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TECHNICAL NOTE

Characterization of eight polymorphic microsatellite loci for the California spiny lobster, *Panulirus interruptus* and cross-amplification in other achelate lobsters

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Abstract Microsatellite sequences were isolated from both non-enriched and enriched genomic libraries of California spiny lobster, *Panulirus interruptus.* Eight consistently amplifying, scorable and polymorphic loci were characterized for 79 individuals collected at Santa Cruz and San Clemente Islands, California, and tested for cross-species amplification in four closely related *Panulirus* spp., as well as four other species of the order Achelata. The number of alleles observed per locus ranged from three to 54 and observed heterozygosities ranged from 0.57 to 0.98. Quality control testing shows that all loci were reliably scorable, independently segregating, inherited in Mendelian ratios, and had low to moderate ($\leq$14.4%) frequencies of null alleles and high statistical power for detecting fine scale genetic structure.

Keywords Crustacea · Microsatellite · *Panulirus interruptus* · Spiny lobster

The California or red spiny lobster, *Panulirus interruptus*, occurs in shallow, rocky coastal areas from Monterey Bay, California to Manzanillo, Mexico, with a small, isolated population in the northern Sea of Cortez (Barsky 2001); however, the majority of the population occurs between Point Conception, California and Bahia Magdalena, Mexico (Duffy 1973). There is significant potential for individuals to disperse across much of this range. After hatching in nearshore waters phyllosome larvae spend seven to nine months drifting in prevailing currents before metamorphosis to the puerulus stage (Serfling and Ford 1975; Engle 1979; Booth and Phillips 1994). Following metamorphosis, pelagic puerulus larvae remain in surface waters for an additional two and a half months before settling to inshore benthic habitats (Shaw 1986). Due to lucrative commercial and recreational fisheries for this species in the United States and Mexico, and the potential impact of these fisheries on the sustainability of lobster populations outside regional management jurisdictions, there is considerable interest in understanding patterns of dispersal and connectivity (Perez-Enriquez et al. 2001; Iacchei et al. 2005). Here we report development of polymorphic microsatellite markers to assess gene flow and infer patterns of connectivity in this species.

Microsatellites were drawn from two different sources. First, a series of 15 potential loci were developed following the Microsatellites for Ecologists protocol (Toonen 1997) as described in Toonen et al. (2004). From roughly 2500 colonies in the initial library, 56 colonies positive for microsatellite-containing inserts were isolated and sequenced, yielding 15 putative microsatellite loci. Of these, 11 contained perfect dinucleotide repeats and four contained compound dinucleotide and/or trinucleotide repeats. The remaining sequences lacked sufficient flanking sequence around the repeat motifs to design primers. PRIMER 3 (Rozen and Skaletsky 1999) was used to develop primers, and unlabelled primers were ordered from

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Operon Inc., Alameda, CA, USA. These 15 primer sets were tested on 5 individuals from each of 5 sites (Anacapa Island, CA, Santa Catalina Island, CA San Clemente Island, CA San Diego, CA, and Bahia Tortugas, Mexico), and four of the loci were found to be polymorphic, amplify consistently, and be reliably scorable (Pin10, Pin29L, Pin189, Pin244).

The other polymorphic loci were developed from four microsatellite-enriched libraries constructed by Genetic Identification Services (GIS: Chatsworth, CA, USA) using pooled genomic DNA extracted from three adult *P. interruptus* collected off Isla Vista Reef, CA. DNA was isolated from leg tissue stored in 70% ethanol using a phenol-chloroform-isoamyl alcohol protocol followed by isopropanol precipitation (after Toonen 1997). Isolated DNA pellets were washed with 70% ethanol and re-suspended with 50 µl of sterile 1X TE buffer, pH 7.2. Methods for DNA library construction, enrichment and screening followed Jones et al. (2002). A total of 34, 16, 16, and 34 colonies positive for microsatellite inserts were isolated and sequenced from $(GA)_{15}$, $(CATC)_8$, $(TACA)_8$, and $(TAGA)_8$ enriched libraries, respectively yielding 19, 6, 5, and 14 putative microsatellite loci. PCR primer pairs were designed for these 44 loci using DESIGNERPCR version 1.03 (Research Genetics, Inc.) and unlabeled primer pairs were ordered from Sigma Proligo, St. Louis, MO, USA. Polymerase chain reactions were performed in 10 µl volumes containing 20 ng template DNA, 1x Colorless GoTaq Reaction Buffer (containing 1.5 mM $MgCl_2$ pH 8.5; Promega Corp, Madison, WI, USA), 2 µM dNTPs, 6 µM forward primer, 6 µM reverse primer, and 0.01U/µl GoTaq polymerase (Promega Corp.) using a Bio-Rad DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR conditions were as follows: 3 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at annealing temperature (see Table 1 for each locus), 40 s at 72°C, and a final extension step of 30 min at 72°C. Fourteen primer pairs amplified products of appropriate size and were selected for further study.

For genotyping, PCR amplification was carried out using the conditions described above with forward primers fluorescently labeled with WellRed D2, D3, or D4 dye (Beckman-Coulter Inc., Fullerton, CA, USA; see Table 1 for each primer label). PCR products were sized on a Beckman-Coulter CEQ 8000 capillary sequencer (Beckman-Coulter Inc.). Size markers (Size Standard 400; Beckman-Coulter Inc.) were included in the lane with PCR products and alleles were scored using a CEQ 8000 genetic analysis system (Beckman-Coulter Inc.). Four loci were found to be consistently amplifying, scorable and polymorphic (PinA5, PinA102r, PinA110, PinD110).

These eight loci were tested for conformation to Mendelian expectations using four *P. interruptus* mothers collected at Santa Catalina Island, California and ≥8 attached offspring per mother. A composite chi-squared analysis was used to test for deviations from expected equal inheritance of maternal, heterozygote alleles by offspring. When maternal genotypes were found to be homozygous, we tested for obligate inheritance of the maternal allele in all genotyped offspring. All eight loci passed the composite chi-squared test ($\alpha = 0.05$) and, when applicable, displayed obligate inheritance of maternal homozygote alleles.

Here we report the combined results (Table 1) from two population samples consisting of 79 adult *P. interruptus* collected from Santa Cruz Island and San Clemente Island, California in 2006 and 2007. MICRO-CHECKER (van Oosterhout et al. 2004) found no evidence of scoring errors due to stuttering or large-allele dropout. FREENA (Chapuis and Estoup 2007) estimated low (<10%) to moderate (10–15%) frequencies of null alleles in all loci except PinA5 and Pin10. The presence of low to moderate frequencies of null alleles in these loci is not surprising as this is commonly observed in a variety of abundant marine invertebrate species (Kaukinen et al. 2004). Five loci had significant heterozygote deficiencies (tested in ARLEQUIN 3.11; Excoffier et al. 1995). The severity of the deficits (F_{is}; FSTAT 1.2; Goudet 1995) at all loci showed a very strong positive correlation with their average frequency of null alleles estimated in FREENA ($\rho = 0.98$, $P < 0.001$). This suggests that observed heterozygote deficits result from low to moderate frequencies of null alleles. There was no significant evidence for linkage disequilibrium among pairs of loci following corrections for multiple comparisons (FSTAT 1.2; Goudet 1995).

Cross-amplification was tested in four congeneric species in the *Panulirus* genus, as well as four phylogenetically distant achelate lobsters (Table 2). Of the eight loci tested, five cross-amplified in other lobster species and were found to be polymorphic. All scored alleles in these samples were within the size range of scored alleles observed in *P. interruptus*. One locus, Pin189, was found to be monomorphic in the phylogenetically distant *Parribacus antarcticus*, and the scored allele in these samples was outside the size range of alleles found in *P. interruptus*. Cross-amplification was otherwise only observed in *Panulirus* spp.

The capacity for these loci to detect fine-scale patterns of population structure was evaluated using the simulation-based power analysis POWSIM (Ryman and Palm 2006). Simulation results estimate 94% statistical power in detecting fine-scale population structure ($F_{ST} = 0.01$) with 30 individuals sampled from each subpopulation.

Table 1 Locus name, repeat motif, dye label, primer sequence, annealing temperature of the PCR reaction (T_a), number of individuals successfully genotyped out of an initial sample size of 79 (N), observed number (N_a) and size range of alleles, observed (H_O) and expected (H_E) heterozygosity, Hardy–Weinberg Equilibrium *P*-value († locus is out of Hardy-Weinberg Equilibrium), null allele frequency, F_{IS}, and GenBank accession $^{\#}$ for each locus

Locus name	Repeat motif	Well Red dye	Primer Sequence (5′-3′)	T_a (°C)	N	N_a	Allele size range	H_O	H_E	P_{HWE}	Null freq.	F_{is}	GenBank accession$^{\#}$
PinA5	(CT)29	D4	GC A A ATT CT CT C A A GGA A GGTT GGC A C GAT AT AC A CTT G GTTT G	56	79	25	116–178	0.97	0.93	0.315	0.000	−0.048	EU735032
PinA102r	(GA)34	D4	T GC A AC AC A GT GGT AT CT CT A A AC GTT C CT AT C AC ATT TT CTT G	48	79	22	271–323	0.86	0.93	0.001†	0.028	0.072	EU735033
PinA110	(CT)26	D3	C C AC GA G A CCA A GGA A ACT A C GA AT GC TT GT GTC GC T A ACT G	54	79	3	170–174	0.57	0.58	0.918	0.002	0.010	EU735034
PinD110	(GATA)13(GA)7	D4	GG G A ATCT CT A A GGA AT GT TC G AGC C A A A GC C A C GA C A A G	60	63	49	243–409	0.68	0.97	0.000†	0.144	0.302	EU735035
Pin29L	(GT)22	D3	TTT ATC C GC AT G GAT G A C GC AGAT GG G GT C CCA A GGT GT G	60	79	44	189–363	0.85	0.97	0.000†	0.057	0.129	EU735036
Pin189	(CA)18	D4	AACACCCCTCCTACCCCC C CC CAT C A A A ACCT CT G GA C	60	79	27	261–321	0.87	0.94	0.036†	0.028	0.073	EU735037
Pin10	CTA)16	D3	C GA AT CAT GGC T GTT C GA GG GGA CT C A GT G GT CT GT A ATT GT	62	79	19	126–182	0.90	0.92	0.601	0.000	0.021	EU735038
Pin244	(GT)36	D2	TC A GT G GA T GA A GGTT A C G AC A GCC TTT CT GT GGA ATT A	56	79	54	93–327	0.90	0.97	0.006†	0.036	0.076	EU735039

Table 2 Cross-amplification of the eight microsatellite loci in eight additional lobster species

	PinA5	PinA102r	pinA110	pinD110	Pin29L	Pin189	pin10	Pin244
Panulirus marginatus (4)	0	3	0	2	4	4	0	0
Arctides regalis (4)	0	0	0	0	0	0	0	0
Scyllarides haanii (4)	0	0	0	0	0	0	0	0
Panulirus penicillatus (4)	0	0	0	0	3	4	4	0
Panulirus inflatus (1)	0	0	0	0	0	0	0	0
Parribacus antarcticus (4)	0	0	0	0	0	2*	0	0
Scyllarides squammosus (4)	0	0	0	0	0	0	0	0
Panulirus versicolor (2)	0	0	0	0	2	0	0	0

The number of individuals tested for each species is indicated by the number in (). Numbers in columns indicate the number of samples amplifying polymorphic products of comparable size to that amplified from *P. interruptus*. 0 indicates no amplification of any products. * indicates amplification of monomorphic products outside the size range of products amplified from *P. interruptus*

Statistical power is increased to 99% when sampling is increased to 50 individuals from each subpopulation. Measures of population genetic structure, such as F_{ST}, may be overestimated in the presence of null alleles when populations are substantially differentiated. This bias diminishes as gene flow between populations increases (Chapuis and Estoup 2007). Larval exchange among populations of *Panulirus interruptus* is potentially extensive, and consequently, patterns of genetic differentiation are likely to be subtle. These eight loci therefore provide sufficient variability and statistical power to address this fine-scale and complex population structure, providing a useful set of molecular markers to address questions of connectivity in this highly valuable fishery species.

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