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PRIMER NOTES

Microsatellite loci isolated from *Odontesthes argentinensis* and the *O. perugiae* species group and their use in other South American silverside fish

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Odontesthes (Atheriniformes: Atherinopsidae) is a diverse and widespread genus of silverside fish, containing between 15 and 22 recognized species distributed across marine, estuarine and freshwater environments of temperate South America (Dyer 1993). The marine-estuarine *O. argentinensis* ranges from the south-east coast of Brazil (25°S) to Chubut, in Argentina (43°S) (Dyer 1993). It is an economically significant resource for local fisheries in Brazil, Uruguay and Argentina (de Buen 1953) and a suitable organism for bioassay studies (Phonlor & Cousin 1997). The freshwater *O. perugiae* species group is composed of morphotypes mostly distributed along the complex system of lagoons of the coastal plain of southern Brazil (Dyer 1993).

We are using molecular markers to understand historical and recent biogeographic scenarios that have shaped the evolution of *Odontesthes*. Results based on sequences of three mitochondrial DNA genes revealed that *O. argentinensis* and *O. perugiae* are closely related and have recently radiated in the coastal areas of southern Brazil (L. Beheregaray, unpublished). We developed microsatellites for these taxa to identify population structures, clarify taxonomic problems and reconstruct their phylogeographic history. Evidence for the application of these primers in a wide range of Neotropical silverside species is presented. To our knowledge, these are the first microsatellite loci isolated from fish native to South America, a continental area with one of the most diverse ichthyofaunas of the planet.

Genomic DNA of *O. argentinensis* and the *O. perugiae* species group was extracted by salting-out (Sunnucks & Hales 1996), pooled together, and digested with *Hae*III, *Eco*RV and *Alu*I. Fragments ranging between 430 and 550 bp were cloned into *Sma*I cut pUC18 and a resulting library of approximately 25 000 colonies was screened with radiolabelled CA/GT and GA/CT co-polymers according to Taylor *et al*. (1994).

Putative positive clones were rescreened, and 31 clones encompassing different hybridization intensities were selected for sequencing. This strategy increased our chances of choosing both long and short repeats as they are expected to inform at different levels of divergence (see below). Thirteen of the clones sequenced contained microsatellites with appropriate flanking regions for primer design.

Variation at each of the 13 loci was assessed by the polymerase chain reaction (PCR) using a 10 μL radiolabelled reaction containing 50–100 ng of template DNA, 12 pmol of each primer, 0.5 units of *Taq* DNA polymerase (Promega), 200 μM of dCTP, dGTP, and dTTP, 20 μM of dATP, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 0.05 μL [α-^{33}P] dATP at 1000 Ci/mmol overlaid with mineral oil. PCR amplifications were performed in an MJ Research PTC100 thermocycler starting with 94 °C for 3 min, followed by a 'touchdown' (32 cycles at 94 °C/20 s, annealing/45 s and 72 °C/60 s), and a final step of 72 °C for 4 min. The annealing temperature of the 'touchdown' PCRs decreased two degrees per cycle until reaching the fifth cycle (Table 1). PCR products were separated by 6% polyacrylamide gel electrophoresis and visualized by autoradiography following Taylor *et al*. (1994).

Twelve loci were successfully amplified and showed high levels of polymorphism and heterozygosity (Table 1). Number of alleles per locus ranged from 6 to 33 and expected heterozygosities from 0.42 to 0.94. An ongoing screening shows that loci with short and less variable repeats (e.g. Odont16) better differentiate between marine and freshwater lineages, while loci with long repeats and higher polymorphism (e.g. Odont07) are useful to reveal fine-scale population structures (L. Beheregaray, unpublished). Marine populations have shown a significant larger number of alleles at all loci than freshwater populations (202 and 120 alleles, respectively, *P* after a Wilcoxon rank test = 0.002). This difference in genetic variability was expected due to the relatively reduced subpopulation sizes of freshwater fish (Ward *et al*. 1994).

All primer sets were also tested without extra optimization in several *Odontesthes* species (including the commercially important *O. bonariensis*) and in the diverged *Basilichthys semotilus*, a silverside endemic to freshwater environments west of the Andes Mountains. Amplification and polymorphism was extensive (Table 2), indicating that these markers are potentially useful for a wide range of population studies of wild and captive South American silverside species.

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Table 1 Primer sequences and characteristics of 12 *Odontesthes argentinensis* and *O. perugiae* microsatellite loci*. Repeat structure is based on sequence of cloned allele. T_a is the annealing temperature used in the 'touchdown' polymerase chain reactions (PCRs) (see text for details). (*n*) is number of *O. argentinensis* and *O. perugiae* individuals screened in populations from southern Brazil and H_E is expected heterozygosity

Locus	Primer sequences (5′–3′)	Repeat structure	T_a (°C)	No. of alleles and (*n*)	Size range (bp)	H_E	GenBank accession no.
Odont02	F: CGGTCACCTGATGGTTGGTC R: AACCCTCAACCCGGCGCATC	$(GA)_{22}$	61 → 53	30 (204)	129–191	0.94	AF177431
Odont07	F: GTCTGAAAGCATCGAGTGAC R: TCTTTTTCTACATCCCAAGC	$(CA)_{27}$	55 → 47	26 (402)	153–209	0.94	AF177432
Odont08	F: TTTCTTTATCCCGCTATGTC R: TGCTCTTGCCCTTGTGTATG	$(CA)_{17}$	55 → 47	25 (402)	126–178	0.93	AF177433
Odont09	F: CCCCACAGAGATAGGCACAC R: GGAGGGAAAAGGTCCAACAG	$(CA)_{17}$	59 → 51	18 (402)	149–193	0.82	AF177434
Odont11	F: ACACTGGTCCTCCTTACCTG R: CAGAATGTGATTGGTGGAGC	$(CA)_{14}$	59 → 51	18 (402)	128–164	0.75	AF177435
Odont16	F: CTGTGCAGACTTTCACTCTC R: CCTGTGGTAAGACCTTTCTG	$(GA)_{11}$	55 → 47	7 (402)	120–138	0.42	AF177436
Odont23	F: TAAGATGGAGGTGCTG R: TTCTCCCCTTCGTCCATAAC	$(GA)_{23}$	59 → 51	33 (204)	108–180	0.92	AF177437
Odont25	F: AAACGGCTCCCATAGACATC R: TAACATCACTGGGGTCACAC	†	61 → 53	12 (10)	102–180	0.80	AF177438
Odont27	F: CCTAATGAAGGTGAAATGC R: GTAAAGGGAGAGGATGGAG	$(CA)_{10}$	59 → 51	6 (15)	185–199	0.87	AF177439
Odont29	F: GAGAAAGCGACCAAAACAGC R: ACAGGAGCTGGGATGGGATG	$(CA)_{9}$	61 → 53	6 (15)	123–135	0.67	AF177440
Odont38	F: ACTAAACGCCCTGATTGTCC R: TCAGTCCATCCATCCAAGAG	‡	61 → 53	11 (12)	104–150	0.85	AF177441
Odont39	F: CTCCCACTAATCCCATTTG R: TACGTGAGCATGTGTGTTG	§	59 → 51	18 (402)	163–199	0.85	AF177442

*OdontO9 and 38 were isolated from *O. perugiae* and the others from *O. argentinensis*. †$(CACG)_4(CA)_7$,$(CA)_7$,$(CA)_{13}$; ‡ $(CA)_{11}$,$(AGAC)_6$; §$(CT)_5$,$(CA)_9$.

Table 2 Number of alleles from cross-species amplification in seven South American silversides. *n* is the number of individuals tested

		Locus (Odont)											
Species	*n*	02	07	08	09	11	16	23	25*	27*	29	38*	39
Odontesthes incisa	10	9	10	8	8	9	1	5	8	5	7	8	6
O. bonariensis	15	7	6	5	5	6	2	11	3	7	4	5	3
O. retropinnis	3	4	4	5	3	2	2	1	2	3	2	4	2
O. humensis	3	5	5	5	2	2	2	2	2	4	4	4	4
O. mirinensis	40	15	10	13	12	9	2	12	9	6	5	9	4
O. hatcheri	3	3	5	3	2	1	2	2	2	3	4	3	2
Basilichthys semotilus	1	1	1	2	2	1	1	1	1	1	2	1	2

*extra optimization is advisable to facilitate scoring these loci.

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Microsatellite characterization in the rainbow wrasse *Coris julis* (Pisces: Labridae)

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The rainbow wrasse, *Coris julis* (Linnaeus 1758), is one of the most common fish in shallow coastal waters within its geographical distribution (e.g. Sanchez Delgado 1981; Lejeune 1987) that includes the Mediterranean, southern Black Sea and the North-Eastern Atlantic (Porteiro *et al*. 1996). Dispersion is apparently limited in the rainbow wrasse, giving that it shows sedentary habits and that migration movements have not been described to date in this species (Lejeune 1987). Thus, it constitutes a good model for investigating genetic drift and differentiation in marine fishes. In this study, we have developed microsatellite markers to analyse the genetic population structure of *Coris julis* throughout its distribution area.

A $(CT)_n$-microsatellite enriched partial genomic library was constructed following a modified protocol of Kijas *et al*. (1994). Fragments ranging from 300 to 600 bp of *Sau*3A-digested DNA were ligated into annealed *Sau*3A adaptors (AdapF: 5′-CTCTTGCTTACGCGTGGACTC-3′ and AdapR: 5′-GATCGAGTCCACGCGTAAGCAAGAGCACA-3′). After denaturation, single stranded DNA was enriched for microsatellites through the hybridization with 5′-biotinylated, 3′-aminated $(CT)_{15}$ oligonucleotides bound to streptavidin-coated magnetic beads (Magnesphere, Promega, Madison, WI). The enriched DNA was eluted twice in 10 μL water for 20 min and then used as a template for a polymerase chain reaction (PCR) using AdapF as a primer to recover double stranded DNA fragments. The PCR product was directly ligated overnight at 4 °C into pGEM-T easy vector (Promega, Madison, WI) using a 1:1 insert:vector molar ratio. The ligation was performed in 10 μL using 50 ng vector, 8 ng purified PCR product, and three units of T4 ligase (Promega). One tenth of the ligation was transformed into 50 μL supercompetant cells (X11-Blue MRF′, Stratagene, La Jolla, CA). Eighty recombinant clones were screened for the presence of $(CT)_n$ microsatellites and 16 for $(GT)_n$ microsatellites using the PCR method described by Waldbieser (1995). Approximately 40% of the clones were positively scored for both the CT and GT motifs.

From 35 clones sequenced, using the dideoxy chain termination method (Sanger *et al*. 1977), 28 contained at least one microsatellite and three sequences were identical. Nineteen clones contained a CT-motif, four a GT-motif, three both CT- and GT-motifs, one an AAAC-microsatellite, and one an ACG-microsatellite. The two last clones were screened positively for the GT motif. These results seem to indicate that the enrichment procedure and the PCR screening are not highly specific to the motif used. Due to the positions of the microsatellites within the inserts, we could define primer pairs for 21 different loci. Seventeen loci were successfully amplified using the following conditions: a total volume of 20 μL contained 10 ng DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, between 0.75 and 2.5 mM $MgCl_2$, 100 μM dATP, 60 μM each of the other dNTPs, 2.4 μM each primer, one unit of *Taq* DNA polymerase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD), and 0.16 μL [α-^{35}S]dATP (12.5 mCi/mL, 1250 Ci/mmol). PCR was performed in a Stratagene Robocycler (Stratagene Cloning Systems, La Jolla, CA) and consisted of a first denaturation step at 94 °C for 4 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 47 or 50 °C depending on the locus, and 45 s at 72 °C, with a final extension of 10 min at 72 °C. Ten loci were polymorphic and showed a number of alleles ranging from 2 to 23. Eight of them were easily scorable with the conditions showed in Table 1.

These eight microsatellites were scored for 20 individuals from Farol Island, Portugal. None of the loci showed a significant departure from Hardy–Weinberg equilibrium (GENEPOP 3.1b; Raymond & Rousset 1995), and no linkage disequilibrium was detected between each pair of locus. Therefore, these eight loci seem highly informative for populations studies.

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Table 1 Microsatellite loci from *Coris julis*. Number of repeats, repeat motif and size of the amplification product from the clones are indicated. Annealing temperatures and $MgCl_2$ concentrations, number of alleles obtained and expected (H_E) heterozygosities are also indicated. Number of alleles and heterozygosities are based on a sample of 20 individuals from Farol Island. GeneBank accession numbers for the cloned sequences are AF190802–AF190809

Locus	Repeats	Primer sequence (5′–3′)	Size (bp)	Anneal. temp. (°C)	$MgCl_2$ (mM)	No. of alleles	H_E
D-11	$(CAAA)_2TAAA(CAAA)_7$	F: GGACACTTCGACCACGAACC R: GTCACTCTCTGAGCTAACTGTGC	176	50	1.25	6	0.74
D-3–3	$(CT)_6$	F: GAAGCACCTTTCCAGGGGAT R: CAGAGGATTCTGTGGTAAAC	85	47	0.75	3	0.55
6–1	$(TG)_3TAA(GT)_3(GA)_2AAG(AC)_6CCTC(AC)_4$	F: GGTCACCGTTGTTGTTTGTC R: GATCCCGTATGCCAAACAC	159	47	1	2	0.18
A-1	$(CT)_8$	F: GTGCTGCTGAGACACTGCGA R: TGTCTTCAGCTCATGCCTCC	230	50	0.75	4	0.65
F-7	$(TG)_3C(GT)_9$	F: GCTCAGTGAAGTAAACGGAGAGG R: TATCAGGAAGCGGCCAGTGTTG	243	50	0.75	6	0.67
G-2–2	$(CT)_7(TC)_2CC(TC)_5$	F: AACATCCTTGTGAACACACG R: AGAACCTGCTGCCTCTCTGC	122	50	0.5	5	0.32
F-2–4	$(GA)_{19}…(TC)_{13}…?…(TC)_5…(TC)_5…(TC)_{12}$*	F: AGGAGAACCAGAACATTACG R: CTGAACTCAAAATAAAGTCG	~ 250**	47	2	4	0.64
E-4	$(CT)_{17}$	F: ACTTCCTTGCACTCACACTC R: GGCTCAGACTTGAGGCTTAG	232	50	0.75	15	0.81

*The question mark corresponds to an unknown sequence of about 25 bp. **Approximate size as revealed from an agarose gel electrophoresis.

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Characterization of microsatellite loci developed for the wattled curassow, *Crax globulosa*

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The family Cracidae comprises 50 species of curassows, guans, and chachalacas (Monroe & Sibley 1993). All are large bodied Neotropical birds, and most are forest dwelling frugivores (Delacour & Amadon 1973). These characteristics mean that species face double jeopardy; they are extensively hunted in addition to suffering from habitat destruction. As a consequence, 13 species are considered threatened species, including the wattled curassow (Collar *et al*. 1992). We developed and characterized six microsatellite loci in the wattled curassow as part of an effort to breed the birds in captivity. Additionally, we examined the polymorphism of these loci in six other members of the Cracidae.

DNA was extracted from blood or tissue using a proteinase K, phenol–chloroform extraction procedure modified from Müllenbach (1989). Size selected genomic DNA digested with *Dpn*II was cloned into Lambda Zap Express, Stratagene, La Jolla, CA (Hughes *et al*. 1998). We screened ≈ 125 000 clones with the oligo $(AAT)_{10}$ (Hughes & Queller 1993), sequenced 31 positives (Sequenase ver 2.0 or Thermosequenase, USB), and developed primers for 10 clones containing ≥ 6 uninterrupted repeats of the sequence AAT.

Polymerase chain reactions (PCRs) (5 μL) contained ≈ 5 ng of DNA, 50 mM KCl, 10 mM Tris/Cl pH 8.3, 1.5% mM $MgCl_2$, 0.1% NP40, 100 mM each dNTP, 0.25 U *Taq* DNA polymerase (Perkin Elmer), 2.5 pmol each primer, and 0.05 μL ^{35}S dATP. Reactions were cycled using the 'tube-control' function of a Hybaid thermal cycler: 90 s at 92 °C, then 5 s at a suitable annealing temperature for each primer pair (Table 1), 5 s at 72 °C, 5 s at 92 °C, 30 times, and finally 90 s at 72 °C. Amplified fragments were resolved using 6% denaturing polyacrylamide gels. Allele length was determined by comparison to the sequencing products of M13.

Six loci tested were found to be polymorphic in the wattled curassow (Table 1). Original clones of these had

Table 1 Polymorphic AAT-repeat microsatellite loci developed for the wattled curassow. H_O, is proportion of observed heterozygotes in a sample of 23 individuals, some known to be related. Expected heterozygotes (H_E) = $1 - p^2_i$. GenBank accession numbers: AF189271–AF189276

Locus names	Repeat sequence	Primer sequences, 5′–3′	H_O	H_E	Product size range	No. of alleles	Suitable T_a °C
CgAAT11	AAT_{13}	F: GGCCATTTGTTGCACAGTAG R: GATCTGGAGCTGCTTTTATTA	0.52	0.69	228–237	4	60–65
CgAAT32	AAT_{14}	F: GTGCCCCAGCAGTAATAATA R: CCATTGTACCAAAGTCACAGTA	0.76	0.66	146–175	6	55
CgAAT62	AAT_6 $GATAAT_7$	F: CTGCTGGCCACGATTCTTC R: TCACGTACTTTTGTCCGTATTGTA	0.78	0.75	112–127	6	55
CgAAT82	ATT_{16}	F: GGTCCCTTCCAAGTTGAATCAT R: AAGCCAAGCATGGAAAGAAAATA	0.67	0.78	97–118	6	60–65
CgAAT85	AAT_{16}	F: CCATAGGTGGGTTGTATTA R: AGCAGAGCCAATATGAAGTAA	0.59	0.71	190–232	6	60–65
CgAAT190	AAT_{13}	F: TCACCACCATTTCCCAACAG R: ATGAGATTTACCTTCAGTTCT	0.60	0.71	192–222	6	55–60

T_a, annealing temperature.

Table 2 Cross species amplification using the primers developed for wattled curassows. Successful amplification of appropriate sized product indicated as P, polymorphic or M, monomorphic. Unsuccessful amplification, —

Species	Sample size	Locus 11	32	62	82	85	190
Crax daubentoni, yellow-knobbed curassow	5	M	P	P	—	P	P
Crax fasciolata, bare-faced curassow	2	M	P	P	P	P	P
Crax rubra, great curassow	5	P	P	M	M	P	P
Mitu tomentosa, crestless curassow	3	M	P	P	—	M	P
Ortalis vetula, plain chachalaca	2	M	P	P	—	P	P
Aburria pipile, Trinidad piping guan	2	—	M	M	—	M	P

contained over 10 AAT repeats. Four loci with 6, 6, 7, and 8, repeats of AAT were monomorphic.

The six polymorphic loci were used in PCR reactions containing DNA from six other species of Cracidae. PCR conditions were those described above. Our primers had less utility in these species (Table 2), although some will be useful in studies of other curassows and chachalacas. These results are consistent with previous studies of primer pair utility across taxa (Primmer *et al*. 1996; Hughes *et al*. 1998). DNA-DNA hybridization shows curassows and chachalacas to be closely related, and these genera to be less related to the guans (Sibley & Ahlquist 1990). The hybridization data however, show the Cracidae to be distantly related to other birds; so distantly, that these primers are unlikely to be of use outside this family.

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Microsatellites in rockfish *Sebastes thompsoni* (Scorpaenidae)

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The rockfish, *Sebastes thompsoni*, is an important commercial and recreational fish. Around Japan, the rockfish is mainly distributed in the northern coast of the Sea of Japan (Amaoka 1984; Ikehara 1989). Nagasawa & Kobayashi (1995) reported that larval rockfish is carried by the Tsushima warm current and widely dispersed in the Sea of Japan, however, their population structure and recruiting dynamics into regional populations around Japan are still unknown. For evaluation and effective use of natural stocks, it is necessary to know genetic population structure and gene flow, and an efficient tool is required. Microsatellite markers are highly polymorphic and quite sensitive genetic markers to study fish populations (McConnell *et al*. 1995). We, therefore, developed microsatellite markers in the rockfish and examined their application to other *Sebastes* species.

A $(CA)_n$-enriched library was constructed using the method of Takahashi *et al*. (1996) with modifications. Genomic DNA was extracted from fin tissues according to Asahida *et al*. (1996). To obtain nonbiased DNA fragments, genomic DNA was fragmented by sonication. Sonicated fragments were blunted by mung bean nuclease (Takara, Shiga, Japan) under the conditions recommended by the supplier. The fragments were electrophored on agarose gel and fragments ranging from 300 to 500 bp were recovered. The fragments were repaired by T4 DNA polymerase (Toyobo, Osaka, Japan) according to the supplier's manual, and then ligated into *Srf* I site of pCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA, USA). Recombinant plasmid vector was transformed into XL2-Blue MRF′ ultracompetent cells (Stratagene), and single-stranded DNA was prepared according to Takahashi *et al*. (1996). Selective second-strand DNA synthesis was employed using $(CA)_{12}$ oligonucleotide and cloned *pfu* DNA polymerase (Stratagene) according to Takahashi *et al*. (1996). Single-stranded DNA remaining in the reaction mixture was digested with mung bean nuclease. The resultant double-strand DNA was transformed again into XL2-Blue MRF′ cells and these transformants were referred to a $(CA)_n$-enriched library. From the library, 35 clones were randomly chosen, and plasmid DNAs were purified. The DNA sequences were determined using Thermo Sequenase™ cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) in combination with KS and T3 primers and subjected to a DNA sequencer (ALFexpress, Amersham Pharmacia Biotech).

Of the 35 clones, 21 CA-repeat microsatellites were detected. The length of CA-repeat units varied between 4 and 30 units with a mean value of 12.1. We developed 16 PCR primer pairs using a software (Primer Premier, Premier Biosoft International, Palo Alto, CA, USA). Five clones were discarded because they had insufficient sequence information to develop primers and/or the CA-repeat arrays were too short. To detect microsatellite polymorphisms, amplification was performed in a 20 μL reaction volume, which included 20 pmols of each primer (one of a pair primer was 5′ end-labelled with Cy5), 100 μM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5 units of Ampli Taq Gold™ (Perkin Elmer, Foster City, CA, USA), and approximately 200 ng of template DNA using PC-960G Gradient Thermal Cycler (Corbett Research, Mortlake, N.S.W., Australia). Polymerase chain reaction (PCR) was performed: 10 min at 94 °C, 25–30 cycles of 30 s at 94 °C, 45 s at a primer-specific temperature, and 45 s at 72 °C, and final elongation for 5 min at 72 °C. Analyses of PCR products were performed using ALFexpress sequencer and a software (Allelelinks, Amersham Pharmacia Biotech). Of the 16 primer pairs, 15 were available for PCR amplification, out of which we finally chose eight primer pairs (the remaining seven having been rejected because their polymorphisms were low and/or they produced unexpected PCR products) and examined further microsatellite polymorphisms in natural rockfish from the northern coast of the Sea of Japan (see Table 1).

As shown in Table 1, the number of alleles per locus ranged from 3 to 22, and the observed and expected hetrozygosity ranged from 0.30 to 0.92, and from 0.30 to 0.88, respectively. Genotype frequencies observed that all loci were in agreement with Hardy–Weinberg's expectations in the Markov-chain method (parameters used; Markov-chain steps: 100 000; dememorization steps: 10 000), using the Arlequin ver.1.1 software (Schneider *et al*. 1997).

We attempted cross-species amplification in three other *Sebastes* species; *S. inermis*, *S. joyneri*, and *S. vulpes*, known to be commercially important fishes. All primer pairs could be used for all species under the same PCR condition, and each locus showed polymorphisms (Table 2). These data suggest that microsatellite markers developed from a *Sebastes* species can be applied to other *Sebastes* species. The microsatellite markers in this study will contribute to phylogentic studies in the *Sebastes* species besides intraspecies population studies.

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Table 1 Core repeat and primer sequences, PCR amplification conditions, and results of variability of eight microsatellite loci in a *Sebastes thompsoni* population

Locus	Core repeat sequence (5′–3′)	Primer sequence (5′–3′)	Anneal °C	No. of samples	No. of alleles	Size range (bp)	H_O	H_E (p*)	GenBank accession no.**
Sth 3A	$(CA)_{10}$	F-ATGGTGACAAGCTAGCAGTGCATTC R-GACAATGTCCATCTAGGCATGACTG	56	50	5	113–121	0.60	0.67 (0.29)	AB033424
Sth 3B	$(CA)_{13}$	F-GTCATGCCTAGATGGACATTGTCTAC R-GAGATAAGAGGAGTTTGAAGGCAGAG	55	50	14	147–178	0.92	0.85 (0.77)	AB033425
Sth 24	$(CA)_8$	F-AGGACAGGATGTGCCCTTTTACCA *R*-GCCTCAGAGGCCGATTTCCTTATT	56	50	4	133–139	0.46	0.53 (0.41)	AB033426
Sth 37	$(CA)_{16}$	F-TACAGGAAACAAGACCACGGGTACAG R-GCAACATCCCTTTAAGTCACCTGCAG	57	49	10	221–243	0.88	0.88 (0.28)	AB033427
Sth 45	$(CT)_{13}X_5(CT)_2X_3(CA)_5(GA)_2A(CA)_4$	F-CTGGGACCTAGCCTGATTACAGCA *R*-AAACTCAGCGACAGCAGACCACA	57	49	10	192–212	0.71	0.76 (0.50)	AB033428
Sth 56	$(TA)_2(CA)_{24}$	F-CAGCAGCTCCAGTTCAGTGTATGT R-GGATTGATCCTCATGTGGTGCTCT	55	50	22	164–250	0.74	0.75 (0.20)	AB033429
Sth 86	$(CA)_6CG(CA)_5C_2(CA)_3$	F-ACCATCACCCACTGTAAACTGCA *R*-TACCAGGAAACGTCGTGTCTCAA	54	50	10	189–211	0.82	0.79 (0.40)	AB033430
Sth 91	$(CA)_6(TA)_3G_2A(CA)_4$	F-TTTCGATATGCTTCGCTAGGGTGTT R-CCATCAAACTGCACCAACAAAGACA	57	50	3	216–220	0.30	0.30 (0.19)	AB033431
				mean	9.8		0.68	0.69	

*p is the exact P-value estimated by a test anologous to Fisher's exact test described by Schneider *et al*. (1997). Signifficant differences between H_O and H_E were not observed at all loci.
**The nucleotide sequence data will appear in the DDJB/EMBL/GenBank nucleotide databases with the accession numbers.

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Table 2 The results of cross-species amplifcation of the eight microsatellites in the three *Sebastes* species

	Species		
Locus	*S. inermis*	*S. joyneri*	*S. vulpes*
Sth 3A	4 (15)	2 (2)	4 (6)
Sth 3B	6 (15)	1 (2)	5 (6)
Sth 24	1 (9)	4 (2)	1 (6)
Sth 37	9 (15)	4 (2)	2 (6)
Sth 45	2 (16)	1 (2)	3 (6)
Sth 56	17 (12)	4 (2)	9 (6)
Sth 86	2 (16)	1 (2)	2 (6)
Sth 91	3 (15)	1 (2)	2 (6)

The number of alleles detected are given. The number of samples examined for each species is given in parenthesis.

Characterization of microsatellite loci in the endangered freshwater crayfish *Austropotamobius pallipes* (Astacidae) and their potential use in other decapods

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Conservation genetics is an applied science whose goal is to describe explicitly the composite genomes of small endangered populations to help in their management. In the white-clawed crayfish *Austropotamobius pallipes*, endemic to western European fresh waters and now confined to headwater river systems, the resolution of available molecular markers such as allozymes and mitochondrial DNA (mtDNA) is of limited usefulness for conservation because of their inability to reveal genetic variability among populations over its range (Grandjean *et al*. 1997; Santucci *et al*. 1997; Lörtscher *et al*. 1998). The use of genetic markers with higher resolution, therefore, appears to be essential in order to understand the genetic structure of *A. pallipes* and then to define concrete management plans. Here we report the isolation and characterization of six microsatellite loci from *A. pallipes*. We examined the polymorphism within this species and two closely related taxa, *A. italicus italicus* and *A. i. carsicus*, described by Brodsky (1983). Because of the deficit of genetic markers available in decapods, we also examined the conservation of these loci in six species of freshwater crayfish encountered in Europe and in four marine decapod species.

We developed microsatellite markers following the methods described by Estoup & Martin (1996). DNA for cloning was isolated from abdominal muscles of one *A. pallipes* specimen using phenol–chloroform (Kocher *et al*. 1989), and 10 µg were digested with *Sau*3AI. Fragments, 400–900 bp, were purified from 1.7% agarose using a DEAE NA45 cellulose membrane (Schleicher and Schuell) and ligated into the pUC18/*Bam*HI plasmid (Pharmacia). XL1-Blue competent cells (Stratagene) were transformed with ligation products. Transformed cells were grown on LB agar, transferred onto Hybond-N + membranes (Amersham) and screened using digoxigenin-end-labelled $(GT)_{10}$ $(TC)_{10}$ and $(CAC)_5(CA)$ probes. Among the 942 clones screened, 58 were identified as positive and isolated. DNA from 40 clones was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems) and analysed with an ABI PRISM 310 automated sequencer. Eleven primer sets were developed using the progam primer 3 by Rozen and Skaletsky (code available at http://www.genome.wi.mit.edu/genome_software/other/primer.html) but five failed to amplify a polymerase chain reaction (PCR) product. Characteristics of the remaining six primer pairs are described in Table 1.

PCR reactions were performed in a final volume of 12.5 µL (1× *Taq* DNA polymerase buffer (Promega: 50 mm KCl, 10 mm Tris-HCl (pH = 9.0), 0.1% Triton X-100), 1.2 mm $MgCl_2$, 60 µm each dNTP, 5 pmol each primer, 0.25 U *Taq* DNA polymerase (Promega) and 15 ng of DNA template) using a Trio-Thermoblock (Biometra). For each reaction, one primer of each pair was end-labelled with one fluorescent phosphoramidite (TET, HEX or 6-FAM). Amplification conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, specific annealing temperature (Table 1) for 30 s, 72 °C for 45 s and a final extension of 72 °C for 10 min. PCR products were run with the internal size standard GeneScan-500 [TAMRA] (PE Applied Biosystems) on an ABI PRISM 310 automated sequencer. Their size was determined using GeneScan Analysis 2.1.

All these microsatellites appeared polymorphic in the white-clawed crayfish (Table1): Ap2, Ap3, Ap5, Ap6 for *A. pallipes*, Ap1, Ap2, Ap3, Ap6 for *A. i. italicus*, and Ap1, Ap3, Ap4, Ap6 for *A. i. carsicus*. However, allelic diversity appeared lower in *A. pallipes* than in *A. italicus*. Locus Ap5 failed to be amplified in *A. italicus*. Amplification products from loci Ap4 for the *italicus* subspecies and Ap2 for the *carcicus* appeared longer than the limit of 500 bp required by the size standard to be analysed.

Concerning cross-species amplifications, annealing temperatures were decreased until 48 °C depending on loci and

Table 1 Characteristics of white-clawed crayfish (*Austropotamobius pallipes*) microsatellites (Tot., total number of alleles per locus; T_a, annealing temperature of primers in C; H_O, observed heterozygosity; H_E, expected heterozygosity; *, 5′ end-labelled primers; –, nonamplification; ?, not assessed). GenBank accession numbers for cloned sequences are AF204815–AF204820

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	Tot.	No. of alleles (T_a) [H_O/H_E] *A. pallipes* n = 30	*A. i. italicus* n = 15	*A. i. carsicus* n = 10
Ap1	F: TCTTGGGGATTGGCTAGTTG R: CCTGAACTAAAAGGTGCTTTGG*	$(CA)_{13}(CG)_2(CA)_6$	123–141	3	1 (60) [0/0]	2 (60) [0.08/0.08]	2 (60) [0.22/0.21]
Ap2	F: TTCGATATAACCGTTTGACCTG* R: TCAGACTTTGGCCATTGAAG	$(CA)_{31}$	151–191	7	3 (60) [0.35/0.50]	4 (58) [0.45/0.61]	? (58) ?
Ap3	F: CGCCTATCTAACCTTGGTTGTC R: GGACTTGGGAAGCCTTGTG*	$(CA)_{25}$	128–216	12	3 (60) [0.43/0.49]	4 (52) [0.51/0.72]	6 (52) [0.40/0.72]
Ap4	F: GCGGTTCTTATGTTGGTC* R: GAGTGTAAGACAATTTTTGG	$(CA)_{14}CC(CA)_9AT(CA)_{37}AA(CA)_{18}$	301–323	4	1 (52) [0/0]	? (48) ?	4 (52) [0.58/0.79]
Ap5	F: CGGCTGAAAGGTCAAAATG R: TGTCTTCCAGGGACGTGAG*	$(CG)_3(TA)_4(CA)_{50}CC(CA)_3(CT)_2(CA)_{52}CG(CA)_{18}$	389–395	3	3 (58) [0.33/0.55]	—	—
Ap6	F: GCTGTGTGGGATGGAGGT* R: CACTAGCGTATTCAAGCAACT	$(TG)_7GGGT(TG)_8GG(TG)_{40}TT(TG)_9TT(TG)_7CA(TG)_3$	346–370	9	2 (56) [0.00/0.13]	4 (54) [0.00/0.35]	5 (54) [0.50/0.56]

Table 2 Cross-species amplification of six white-clawed crayfish microsatellites in decapods (+ , amplification; –, non-amplification; *, multibanding pattern). For each species, only one specimen was analysed

Species	Infra-order	Ap1	Ap2	Ap3	Ap4	Ap5	Ap6
Austropotamobius torrentium	Astacidea	+	+	–	–	–	+
Astacus astacus	Astacidea	+	+	–	–	–	*
Astacus leptodactylus	Astacidea	+	+	+	+	–	*
Pacifastacus leniusculus	Astacidea	+	*	+	+	–	*
Procambarus clarkii	Astacidea	–	+	+	–	–	*
Orconectes limosus	Astacidea	+	+	–	+	–	*
Nephrops norvegicus	Astacidea	–	–	–	–	–	–
Homarus gammarus	Astacidea	*	*	–	–	+	*
Cancer pagurus	Brachyura	+	–	–	–	+	*
Galathea strigosa	Anomura	+	+	–	–	–	–

species. Table 2 reveals that conservation of these loci appeared relatively low between studied decapod species (38%), with no information about the extent of polymorphism.

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Microsatellite loci from common and thick-billed murres, *Uria aalge* and *U. lomvia*

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Murres (guillemots) are colonial seabirds and important components of arctic and subarctic marine ecosystems. Thick-billed murres (*Uria lomvia*) generally occupy arctic latitudes while common murres (*U. aalge*) generally are found at boreal and subarctic latitudes (Gaston & Jones 1998). To investigate the hypothesis that natal philopatry has lead to genetic structuring both within and among breeding colonies (Birt-Friesen *et al*. 1992; Friesen *et al*. 1996), we isolated and characterized microsatellite loci from these species.

Birds from both species were sampled throughout their respective ranges and DNA was prepared from either blood or solid tissues using standard Proteinase-K digestion followed by phenol–chloroform extraction. Microsatellites were isolated using protocols similar to those reported by Gibbs *et al*. (1998). Genomic libraries were made by digesting 10 μ*g* of DNA (2 μ*g* from each of five birds for each species) with *Alu*I, *Hae*III and *Rsa*I. Following electrophoresis through 2% agarose gels, DNA fragments ranging in size from approximately 250–450 bp were purified and ligated into the *Sma*I site of pUC18. Colony lifts were then probed with dinucleotide repeat tracts (AC, GT, AG and CT) labelled with [α^{32}P]-dCTP. Positive clones were sequenced and polymerase chain reaction (PCR) primers were designed from sequences flanking repeats.

Optimal PCR conditions varied slightly among the five loci subsequently found to be polymorphic. In general, amplifications were done in 10–15 μL reaction cocktails containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM dNTPs (each), 4 pmol primers (each) and 0.3 U *Taq* DNA polymerase (Boehringer Mannheim). One primer in each pair was end-labelled with [γ^{33}P]-dATP using polynucleotide kinase. Resolution of alleles for locus ulo12a12 in common murres was improved by increasing $MgCl_2$ concentration to 2.0 mM. Optimal annealing temperatures used also varied among loci (Table 1). Amplified products were resolved in 6% denaturing polyacrylaminde gels (7 M urea) and visualized using BIOMAX X-ray film (Kodak).

All five microsatellites developed from *U. lomvia* (three markers) and *U. aalge* (two markers) amplify and are polymorphic in both murre species (Table 1). Numbers of alleles detected vary from 4 to 32, although sample sizes also vary considerably among loci and species. Heterozygosity estimates are similar to those reported for other avian microsatellites (e.g. Primmer *et al*. 1995; Dawson *et al*. 1997). Generally, observed heterozygosities are similar to expected values except for locus ulo12a12 in common murres, in which

Table 1 Primer sequences, sizes of amplified products and variabilities of murre microsatellites. Loci prefixed by *ulo* are from thick-billed murres; those prefixed by *uaa* are from common murres. Variabilities for thick-billed murres were derived from birds sampled at Coats Island (Hudson Bay, Canada); common murres were sampled from several breeding colonies in southern Alaska and the Aleutian Islands. Expected heterozygosities (H_E) were calculated as $(1 - \Sigma x_i^2)$, where x_i is the frequency of the *i*th allele. GenBank accession numbers for clone sequences are AF195180–AF195184

			Thick-billed murre						Common murre					
Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	No. of alleles	Size (bp)	*n*	H_E	H_O	T_a (°C)	No. of alleles	Size (bp)	*n*	H_E	H_O
ulo12a12	$(CA)_{14}$	F: TCTACGATTCTATGATTCCACA R: GATCTCTACCACATTCTCCCTA	58	26	98–162	296	0.917	0.903	58	10	100–124	128	0.784	0.469
ulo12a22	$(CA)_{12}(TG)(TC)_3$	F: TGAATGCAGTGTCAGTCAAG R: TATAGGCTTATGCCAGAGAGAC	58	6	139–151	43	0.777	0.744	54	4	137–143	128	0.441	0.391
ulo14b29	$(CA)_4(TA)(CA)_7(TA)(CA)_2$	F: GTATTATGTTCCGGAAAACTGT R: TACCCCTATATACAAACCCAAG	57	21	120–172	296	0.804	0.801	58	20	127–161	128	0.737	0.727
uaa1–23	$(CT)_5(TT)(CT)_2(TT)(CT)(TT)(CT)_{11}$	F: CCTGTGTTGAAAATAGAACAGA R: TTTAGCTGGTGAAGTTAGTCAG	57	32	146–198	296	0.912	0.892	58	20	155–197	128	0.773	0.779
uaa5–8	$(AC)_{14}$	F: CAGTTTCTTTAAGTCGTGCCAG R: CACTTAGGTCCAAAACCTAACC	57	8	108–126	43	0.677	0.721	53	9	106–122	128	0.716	0.719

T_a, annealing temperature; *n*, number of individuals sampled; H_O, observed heterozygosities.

expected heterozygosity exceeds observed heterozygosity. The most likely explanation is the presence of one or more null alleles at this locus (Pemberton *et al*. 1995), but this explanation cannot be verified from the present data set.

Primers developed to amplify ulo14b29 in murres generate PCR products in marbled murrelets (*Brachyramphus marmoratus*), although only two alleles were detected among 121 birds (Congdon *et al*., in preparation). Otherwise the utility of these loci with other species has not been explored.

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Development of polymorphic microsatellite markers in a tropical tree species, *Melaleuca cajuputi* (Myrtaceae)

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The tropical tree species, *Melaleuca cajuputi*, belongs to the Myrtaceae, and is widely distributed from northern Australia through Malay Peninsula to Indochina. *M. cajuputi* trees frequently form populations in peat swamps and sandy places in Narathiwat, southern Thailand, where other tree species rarely grow. Therefore, *M. cajuputi* is expected to be a suitable tree species for reforestation in tropical deteriorated lands. The trees enlarge their population both by sexual and vegetative reproduction. Although knowledge about the reproduction of tree species which may be suitable for reforestation is of general importance, the reproductive pattern of *M. cajuputi* in the field is not well understood. In the present study, polymorphic microsatellite markers were developed to investigate the balance between sexual and vegetative reproduction, and between autogamy and allogamy in natural populations of *M. cajuputi*.

We isolated microsatellite regions from total DNA of *M. cajuputi* using a modification of the enrichment technique described by Edwards *et al*. (1996). DNA was extracted from *M. cajuputi* leaves dried with silica gels by a modified CTAB method (Zhou *et al*. 1999), and digested with a restriction enzyme (*Hin*dIII, *Eco*RI, *Xho*I or *Sal*I). The 5′ projecting ends of the DNA fragments were blunted by a DNA Blunting Kit (Takara Shuzo Co., Tokyo), and ligated with an *Eco*RI/*Not*I/*Bam*HI adaptor (Takara Shuzo Co.) by a DNA Ligation Kit version 1 (Takara Shuzo Co.). The adaptor-ligated fragments were amplified by polymerase chain reaction (PCR) using a primer designed on the basis of the sequence at their ends. Several kinds of oligonucleotides among $(GCC)_{10}$, $(CAA)_{10}$, $(CTG)_{10}$, $(GTG)_8$, $(GC)_{15}$, $(GA)_{15}$, $(GT)_{15}$ and $(AT)_{15}$ were attached to the membrane filter for enrichment. Microsatellite-containing fragments were cloned into pT7Blue vectors using a pT7Blue Perfectly Blunt Cloning Kit (Novagen Co., WI, USA), and propagated in *Escherichia coli*, XL1-Blue MRF′ strain. Sequencing reactions were performed using a Texas Red T7 primer (Hitachi Instruments Service Co., Tokyo) and a Thermo Sequenase Premixed Cycle Sequencing Kit (Hitachi Instruments Service Co.) according to the manufacturer's instructions. Extension products were separated by a sequencer (SQ-5500, Hitachi Co., Tokyo). Primer pairs for amplification of the microsatellite regions were designed on the basis of the sequences flanking the obtained microsatellites.

To characterize each microsatellite loci, the regions in the DNA extracted by the above method from dried leaves of 31 *M. cajuputi* ramets growing in a sandy place in Narathiwat, southern Thailand, were amplified by PCR using the designed primer pairs. The amplification was performed in 10 μL of a reaction mixture containing 10 ng DNA, 0.4 mM of each dNTP, 0.2 μM of each designed primer pair, 1 × GC buffer I (Takara Shuzo Co.; ingredients are unavailable) in which 2.5 mM of Mg^{2+} were included, and 0.5 U of LA *Taq* DNA polymerase (Takara Shuzo Co.), where the reverse primer was labelled with Texas Red, by a PCR thermal cycler (TP3000, Takara Shuzo Co.). The reaction schedule was as follows: (i) 94 °C for 1 min, annealing temperature (*T*a) shown in Table 1 for 1 min,

Table 1 Primer pairs for amplification of 11 polymorphic microsatellite regions in *Melaleuca cajuputi* and some characteristics of the microsatellites

Microsatellite locus*	Primer pair sequence (5′–3′)	Repeat type†	Size range (bp)	*T*a (°C)‡	Allele number	H_O§	H_E¶
Eco-1	R:CCATGGGTAATGTCGGAAGTG	$(GGC)_7(GAT)(GGC)$	115–124	57	3	0.00	0.56
(AB034970)**	F:GCCATACATGCGACTGAGTAC						
Hin-2	R:ACCGTCAACCACCACTGTTTG	$(GCC)_5(GCT)(GCC)_4$	116–130	57	5	0.45	0.66
(AB034971)	F:GCCAGCAGTGATTAGAGCATC						
Hin-4	R:TTTGGCGTGCTCAGAGCTCT	$(GA)_{10}AA(GA)_4$	91–110	57	5	0.13	0.67
(AB034971)	F:CACCCCAAATATTCCATCTCTC						
Hin-5	R:GTTTGCCAAATCCATTACGGTC	$(AAG)_3(AAT)(AAG)_6$	146, 134	57	2	0.13	0.12
(AB034972)	F:CAATGATATTCACGTAGTCGGTG						
Hin-6	R:TCATCGACTTCTCTTTCTGAGC	$(AG)_{31}$	108–234	57	9	1.00	0.86
(AB034973)	F:CATCAACACACGCAACCACTC						
Hin-7	R:TCACTACCATGTAGGTGCTCC	$(CT)_{14}\ldots(CA)_{23}$	168–226	57	7	1.00	0.79
(AB034974)	F:TTACAAACATACCTCTGGCCAG						
Sal-1	R:AGTCCCAGTCGTCAACAGAG	$(CGG)_6$	91–97	57	3	0.90	0.60
(AB034975)	F:CCATCAAAGACAAAAGAGCGTC						
Sal-2	R:GAGGATTCTAAGCTGCAGC	$(GGC)_6(GGT)_2(GAT)$	129–139	57	5	0.71	0.71
(AB034976)	F:GAAAGGCCATACGGAATCTCC	$(GGT)(GAT)_7$					
Sal-3	R:GCATCATCATCGAGCTGCATG	$(GT)_{20}(GC)_3GT(GA)_{27}$	139–192	57	10	1.00	0.86
(AB034977)	F:ACCAGTGACTAATCGGGTGTG						
Xho-1	R:AGGTGGTGATGGACGAGCTG	$(GGC)_6(GGT)(GGC)$	103–117	60	7	0.77	0.70
(AB034978)	F:GTCGCATTGACATCCGAAGCG						
Xho-4	R:AATCCGCGACTGTGCAGAGG	$(GT)_{41}(GA)_{23}$	155–283	60	7	0.61	0.71
(AB034979)	F:CTCAAGCCGATGTTCCTCGC						

*Microsaellite loci designated as *Eco*-, *Hin*-, *Sal*- and *Xho*- mean those isolated from the amplified DNA fragments which had been prepared after digestion of DNA with *Eco*RI, *Hin*dIII, *Sal*I and *Xho*I, respectively.
†Sequenced microsatellite repeat type.
‡Annealing temperature for polymerase chain reaction amplification.
§Observed heterozygosity (n = 31 ramets).
¶Expected heterozygosity (n = 31 ramets).
**DDBJ/EMBL/GenBank accession number for the cloned sequences. The loci of *Hin*-2 and *Hin*-4 were found from the same clone.

72 °C for 1 min, 29 cycles; (ii) 94 °C for 1 min, *T*a for 1 min and 72 °C for 5 min, 1 cycle. The PCR products were denatured by heating and separated by the sequencer on a sequencing gel [6% Long Ranger (FMC BioProducts Co., ME, USA), 6.1 M urea and 1.2 × TBE (0.1 M Tris (hydroxymethyl) aminomethane, 3.0 mM EDTA and 0.1 M boric acid)] in 0.6 × TBE. The band sizes were estimated by computer software FRAGLYS version 2 (Hitachi Electronics Engineering Co., Tokyo). Also, observed and expected heterozygosities in each microsatellite locus were calculated by Nei's equation (Nei 1987).

The sequences of 17 microsatellite loci in DNA of *M. cajuputi* were determined. When the DNA was digested with *Eco*RI, *Hin*dIII, *Sal*I and *Xho*I at the first step of microsatellite isolation, the sequences of 3, 7, 3 and 4 microsatellite loci were determined, respectively. Eleven out of 17 loci showed single-locus, polymorphic banding patterns (Table 1). These polymorphic loci had 2–10 alleles per locus and expected heterozygosities ranging from 0.12 to 0.86. Observed heterozygosities in most loci showed relative consistency with the expected ones. Two loci, however, were not polymorphic, being amplified into only one allele, and in the other four loci, no clear bands were amplified by the designed primer pairs (data not shown).

We confirmed that the 11 polymorphic markers could distinguish genetic differences between several groups of ramets connected to each other by roots. This suggests that the 11 markers are available to identify *M. cajuputi* genets in the field. Because the 11 markers are single-locus and polymorphic, they would also be useful for analyses of gene flow in *M. cajuputi* populations.

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Microsatellite markers for investigating population structure in *Octopus vulgaris* (Mollusca: Cephalopoda)

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Octopus vulgaris, Cuvier 1787, is probably the most widely found cephalopod species, having a worldwide distribution in temperate waters on the continental shelves of Europe, Africa, Asia and the Atlantic coasts of North and South America. Whether it is a truly cosmopolitan species or a species-complex has yet to be investigated by modern molecular methods, but it is nevertheless an important fisheries category with global catches in excess of 100 000 tonnes. Despite its fishery importance, there are currently no methods for the investigation of population structuring or the identification of 'stocks' for fishery management of this important species. Here we report on the isolation and characterization of the first polymorphic microsatellite markers for the study of population structure in *O. vulgaris*.

Genomic DNA was extracted from ethanol preserved arm tips from six individual octopus specimens from Crete, and six from Vigo; these locations represent the extremes of the range of sites from where *O. vulgaris* samples were obtained during this project. A phenol–chloroform extraction procedure modified for use in molluscs following Vernon *et al*. (1995) was used.

Two size selected partial genomic libraries (Rassmann *et al*. 1991), the first 300–600 base pairs (bp) and the second 300–900 bp were constructed by ligating *Sau*3AI digested DNA into dephosphorylated pUC18 vector digested with *Bam*HI (Pharmacia). The first library was probed with a [α-^{32}P] dATP labelled dinucleotide polymers $(AG)_8$ and $(AC)_8$ (2034 recombinant colonies gave 78 positives), whilst the second was probed with [γ-^{32}P] dATP end-labelled tri- and tetra-oligonucleotide repeats, e.g. $(TAA)_4$ $(GATA)_4$ and $(GACA)_4$ (3500 colonies gave 25 positives) at high stringency. A selection of di-, tri- and tetranucleotide positives were sequenced using a Big Dye dye-terminator kit (following manufacturer's protocols), and an ABI 377 automated sequencer (PE Applied Biosystems) was used to detect sequences. Primers were designed from unique sequences flanking the microsatellite repeats, using OLIGO™ Macintosh version 4.1 (National Biosciences Inc., USA).

Amplification efficacy of primer pairs was tested using individuals that were used to construct the partial genomic library, and a few randomly chosen individuals. Amplified samples were run on 1% 1 × TBE agarose gels at 80 V for 15 min and gels stained with ethidium bromide. Polymerase chain reaction (PCR) mixes contained 20 ng template DNA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, 0.04 units of *Taq* DNA polymerase (Bioline), and the manufacturers supplied buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl, pH 8.8, 0.01% Tween-20), in a final reaction volume of 10 µL. Reactions were performed on a Hybaid 'Omn E' PCR thermal cycler using a 'touchdown' protocol (Don *et al*. 1991) which used an initial set of 10 cycles dropping from 5 °C above the supposed annealing temperature by 0.5 °C increments each cycle. Touchdown programmes with annealing temperatures dropping from 60 °C–50 °C gave best results.

PCR conditions were optimized for six loci (reported in Table 1); bright resolvable, polymorphic products were

Table 1 Microsatellite loci in *Octopus vulgaris*

Locus*	Repeat unit	*T*m (°C)†	Mg^{2+} (Mm)	Product size‡	*n*§	No. of alleles¶	H_E	H_O	Primer sequences (5′–3′)
µOct3	$(AT)_{16}(GT)_{15}$	55	2.5	147	12	12	91.6	75.0	F: CTCCCTAGTTTTGAATCACG R: GCCACTAATACACTTTTCAAGG
µOct8	$(TG)_{36}$	56	2.5	160	10	11	92.6	70.0	F: AGGGAGAGAAAATAGAAAAAC R: TAAACTGAATAATACATACATACG
µOv04	$(TTA)^{22}$	44	2.5	126	49	14	72.0	98.0	F: ATACCAGGCCTTGTGCCTTTAG R: CAGCACCGTAATACATCTTCAG
µOv06	$(ATT)^{24}$	45	2.5	146	49	17	69.5	98.0	F: GGGCCTTATTCCTTAAGCAG R: CCATTTGCATTTGAATATTTTTAAAG
µOv10	$(GA)^{14}$	52	2.5	122	62	12	68.7	98.4	F: GCAATAAAGGAGAAAACAAAAACA R: GCTATTGTCACAATAAGGCTCTCC
µOv12	$(GATA)^{20}$	53	2.5	176	61	18	64.7	83.6	F: GCATAATGTGCCGCTAAATGGAAC R: GCCTCGTCGGTATTTCCTCTTTCA

*µOct and µOv, locus nomenclature. µOct loci isolated from first library, and µOv from second. †*T*m(°C) predicted by OLIGO™. ‡Predicted product size in base pairs assuming allele size identical to sequenced allele. §Individuals genotyped from two populations of *Octopus vulgaris*, from Crete, and Vigo ¶Number of alleles at each locus, expected (H_E) and observed (H_O) heterozygosity are given for these *n* individuals. GenBank accession numbers (order listed in table): AF197130–AF197135.

produced in all samples of the predicted size. Forward primers of these primer sets were labelled using fluorescent dyes compatible with the PE/Applied Biosystems Genescan system, and alleles sized from 4% acrylamide denaturing gels run for 1 h at 750 V. Primers were tested on putatively unrelated individuals from two populations, one from Crete and one from Vigo.

All six loci were polymorphic. Some problems with shadow bands were encountered with loci from the first library (μOct loci). This is a common problem with dinucleotide repearts (Litt *et al*. 1993). The second library was probed with tri- and tetranucleotide oligonucleotides, to avoid problems encountered with the first set of dinucleotides.

This suite of microsatellite primers will be a useful tool in understanding population structure in *O. vulgaris*.

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Characterization of microsatellite loci in bannertailed and giant kangaroo rats, *Dipodomys spectabilis* and *Dipodomys ingens*

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Microsatellite genotyping is the current method of choice for analysing genetic structure and pedigrees in animal populations. Here we describe the isolation and characterization of six microsatellite loci in the bannertailed kangaroo rat, *Dipodomys spectabilis*, and three microsatellite loci in the giant kangaroo rat, *D. ingens*. These are the first microsatellite loci to be characterized for any member of the rodent family Heteromyidae. The primers were developed as part of long-term studies using genetic data to address questions regarding between-population dispersal and within-population inbreeding in metapopulations of *D. spectabilis* and *D. ingens* (Keane *et al*. 1991; Waser & Elliott 1991; Good *et al*. 1997).

D. spectabilis microsatellites were isolated from small insert genomic libraries. For each library approximately 50 μg of genomic DNA was isolated from muscle tissue by standard methods (Sambrook *et al*. 1989) and digested to completion with *Sau*3A. The resulting fragments were electrophoresed on a 1% agarose gel and fragments of 150–500 bp were excised and isolated from the gel using QIAquick™ spin columns (QIAGEN). Size selected fragments were ligated into *Bam*HI digested dephosphorylated M13mp18RF (Gibco BRL) followed by transfection into *Escherichia coli* DH5α F′IQ by electroporation (*E. coli* Pulser™, Bio-Rad). Approximately 10 000 recombinant clones were screened with a biotinylated $(GT)_{11}$ oligonucleotide probe, and clones containing putative microsatellites were detected using a nonradioactive detection kit (Gibco BRL). A total of 134 positive clones were isolated. Fifty of these clones were rescreened, and genomic inserts from 39 clones were amplified and sequenced as in Zheng *et al*. (1995). GT dinucleotide repeats in *D. spectabilis* were commonly very long, containing as many as 75 repeat units. Primers were designed using OLIGO (National Biosciences Inc., Version 4.0) with one primer of each set fluorescently labelled with one of 6-FAM, TET or HEX (Applied Biosystems) for putative microsatellites in 30 clones.

In order to assess variation at the loci derived from *D. spectabilis*, six animals from Portal, Arizona were genotyped using all 30 primer sets. Total genomic DNA was isolated from 10 to 20 mg ear snips using QIAquick™ spin columns and approximately 100 ng was used as template in 15 μL polymerase chain reactions (PCRs) also containing 0.16 μM (2.4 pmol) of each primer, 120 μM dNTP, 2 mM $MgCl_2$, 0.3 U of *Taq* DNA polymerase and 1X PCR buffer (10 mM Tris buffer, pH 8.8, 0.1% Triton X-100, 50 mM KCl and 0.16 mg/mL BSA). Cycling was performed in a Perkin Elmer 9600 thermal cycler under the conditions: 1 min at 94 °C, three cycles of 30 s at 94 °C, 20 s at 54 °C, and 5 s at 72 °C, followed by 33 cycles of 15 s at 94 °C, 20 s at 54 °C, and 1 s at 72 °C. Final extension at 72 °C for 30 min preceded ramping to 4 °C. PCR products were resolved on a model 377 DNA Sequencer and analysed using GENESCAN and GENOTYPER software (Applied Biosystems). Many primer sets amplified products which appeared as streaks on the gel image due to excessive slippage of the

Table 1 Repeat motif in allele cloned, number of alleles (N_A), size of polymerase chain reaction products in bp, observed (H_O) and expected (H_E) heterozygosity in *Dipodomys spectabilis* (*Ds*) and *D. ingens* (*Di*), and primer sequences (5′–3′) for six microsatellite loci developed in *D. spectabilis* and three loci in *D. ingens*. The number and size of alleles, as well as heterozygosities, at each locus are based on the analysis of 29 *D. spectabilis* and seven *D. ingens* individuals for *D. spectabilis* primers (*Ds*xx), and on 10 *D. spectabilis* and 50 *D. ingens* for *D. ingens* primers (*Di*xx)

Locus	Repeat motif	N_A	Size range	H_O	H_E	Primer sequences	Accession no.
*Ds*1	$(GT)_{17}(AG)_{19}$	*Ds*:8	202–218	0.72	0.82	F: GATCAACCACCCAGCTCTAT	AF186399
		Di:9	170–214	0.86	0.87	R: GCAAAGCCCTGAGTTCAAAAG	
*Ds*3	$(GT)_{16}$	*Ds*:7	170–182	0.52	0.73	F: TCAAGCTCCAGGACAGCACAAG	AF186400
		Di:4	174–184	0.43	0.64	R: GTTTCCATTGATGCCCAGCAGATTT	
*Ds*19*	$(GT)_{36}$	*Ds*:11	94–128	0.48	0.86	F: ATCCTCAGAACCTTCATTCA	AF186401
		Di:8	102–122	0.57	0.87	R: AATCTATGAGTGAGCCAACAG	
*Ds*28	$(GT)_{15}$	*Ds*:7	183–209	0.76	0.79	F: GATCTTTATTTATTTGTGTGTTT	AF186402
		Di:6	188–210	0.57	0.79	R: ATTAGAGTCAGAAGCCAGAGCAG	
*Ds*30	$(GT)_{15}$	*Ds*:20	246–318	0.97	0.95	F: ATCCTTCCTCCCAATGTTGTAG	AF186403
		Di:9	219–267	0.71	0.86	R: GTTTCCCAGGCAGAAAAGTTTATGATA	
*Ds*46	$(GT)_{14}GCG(GT)_{11}$	*Ds*:7	174–188	0.69	0.70	F: CTAATCACCGAGCCAAA	AF186404
		Di:5	147–157	0.71	0.76	R: GTTTATCAACTATAAAAATCATAGAAAA	
*Di*5	$(CAC)_{11}CAA(CAC)_4$	*Ds*:4	175–199	0.30	0.65	F: TGGTTCCATGTAGTAGCCCTGAGC	AF187992
		Di:2	179–182	0.22	0.31	R: TCCCAGAGTTCCAAGTACCTCC	
*Di*12E	$(CA)_{16}CCC(CAC)_{18}$	*Ds*:4	210–234	0.60	0.67	F: GTAGCTGGGCTTACAGACATGAG	AF187993
		Di:7	195–225	0.52	0.68	R: GGCAGGTCTCCTTCTGAGATGGC	
*Di*12F	$(AGC)_{14}(CAC)_{11}$	*Ds*:1	—	—	—	F: CCTTGAGTGATAAGGCTCAG	AF187994
		Di:2	163–214	0.18	0.16	R: CAGTCTCCTGAGTAGCTAGG	

*Locus *Ds*19 is X-linked.

Table 2 Results of cross-species amplification of DNA from six species in the family Heteromyidae using six microsatellite primer pairs designed from the bannertailed kangaroo rat, *Dipodomys spectabilis*. Entries are the size range in number of bp of the polymerase chain reaction (PCR) products detected (number of alleles in parentheses). —, indicates that either no product or a multiband pattern was observed with the PCR conditions used

Locus	*Dipodomys merriami* (n = 10)	*Dipodomys ordii* (n = 3)	*Perognathus flavus* (n = 6)	*Chaetodipus baileyi* (n = 2)	*Chaetodipus hispidus* (n = 4)	*Chaetodipus penicillatus* (n = 4)
*Ds*1	168–214 (12)	188–217 (3)	243–261 (5)	176 (1)	—	176 (1)
*Ds*3	174–204 (9)	—	—	—	—	—
*Ds*19	96–122 (8)	—	243–262 (4)	—	—	—
*Ds*28	—	173–198 (6)	—	—	—	—
*Ds*30	242–273 (12)	235–252 (4)	—	—	262–264 (2)	—
*Ds*46	—	159–171 (4)	—	—	—	—

polymerase during amplification over long repeat units and could not be scored. Six loci, with moderate repeat lengths in the sequenced allele, exhibited clean and variable banding patterns and were run on 29 additional individuals from Portal, AZ (Table 1). These six loci had 7–20 alleles; one locus (Ds19) was sex-linked, and the low H_O in another (Ds3) suggested possible null alleles. All six loci were also polymorphic in a sample of seven *D. ingens* individuals.

D. ingens trinucleotide microsatellites were developed according to the enrichment protocol in Fleischer & Loew (1996). $(CAC)_n$ repeats were highly enriched and all of the loci reported here are composed of $(CAC)_n$ or some combination of $(CAC)_n$ and $(GT)_n$. From the *D. ingens* sequences we designed 11 primer sets, three of which resulted in variable products (Table 1). PCR conditions were similar to those described above, but included 1.5 mM $MgCl_2$, 0.5 U of *Taq* Gold DNA polymerase, 200 μM of each dNTP, and 0.5 μM (5 pmol) of each primer in a total reaction volume of 10 μL. The PCR involved a 10-min start at 94 °C followed by 30 cycles of 94 °C for 50 s, 56 °C for 80 s and 72 °C for 30 s. In *D. spectabilis*, two primer sets revealed variable patterns in 10 individuals, but the low H_O in Di5A suggests there may be null alleles present.

We also tested the effectiveness of the *D. spectabilis* derived primer pairs in six other species within the family Heteromyidae (Table 2). These results demonstrate that some of the primer pairs designed from the DNA sequences of *D. spectabilis* may also be useful in other heteromyid species, particularly within the genus *Dipodomys*. However, the higher percentage of heterospecific primer pairs within the genus *Dipodomys* indicates that the conservation of microsatellite flanking regions decreases with increasing phylogenetic distance

within the family Heteromyidae, as has been seen for other organisms (e.g. Primmer *et al*. 1996).

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