briefly, 2 μg of genomic DNA was digested with RsaI and fragments were ligated to Super SNX linkers. Biotinylated probes (mix 2 and mix 4) were used to perform subtractive hybridization. After enrichment, 2 μL of DNA enriched with repeats was amplified using the SuperSNX-24 as primer in a 25-μL reaction. The resulting PCR products were ligated into pMD18-T vector (Takara) and transformed into the bacterial host *E. coli* JM109. Bacteria were ampicillin selected and

54 inserts were PCR amplified using M13 forward and M13 reverse primers. The PCR program
55 consisted of denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 20 s, 50°C for
56 20 s, 72°C for 1 min. Inserts between 150 bp and 1000 bp were gel purified (Qiagen, Hilden,
57 Germany), and were sequenced in both direction using M13 forward and M13 reverse primers
58 (Macrogen). DNA amplification primers were designed from 38 clone sequences displaying
59 suitable repeat motifs, using the program OLIGO 4.05 (National Bioscience), of which 12
60 pairs yielding the best results are presented in Table 1.

61 These 12 microsatellites and 21 loci developed for other loliginid squids, *Loligo*
62 *vulgaris* (Guarniero *et al.*, 2003) and *Loligo forbesi* (Emery *et al.*, 2000; Shaw, 1997), were
63 characterized in 30 *U.* (*P.*) *chinensis* individuals collected in Hong Kong, China. Genomic
64 DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Each
65 PCR consisted of 20 ng DNA, 0.4 μM of each primer, 1xPCR buffer (50 mM KCI, 1.5 mM
66 $MgCl_2$, and 10 mM Tris-HCl), 200 μM dNTPs and 0.5 U *Taq* DNA polymerase (GE
67 Healthcare). PCR conditions consisted of a denaturation stage at 94°C for 3 min followed by
68 34 cycles of 30 s at 94°C, 30 s at the locus-specific annealing temperature (Table 1) and 30 s
69 at 72°C, followed by 72°C for 3 min. PCR products were scored on 5% denaturing
70 polyacrylamide gels following electrophoresis.

71 Primer sequences and genetic diversity statistics for the 12 newly developed loci are
72 presented in Table 1. All primer pairs amplified products of expected size. Expected and
73 observed heterozygosities, deviations from Hardy-Weinberg expectations (HWE), and linkage
74 disequilibrium were estimated using GENEPOP version 3.4 (Raymond and Rousset, 1995).
75 All 12 of the loci screened were polymorphic in *U.* (*P.*) *chinensis*. The number of alleles
76 ranged from five to 27 (Table 1). Expected heterozygosity ranged from 0.70 to 0.97. Nine

77 loci had allele frequencies supporting HWE and three loci significantly deviated from HWE
78 after Bonferroni correction (Table 1). Using Micro-Checker 2.2.3 (Van-Oosterhout *et al.*,
79 2004), we detected the presence of null alleles in these three loci. No pairs of loci
80 significantly deviated from linkage equilibrium. For the investigation of the cross-
81 amplifications of the 21 loci in *U.* (*P.*) *chinensis*, successful amplification and polymorphism
82 was only found in Lfor3 (Shaw, 1997) (Table 1).

83 Cross-species amplification was investigated in two other commercially important
84 *Uroteuthis* species: *Uroteuthis* (*Photololigo*) *edulis* and *Uroteuthis* (*Photololigo*) *duvauceli*.
85 Cross species PCR were run at the optimal conditions identified for *U.* (*P.*) *chinensis*, and
86 products were separated and scored on silver-stained polyacrylamide gels. Polymorphic
87 amplification was only found in the locus Lfor3 (Table 2), which may represent a
88 microsatellite common to a wide variety of squids.

89 This study provides 12 new polymorphic loci that will be valuable in studies of mating
90 system, genetic diversity, genetic population structure and assessments of gene flow in *U.* (*P.*)
91 *chinensis*.

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97 **References**

Emery AM, Shaw PW, Greatorex EC, Boyle PR, and Noble LR (2000) New microsatellite markers for assessment of paternity in the squid *Loligo forbesi* (Mollusca: Cephalopoda). *Molecular Ecology* **9**, 110-111.

Garoia F, Guarniero I, Ramsak A, Ungaro N, Landi M, Piccinetti C, Mannini P, and Tinti F (2004) Microsatellite DNA variation reveals high gene flow and panmictic populations in the Adriatic shared stocks of the European squid and cuttlefish (Cephalopoda). *Heredity* **93**, 166-174.

Glenn TC, and Schable NA (2005) Isolating microsatellite DNA loci. *Methods in Enzymology* **395**, 202-222.

Guarniero I, Garoia F, Placido RD, Ramsak A, Mannini P, and Tinti F (2003) Species-specific microsatellite loci for the European squid (*Loligo vulgaris*). *Molecular Ecology Notes* **3**, 312-313.

Raymond M, and Rousset F (1995) GENEPOP version 1.2: population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**, 248-249.

Reichow D, and Smith MJ (2001) Microsatellite reveal high levels of gene flow among populations of the California squid *Loligo opalescens*. *Molecular Ecology* **10**, 1101-1109.

Roper CFE, Sweeney MJ, and Nauen CE (1984) FAO species catalogue. Vol. 3. Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. *FAO Fisheries Synopsis* **125**, 277 pages.

Shaw PW (1997) Polymorphic microsatellite markers in a cephalopod: the veined squid *Loligo forbesi*. *Molecular Ecology* **6**, 297-298.

Shaw PW (2002) Past, present and future applications of DNA-based markers in cephalopod biology, workshop report. *Bulletin of Marine Science* **71**, 67-78.

Shaw PW, Arkhipkin AI, Adcock GJ, Burnett WJ, Carvalho GR, Scherbich JN, and Villegas PA (2004) DNA markers indicate that distinct spawning cohorts and aggregations of Patagonian squid, *Loligo gahi*, do not represent genetically discrete subpopulations. *Marine Biology* **144**, 961-970.

Shaw PW, Pierce GJ, and Boyle PR (1999) Subtle population structuring within a highly vagile marine invertebrate, tthe veined squid *Loligo forbesi*, demonstrated with microsatellite DNA markers. *Molecular Ecology* **8**, 407-417.

Van Oosterhout C, Hutchinson WF, Wills DPM, and Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**, 535-538.

Voss GL, and Williamson GR (1971) *Cephalopods of Hong Kong* Hong Kong Government Press, Hong Kong.

Table 1 Core repeat, primer sequences (5' to 3'), and optimal annealing temperature (T_a) for microsatellite loci isolated from *Uroteuthis chinensis*. n is the number of individuals successfully genotyped in *U. chinensis*, H_O and H_E are observed and expected heterozygosities, respectively. H_O significantly different from Hardy-Weinberg expectations after Bonferroni correction are indicated by asterisks (*). Data are also given at a locus isolated from *Loligo forbesi* (Shaw 1997).

Locus	Repeat	Primers 5'-3'	T_a (°C)	Allele size range (bp)	GenBank Accession no.	n	No. of alleles	H_E	H_O
Uchi1	$(TATG)_9TATC(TTTC)_2(TATC)_3TTTC(TATC)_3\ldots(TATC)_{10}$	F: TAAAATGTAACTTCCGAAATACC	60	187-317	FJ980010	27	25	0.94	0.78
		R: ACACAAAAGCAAACAGACACAGG							
Uchi2	$(GA)_3(TAGA)_2CA(GATA)_6(GA)_{11}GTGAAC(GA)_2GG(GA)_9$	F: GATGACTAATAAGGATGGTTGGA	60	225-311	FJ980011	27	26	0.97	0.89
		R: TGAGATCAAAACAGGTTGAACTT							
Uchi3	$(GAA)_{27}$	F: CTACGATTATACTGGGGAAGGTG	59	215-278	FJ980012	30	18	0.93	0.83
		R: TAGTCTAATTTGTCTGTATGGTT							
Uchi4	$(TGA)_{13}$	F: GATAAGGACGGGTGGGGAGAAAG	59	166-235	FJ980013	30	19	0.94	0.67*
		R: TCGTGGAAAGAGATTACTGCAAA							
Uchi5	$(GT)_4\ldots(GT)_7\ldots(GT)_6\ldots(GT)_4\ldots(GT)_5$	F: ATGGGTCTATTTTGAAGCCTATG	59	195-201	FJ980014	29	5	0.70	0.38*
		R: GACACAAACAGAGAAAGAGAAAT							
Uchi6	$(AG)_{18}AC(AG)_3AC(AG)_2AT(AG)_3AC(AG)_2AT(AG)_9\ldots(AG)_8$	F: ACGGAGTATTTCGTCTGGCAATG	59	229-287	FJ980015	30	24	0.94	0.97
		R: ATCTGCTGGATGTCCCCTACTTG							
Uchi7	$(CAA)_{16}$	F: AAGTATAAGAAATGAAATGAACC	54.3	132-177	FJ980016	30	14	0.89	0.87
		R: CATTAAAAGTGAAAGATATTGTC							
Uchi8	$(GA)_{35}$	F: AACATATTTCTCAGGGGATTTTC	59	122-166	FJ980017	29	20	0.94	0.93
		R: TACCTTTCAGTGTCATCGCATAC							
Uchi9	$(CT)_{10}\ldots(TC)_{12}$	F: CGAAGTTATTTTCCAAGACATTA	59	233-297	FJ980018	19	15	0.91	0.58*
		R: ATTTCTCTGCATTCCTGCTAAGC							
Uchi10	$(TG)_{20}$	F: GTGTATGTGTGCGTGCGTGGATG	59	259-297	FJ980019	29	20	0.95	0.97
		R: TCTCCCTCAGTTGTTGTTTGAAC							
Uchi11	$(CAT)_{10}(TCTG)_3(TC)_4$	F: TTCTATTTAACCAGGAACGAAAG	54	141-164	FJ980020	25	13	0.86	0.68
		R: AATGATGGATAGGGACGGTTGGA							
Uchi12	$(AC)_{15}\ldots(AAG)_{27}$	F: TGGGGTCATGGAAACACTCAAAG	59	271-331	FJ980021	30	27	0.96	0.90
		R: AAGAGCCGTCAAATTCGAGGATC							

Primers published for *Loligo forbesi*									
Lfor3	$(AAT)_{22}$	F: GGTCATGTCATTCTCTGCAC R: ACATTTATCCATTAACAGAGTAGCA	60	122-161	27	13	0.92	0.93

142 **Table 2** Numbers of alleles and size ranges observed for Lfor3 (Shaw 1997) in two

143 *Uroteuthis* species

Locus	*U.* (*P.*) *edulis*					*U.* (*P.*) *duvauceli*				
	n	Number of alleles	Size range	H_E/H_O	P_{HW}	n	Number of alleles	Size range	H_E/H_O	P_{HW}
Lfor3	8	12	124-181	0.96/1.00	NS	6	4	124-148	0.80/1.00	NS

144 NS = P > 0.05.