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PRIMER NOTES

Microsatellite loci for the social wasp *Polistes dominulus* and their application in other polistine wasps

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The social wasps of the genus *Polistes* are an important model system for understanding the evolution of cooperation. Their relatively simple societies lack the distinct morphological castes which characterize many of the social insects, and newly emerged females possess a variety of reproductive options (Reeve 1991). A female may remain on her natal nest as a helper gaining indirect fitness; usurp a foreign nest and become reproductively dominant; initiate a new nest independently; reproduce on a satellite nest; or initiate a new nest in cooperation with other wasps (Strassmann 1981; Reeve 1991; Mead *et al*. 1995; Cervo & Lorenzi 1996; Queller *et al*. 2000). By characterizing the reproductive payoffs associated with different reproductive strategies, we are better able to understand how cooperative societies are maintained.

Recently, microsatellite genetic loci have greatly extended our ability to characterize the reproductive strategies used by social wasps (Hughes 1998; Queller *et al*. 1993a). Using microsatellite loci we can reconstruct pedigrees, and estimate relatedness. Using this information, unobserved events such as queen death, nest usurpation or past reproductive dominance can be inferred (Queller *et al*. 1993a,b; Field *et al*. 1998; Hughes 1998). In this paper, I describe microsatellite loci isolated from the social wasp *Polistes dominulus*, one of the best studied *Polistes* species.

We followed published protocols for the isolation of microsatellite loci (Strassmann *et al*. 1996) with clarifications and modifications to those protocols as noted below. DNA was extracted from 1 to 1.5 g of pupal thoraces ground in a mortar and pestle which had been chilled in liquid nitrogen. The ground tissue was suspended in grinding buffer (0.1 M NaCl; 0.1 M Tris-HCl, pH 9.1; 0.05 M EDTA; 0.05% SDS), and purified three times with phenol:chloroform:isoamyl alcohol (25:24:1), and then three times with chloroform:isoamyl alcohol (24:1). The purified genomic DNA was then ethanol precipitated, and resuspended in distilled water.

Genomic DNA was digested with *Sau*3aI, and 300–1000 bp inserts were ligated into the pZErO –2 plasmid (Zero Background cloning kit, Invitrogen) digested with *Bam*HI. We transformed TOP10 cells (Invitrogen) to obtain approximately 5000–6000 clones. Nylon replicates of the genomic library were probed with five oligonucleotides (AAT_{10}, AAG_{10}, AAC_{10}, TAG_{10}, and CAT_{10}) which were end-labelled with [γ-^{33}P]-dATP. Probes of the nylon replicates yielded 151 positives and subsequent probing of plasmid DNA on the southern blot confirmed 34 unique positives. Clones which were positive on the southern blot were sequenced on an ABI 377 automated sequencer (Perkin-Elmer), and 19 sets of polymerase chain reaction (PCR) primers were designed from the 28 resulting sequences using Mac Ventor 5.0 (Kodak Scientific Imaging Systems).

We optimized the PCR primers on an MJ Research PTC-100 thermocycler using 10 µL reactions (Peters *et al*. 1998), and assessed within-species polymorphisms for eight species of polistine wasps, using from one to eight unrelated females for each species (Table 1). PCR products were visualized on 6% polyacrylamide/8 M Urea sequencing gels.

Twelve of the 19 loci tested were polymorphic within our *P. dominulus* population and had a mean observed heterozygosity (H_O) of 0.76. Loci with a minimum of five uninterrupted repeats were polymorphic, and heterozygosity increased logarithmically with the number of uninterrupted repeats (Fig. 1; logarithmic regression, $R^2 = 0.454$, $P = 0.0016$). The loci retained much of their polymorphism in other species of *Polistes* with six polymorphic loci for *P. fuscatus* and *P. apachus* which had a mean H_O of 0.48. No polymorphisms were detected outside of the *Polistes* genus, however, it is likely that some polymorphisms went undetected due to the small number of individuals screened in the other species (Table 1).

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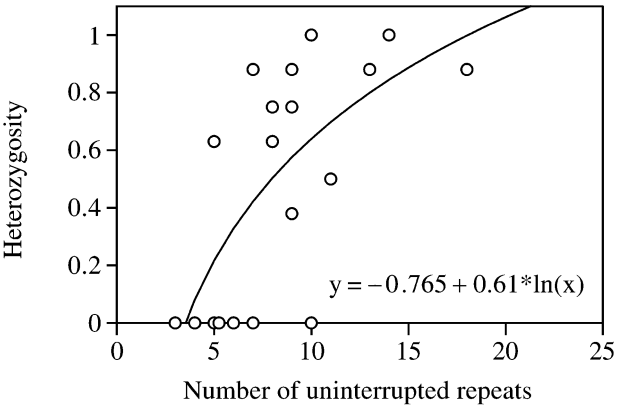

Fig. 1 The relationship between the observed heterozygosity and the number of uninterrupted repeats for 19 microsatellite loci isolated from *Polistes dominulus*.

Table 1 A description of polymorphic microsatellite loci isolated from *Polistes dominulus*, including their utility in related polistine taxa. The sample size (n) for each species is given in the column heading with exceptions for certain primers noted in the table. Where $n \geq 3$, we report the observed heterozygosity for all species, as well as the expected heterozygosity for *P. dominulus*. In all cases we report the observed number of alleles in parentheses. The product size and repeat region data are based on the sequenced allele. NP = no scorable product. GenBank accession nos are AF155596 to AF155623 and include 16 additional loci not summarized in the table

Locus	Size (bp)	T_a (°C)	Repeat	*Polistes dominulus* (n = 8)	*Polistes fuscatus* (n = 4)	*Polistes apachus* (n = 4)	*Protopolybia exigua* (n = 2)	*Brachgastera mellifica* (n = 2)	*Polybia occidentallis* (n = 1)	*Ropalidia excavata* (n = 1)	*Miscocyterus alfkenii* (n = 1)	Primers (5′–3′)
Pdom 1	209	55	$(CAG)_9TAG(CAG)_5$ $(CAT)_5GGCAC(CAG)_3$	H_O = 0.38 (3) H_E = 0.41	0.00 (1)	−(2) n = 1	−(1)	−(1)	−(1)	−(1)	−(1)	F:GGACGCTCGGCTGATTTGTC R:AAGGGATTTTTCCTGAGACTATTCG
Pdom 2	184	51, 48	$(AAG)_8CG(AAG)_2$	H_O = 0.75 (4) H_E = 0.63	0.50 (4)	0.33 (3)	NP	NP	NP	NP	NP	F:CGTCTCTCGAAATATGCTAAAC R:AGAACGGTAAACATTCTTCTATC
Pdom 7	160	54	$(AAG)CAG(AAG)_9$	H_O = 0.75 (5) H_E = 0.73	−(1) n = 2	0.00 (1)	−(1) n = 1	−(1) n = 1	−(1)	NP	−(1)	F:CACTGTATTGTCCTACGGTGGTCC R:GCGAGAACCTGTACTCAAAACAAAC
Pdom 20	236	55, 52	$(CAT)_{18}$	H_O = 0.88 (4) H_E = 0.63	0.75 (6)	1.00 (5)	NP	NP	NP	NP	NP	F:TTCTCTGGCGAGCTGCACTC R:AGATGGCATCGTTTGAAAGAGC
Pdom 25	157	50, 45	$(AAG)_{11}$	H_O = 0.50 (3) H_E = 0.53	NP	NP	NP	NP	NP	NP	NP	F:CATTATAAACGCCGCG R:ACGATGGAAACGTAAGTCC
Pdom 93	131	55	$(AAG)_2ACG(AAG)_2$ $ACG(AAG)_5$	H_O = 0.63 (2) H_E = 0.43	0.25 (4)	0.50 (5)	−(1)	−(1)	NP	−(1)	NP	F:CCATCAGCTGTCCCATTCGC R:AATCGGTTTCGCTCGTCCACCTCC
Pdom 117	260	51, 48	$(AAG)_4AGG(AAG)_2$ $AGG(AAG)_{14}$	H_O = 1.00 (9) H_E = 0.83	0.25 (2)	−(2) n = 2	−(1)	−(1)	NP	NP	NP	F:AAGAAAACCTACTACGTTGTGTGAG R:TTTCAACATTCCATAGGGACAG
Pdom 121	218	54, 50	$(AAG)_8AGGAAC$ $(AAG)_2AAC(AAG)_2$	H_O = 0.63 (6) H_E = 0.78	0.00 (1)	0.00 (1)	NP	NP	NP	NP	−(1)	F:GAGTGGGTATGACGAAGATGATGG R:TGATTATAGCCTGCCGAAACTCTG
Pdom 122	172	46, 48	$(AAT)_{10}GAAAAT$ $(AAT)_2GAAAAT\ (AAT)_8$	H_O = 1.00 (9) H_E = 0.85	0.50 (2)	−(2) n = 1	NP	NP	NP	NP	−(1)	F:CCGAAGAATGATAGTAGGTCC R:AGACCATCTCTCGCACGC
Pdom 127b	119	48	$(AAT)_{13}\ldots(AAT)_6\ AA$ $(AAT)_4AAC(AAT)$	H_O = 0.88 (9) H_E = 0.80	0.00 (1)	0.00 (1)	−(1)	−(1)	−(1)	−(1)	−(1)	F:TCCCCCGTTTTTGGTCCTTG R:GGGAGAGAATCGTGCCTTTTC
Pdom 139	186	48, 45	$(AAC)_7(AAT)_2(AAC)$ $(AAT)_2(AAC)_2$	H_O = 0.88 (6) H_E = 0.72	0.00 (1)	0.00 (1)	NP	NP	NP	NP	− (1)	F:TGACAAAAGACAACAAAATATG R:AGCTTCGGTAGGGCTTCG
Pdom 140	192	55	$(TAG)_9$	H_O = 0.88 (9) H_E = 0.85	0.00 (1)	0.00 (1)	−(1)	−(1)	−(1)	NP	NP	F:GCTTTTCCCTTATTTTCCCG R:CGTGTTCGTATATTCCTGTAACG
Pdom 151	115	52, 50	$(CAT)_2AA(CAT)CAAT$ $(CAT)_3$	H_O = 0.00 (1) H_E = 0.00	0.25 (2)	−(1)	−(1)	−(1)	NP	−(2)	−(1)	F:TGATGTTACCACTGCTTTGAGCG R:TTCAGCACCGTCGTCGTTGTTG

T_a, annealing temperature.

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Characterization of nuclear microsatellites in *Pinus halepensis* Mill. and their inheritance in *P. halepensis* and *Pinus brutia* Ten.

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Nuclear microsatellites, or single sequence repeats (nSSRs), have been characterized in many tree species and are powerful markers for genetic diversity studies in natural populations (e.g. Echt *et al*. 1996; Pfeiffer *et al*. 1997). Although nSSR enrichment protocols have successfully been applied to conifers (Edwards *et al*. 1996), identification of single-locus, reproducible markers is difficult, probably because of their large genome size and complexity (Pfeiffer *et al*. 1997; Soranzo *et al*. 1998). In this study, we report the successful isolation of nSSRs in *Pinus halepensis* Mill. and their Mendelian segregation in both *P. halepensis* and *P. brutia*, two closely related Mediterranean pines.

A microsatellite library enriched for di- (GC, CT, CA), tri- (CAA, GCC) and tetra-nucleotide (GATA, CATA) repeats was constructed for *Pinus halepensis*, following the method described by Edwards *et al*. (1996). A total of 43 clones containing a microsatellite were detected from 47 clones randomly chosen from the library: 16% were repetitions of a single nucleotide (A/T), 77% were repetitions of dinucleotides (CA, CT or compounds CA–TA, CA–GA) and 7% were repetitions of trinucleotides (TAA, GCC). Sequencing reactions were performed using the Pharmacia AutoRead Sequencing Kit, and run on a 6% polyacrylamide gel containing 7 M urea using an ALF Pharmacia automatic sequencer. Primers were designed for the amplification of 25 dinucleotide nSSRs using the computer program Primer (http://www-genome.wi.mit.edu/genome_software/other/primer3.html).

Total genomic DNA extracted from leaf and mega-gametophyte tissue was used for testing the primer pairs. The procedure described by Doyle and Doyle (1990) and the Nucleon Phytopur DNA extraction kit were used for leaf tissue and mega-gametophytes, respectively. Polymerase chain reaction (PCR) was carried out using a Gradient 96 Stratagene Robocycler: the reaction solution (25 μL) contained four dNTPs (each 0.2 mM), 0.25 μM of each primer, 2.5 μL reaction buffer (100 mM Tris–HCl pH 9.0, 15 mM $MgCl_2$, 500 mM KCl), 25 ng of template DNA and 1 unit of Taq polymerase (Pharmacia). After a preliminary denaturing step at 95 °C for 1.5 min, PCR amplification was performed for 35 cycles: 1.5 min denaturing at 94 °C, 1.5 min at annealing temperature (Table 1) and 1.5 min extension at 72 °C, with a final 5 min step at 72 °C. After amplification, PCR products were mixed with a loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol and 10 mM NaOH), heated for 5 min at 95 °C, and then set on ice. Fragments were electrophoretically separated on a 6% polyacrylamide gel and stained using silver nitrate (Rajora *et al*. 2000).

Out of 25 primer pairs, nine (36%) either gave no amplification ($n = 4$) or produced multi-band patterns ($n = 5$). Sixteen produced fragment amplification in the expected size range, of which eight were polymorphic within one or the other species (Table 1). This proportion of functional markers is comparable to what is generally observed in conifers (e.g. Echt

Table 1 Primers and characteristics of seven microsatellite loci that were polymorphic either within *Pinus halepensis* or within *Pinus brutia**

Locus†	Repeat sequence	Primer sequence (5′ → 3′)	Annealing temp. (°C)	$MgCl_2$ (mM)	Expected size (bp)	Number of alleles‡	Heterozygosity (H_O/H_E)§	Number of mega-gametophytes per bi-allelic combination	χ^2 test (P value)	Accession no.
PHAF01	$(CA)_{18}$	F: TTCAGATATGGTCCATGGATG R: GATCACAATGTCATTATCGGG	54	2.5	194	3/3	0.611/0.538	15	0.795	AF195535
PHAF02	$(CA)_{15}$	F: TGGCAATGGAAACCTGATAC R: GCCCCACCATCATATCTCTTTAG	54	2.5	149	3/3	0.550/0.609	15	0.795	AF195536
PHAF05	$(CA)_{17}$	F: TCATAAGCCCTTTGTTTCTTTTC R: TTTTTCGCCCTGTATTTTCTG	56	3.5	125	4/4	0.611/0.624	20, 15, 8	0.655, 0.197, 1	AF195540
PHAF07	$(CT)_{16}$	F: ATCAGCTTAGTAGGTCTCGCC R: AGACACTAAAGGGGAGTCCG	54	2.5	123	3/3	0.700/0.676	13, 9	0.782, 0.739	AF195541
PHAF08	$(CT)_{25}$	F: TTCCACATTGTATTTTGATGCT R: AACTTTGGAAGTGACCAAATGT	53	4.5	150	2/1	0.500/0.479	19	0.251	AF195542
PHAF09	$(CT)_{18}$	F: ACTAAGAAACGGTGTGATGCTG R: CTTCGCATAGGCATGCATAC	59	2.5	198	2/1	0.600/0.505	19	0.819	AF195538
PHAF10	$(CA)_{17}(TA)_3$	F: TCCTTTCTTGTTCTTGGTAACTG R: ACCGCGGATTATAACCTGTG	53	2.5	129	4/4	0.529/0.665	19, 16	0.108, 1	AF195543

*An eighth locus, ITPF4516 (accession AJ012087) tested in *P. pinaster* (Mariette *et al*. 2000), is polymorphic in *P. halepensis* and *P. brutia* (four common alleles in both species). †PHAF, *Pinus halepensis* Avignon Firenze. ‡Values are for *P. halapensis*/*P. brutia*. In loci PHAF08 and PHAF09, *P. halepensis* and *P. brutia* do not share common alleles (sizes 205 and 155 bp respectively). §H_O is the frequency of heterozygotes in the sample and H_E is the unbiased expected heterozygosity (Nei 1978), where $H_E = (2n/2n - 1)(1 - \Sigma p_i^2)$.

et al. 1996; Pfeiffer *et al*. 1997). A single marker was found to be polymorphic in *Pinus pinaster* when the same 25 primer pairs were tested (Mariette *et al*. 2000). Transfer of nSSR markers across species of the same genus is generally difficult in conifers (e.g. Echt & May-Marquardt 1997), and the results thus confirm the close taxonomic relatedness between *P. halepensis* and *P. brutia*.

nSSR polymorphism was screened at population level using 50 *P. brutia* individuals (two populations) and 47 *P. halepensis* individuals (three populations). The maximum number of alleles per locus was four, and the expected heterozygosity per locus was between 0.479 and 0.676 (Table 1), which is lower than observed for other conifers, e.g. *Pinus sylvestris* (Soranzo *et al*. 1998) or *Picea abies* (Pfeiffer *et al*. 1997), but higher than found using isozymes (Schiller *et al*. 1986; Teisseire *et al*. 1995). Mendelian segregation was tested on 1–3 bi-allelic combinations in all polymorphic loci (Table 1). No significant deviation from the expected 1:1 ratio was observed. nSSRs are thus potentially helpful markers for studying population diversity in *P. halepensis* and *P. brutia*.

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Microsatellite markers for behavioural studies in a semi-fossorial shrew (Soricidae: *Anourosorex squamipes*)

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Genetic information revealed by microsatellite markers is useful for inferring social behaviours in animals (Garza *et al*. 1997), particularly for species that lead a secretive life style. The mole shrews (*Anourosorex squamipes*) are semi-fossorial, living underground and digging burrows but also coming to the forest floor to search for food (Hutterer 1985). Yu (1994) suggested that several mole shrews might share the same burrow system, as three or four mole shrews were often caught successively by one trap placed on the same spot. Thus, the mole shrew may have the peculiar social structure and behaviour common to some other subterranean mammals (Nevo 1979). As a preparatory step for studying behavioural genetics, we have characterized 11 microsatellite loci that are polymorphic and suitable for use to address questions regarding social structure in *Anourosorex squamipes*.

Genomic DNA for constructing the partial libraries was prepared according to procedures described by Sambrook *et al*. (1989). Genomic DNA was digested with *Sau*3A and fractioned in a 2.5% NuSieve™ GTG gel (FMC, Rockland, ME, USA). DNA of size range of 300–700 bp was isolated, purified with a GeneClean III kit (Bio101 Inc.) and ligated into plasmid PUC18/*Bam*HI/BAP (Pharmacia, Vista, CA, USA) according manufacturer's protocols. Ligated plasmids were transformed into competent SURE cells or XL-2 Blue ultracompetent cells (Stratagene).

Recombinant clones containing inserts were transferred to Hybond N^+ nylon membranes (Amersham), which were hybridized to a set of six oligonucleotide probes: $(AC)_{10}$, $(TC)_{10}$, $(CAC)_5CA$, $CT(ATCT)_6$, $(TGTA)_6TG$ and $CT(CCT)_5$. Probes were labelled with a DIG Oligonucleotide 3′-End Labelling Kit (Boehringer Mannheim). Hybridization was performed at 45 °C for 16 h in a standard hybridization buffer consisting of 5 × SSC, 0.1% *N*-lauroylsarcosine, 0.2% SDS and 1% blocking reagent (Boehringer Mannheim). The membranes were washed twice for 5 min at 45 °C, with a solution of 2 × SSC, 0.1% SDS, and then twice for 15 min at 65 °C with a solution of 0.1 × SSC, 0.1% SDS. Chemi-luminescent detection was performed with a DIG Luminescent Detection Kit (Boehringer Mannheim). The exposure time ranged from 15 to 30 min.

Positive clones were chosen for sequencing to confirm suitable length and base composition. The sequencing reactions were performed with a Big Dye dye-terminator kit, following the manufacturer's protocols, and analysed on polyacrylamide gels with an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems). The online program

Table 1 Characteristics of 11 polymorphic microsatellite loci in *Anourosorex squamipes*, including repeat motif, primer sequences, annealing temperature, allele size range, number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E)

Locus*	Repeat motif	Primer sequences (5′ → 3′)	Annealing temp. (°C)	Allele size range (bp)	Number of alleles	H_O	H_E
AS1	$(AC)_{15}$	GGATTCTATTTCATTCTTGAGTCAC GTAAAACTCTGGCTGGTGCC	53	129–155	10	0.75	0.88
AS2	$(TC)_9(TG)_6$	CCTGGTTTGACCTCATGTTTGG GACAGAGAGAGATGGGTGGGG	58	136–166	15	0.56	0.89
AS3	$(TG)_3TA(TG)_{18}$	TTCCGCCTTGTACTTTGCTG CCCCGGGGATCCAGTGTCTTAC	56	118–138	20	0.67	0.93
AS4	$(TGTC)_5(TC)_{11}(AC)_6$	GGATCCTTCCAGCGTTCTCTCTC GCAGCATGTTTCCCCAGTGTC	53	140–164	11	0.78	0.89
AS5	$(CA)_{17}$	AGGCAAACGCTTTACCCTTG TGTAGAAGGCTGGAGAGACAGTG	56	94–112	17	0.58	0.89
AS6	$(AC)_{13}$	GGTATGGAGGCACACAACGG TGCTTGCCAGTCTTCTCTGCG	56	96–126	13	0.56	0.86
AS7	$(TG)_{14}$	CGCATGCGTGTGTGTGAATC CCAGGTGTGCCCTTGAAACC	53	120–150	13	0.36	0.82
AS8	$(TG)_{12}$	TGCTCAAAAGCAATGCTAGCTG GTTCCAAGGACAATGCACGG	52	112–138	13	0.53	0.88
AS9	$(TG)_{12}$	CGCACTTTTGTTGTTGTATGCG TTCCTGGCGCCCCATAATAG	50	126–148	19	0.58	0.93
AS10	$(CA)_{26}$	GGGGCCTATTCCCCTGTTTC GGATGAGGGAATCCAGAAGACG	56	79–111	20	0.92	0.96
AS11	$(CCA)_6CCG(CCA)_8$	AGCCACAGGTTTCCACCCAC TTCCGCCTGTCTGCTTCTCC	56	80–119	19	0.33	0.88

*GenBank accession nos (order listed in table): AF261959–AF261969.

Primer 3.0 (http://www.genome.wi.mit.edu) was used to design primers from flanking regions of microsatellite DNA loci that contain more than 10 repeat units.

Individual genotypes were determined by polymerase chain reaction (PCR). PCR reactions were performed either with non-radioactive primers or radioactive primers. For non-radioactive PCR, 25 μL reactions were performed, containing 200 ng template DNA, 10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100, 0.75 mM Mg^{2+}, 0.15 mM dNTP, 0.5 μM of each primer and 2 units Taq DNA polymerase (Promega). Amplification was carried out according to the thermal profile: 95 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, optimal annealing temperature (Table 1) for 30 s and 72 °C for 30 s, with a final extension step at 72 °C for 7 min. PCR products were run on 6% native polyacrylamide gel, stained by ethidium bromide and visualized on a UV light box. The non-radioactive PCR was used to screen for polymorphic loci and the initial round of genotyping.

For radioactive PCR, one primer from each pair was 5′ end-labelled with [γ^{32}P]-ATP (NEN) and T4 polynucleotide kinase (Promega, Boston, MA, USA), following the manufacturer's protocols. Each PCR reaction totalled 10 μL, containing 200 ng template DNA, 10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100, 0.25 mM dNTP, 0.2 μM of each unlabelled primer, 0.6 mM Mg^{2+}, 0.25 units Taq DNA polymerase (Promega) and 0.5 pmol [γ^{32}P]-ATP labelled primer. Amplification was carried out according to the thermal profile: 95 °C for 3 min, followed by 25 cycles of 95 °C for 15 s, optimal annealing temperature (Table 1) for 2 min and 72 °C for 2 min, with a final extension step at 72 °C for 7 min. PCR products were run on a regular denaturing 6% polyacrylamide sequencing gel. The sizes of alleles were estimated by using control DNA (PUC18) from a Thermo Sequenase Cycle Sequencing Kit (Amersham) as markers. The radioactive PCR was used for a second round of screening: all the alleles of different sizes detected in the first round of screening were run on comparison gels to accurately determine their sizes. Running radioactive PCR products on denaturing gels also helps reduce the confusion caused by the heteroduplex bands that sometimes appeared in the first round of screening.

Eleven clones were confirmed to be polymorphic (Table 1) by typing 36 mole shrews collected from Taiwan. The number of alleles per locus ranged from 10 to 20, and the observed and expected heterozygosities ranged from 0.33 to 0.92 and from 0.82 to 0.96, respectively. The observed genotypes deviated from Hardy–Weinberg expectation at the 11 loci (all $P < 0.05$), resulting from heterozygote deficiency, which may be caused by combining samples from various disparate localities (Wahlund's effect).

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Isolation and characterization of microsatellite DNA markers in the Florida manatee (*Trichechus manatus latirostris*) and their application in selected Sirenian species

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The West Indian manatee (*Trichechus manatus*) inhabits subtropical and tropical waters of the Caribbean Sea from the southern USA to Brazil's north-east coast. Two sub-species are recognized, the Florida manatee (*T. m. latirostris*) and the Antillean manatee (*T. m. manatus*) (Domning & Hayek 1986). Abundant biological and ecological data for the Florida manatee have been collected, and the information has formed the basis for management and conservation programmes. However, to plan and implement biologically sound management programmes for this marine mammal, knowledge of the amount of genetic diversity present and a thorough understanding of the evolutionary relationships among geographical populations are essential. Genetic studies employing allozymes (McClenaghan & O'Shea 1988) and mitochondrial DNA (Bradley *et al*. 1993; Garcia-Rodriguez *et al*. 1998) have identified low levels of genetic diversity, and failed to resolve population structure for the Florida manatee. A technique with a higher resolution of genetic population structure and pedigree analysis is needed. We report the development and characterization of microsatellite DNA markers in the Florida manatee and test the utility of these markers in three closely related Sirenian species.

Two methodologies were used to generate microsatellite-enriched libraries for *T. m. latirostris*. Four enriched libraries were produced by Genetic Identification Services (Chatworth, California, USA) using proprietary magnetic bead capture technology. An additional library was constructed and screened for polymorphic loci following a protocol modified from Armour *et al*. (1994). For this protocol, approximately 50 μg of manatee genomic DNA were digested with *Sau*3AI (Life Technologies, Rockville, Maryland, USA), gel-fractionated to isolate 0.4–1.0 kbp fragments, and ligated to *Sau*3AI linkers. Polymerase chain reaction (PCR) amplifications were performed in a 100 μL volume containing 15 ng of purified DNA, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 0.25 mM $MgCl_2$, 0.2 mM dNTPs (Applied Biosystems, Foster City, California, USA), 1 μM *Sau*-L-A primer and 2.5 units of Taq DNA polymerase (Promega, Madison, Wisconsin, USA). The following amplification programme was used: 94 °C for 3 min, 30 cycles of 94 °C for 45 s, 68 °C for 45 s and 72 °C for 1.5 min, followed by 72 °C for 10 min. Purified PCR products were denatured by alkali treatment and hybridized to nylon filters containing $(CA)_n$ oligonucleotide repeats. Hybridization was performed overnight at 65 °C, and 5 μL of the recovered hybridized molecules were used for a 100 μL PCR amplification of microsatellite-enriched genomic DNA fragments following the amplification and PCR conditions described above. PCR products were directly ligated to pCR®2.1 (Invitrogen, Carlsbad, California, USA) followed by transformation into INVαF′ One Shot™ competent cells (Invitrogen). A total of 186 colonies were screened for $(CA)_n$-containing inserts using alkaline phosphatase-conjugated $(TG)_n$ oligomer and a chemi-luminescent detection system (FMC BioProducts Corp., Rockland, Maine, USA). Following a secondary screening, 60 positive colonies were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) employing M13 forward and reverse primers. Sequencing reactions were electrophoresed on an ABI 377 automated sequencer (Applied Biosystems).

From the two sets of libraries, primers were designed in the flanking regions of 61 microsatellite-bearing clones using OLIGO 5.1 (National Biosciences, Molecular Biology Insights Inc., Cascade, Colorado, USA). Microsatellite DNA amplification reactions consisted of 200 ng DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.20 mM dNTP, 5 pmol of forward and reverse primer and 1.0 U Taq DNA polymerase (Promega) in a total volume of 20 μL. The forward primer was 5′ modified with either TET, FAM or HEX fluorescent labels (Applied Biosystems). Amplification was performed in a Biometra® UNO II thermal cycler using the following conditions: 94 °C for 2 min, 34 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Amplified fragments were subjected to fragment analysis on an

Table 1 Expected size of fragment (bp), repeat type, number of alleles detected, observed and expected levels of heterozygosity, primer sequence, and GenBank accession nos for 14 *Trichechus manatus latirostris* microsatellite DNA markers surveyed in 50 animals collected throughout Florida, and the results of cross-species amplification of these markers in three other Sirenian taxa

			Manatee species							
			Florida				Antillean	Amazonian	Dugong	GenBank
Locus	Size	Repeat type (and length)	*N*	H_O	H_E	Primer sequences (5′ → 3′)	*N*	*N*	*N*	accession no.
TmaA01	107	$(TA)_3(CA)_3CG(CA)_7$	1	0.00	0.00	F-CAGAAGGGATACATATACA R-CAGCCCCTGGCTGTCTCTTGTC	2	2	2	AF223649
TmaA02	247–251	$(CACT)_2(CA)_{16}$	3	0.51	0.54	F-CTCAGTCCAAACAGCTAATG R-TAGTCATTTGTGCAGAGTGC	3	5	2	AF223650
TmaA03	163–183	$(GACA)_4$	2	0.30	0.40	F-ACATGTGTTCCCTGCTGTAT R-GATTTTTGGAGCAGTTGTCA	4	3	1	AF223651
TmaA04	204	$(CT)_2(GT)_{12}AT(GT)_7AT(GT)_2$	1	0.00	0.00	F-GAACACAAGACCGCAATAAC R-TGGTGTATCACTCAGGGTTC	3	1	3	AF223652
TmaA09	150	$(GT)_{15}$	1	0.00	0.00	F-GATGGGATACTGGGTTATGC R-ATGCAGACACTGGACATAGG	1	4	3	AF223653
TmaE02	172–174	$(GT)_{13}$	2	0.44	0.46	F-GTCTCTACGGCCTAGAATTGTG R-TTTCTCTACCTCTCCTCACACG	2	1	3	AF223656
TmaE08	149–165	$(CA)_{13}TA(CA)_5$	3	0.47	0.55	F-GAATAGAGACTGGGCTAGAATCC R-GCCTTTTGGAGGGATAGAAGTAG	4	2	3	AF223657
TmaE11	177–197	$(CA)_{13}$	6	0.58	0.63	F-ACACACAACATCACTCATCCAC R-AAGCTGCGTTCTACTTCATATAATC	8	1	3	AF223658
TmaE26	199–201	$(CA)_8C(CA)_{17}$	2	0.24	0.26	F-CATTCCTGATCCACAAAATC R-CCTGTCTTCTCTCTGTTTCTCC	5	3	2	AF223659
TmaF14	204–206	$(TC)_6(TG)_2TA(TC)_5TG(TC)_3$	2	0.24	0.32	F-CTAAGACATTGCTCCAAAAGC R-GGGCAGTGGGATTTGAGATG	2	2	1	AF223660
TmaF34	271	$(TCTCTCTCTTTCTG)_2(TC)_4$ $TT(TC)_3$ $(AC)_8AT(AC)_9$	1	0.00	0.00	F-CATGAGAGACTATGCTCCCTTC R-CAGGTAGGAAGATGATGAGGAC	1	2	—	AF223661
TmaH1I	298	$(TCTG)_4(TCTA)_5CCTGTCTATCCA$ $(TCTA)_3CCTG(TCTA)_8CCTG(TCTA)_5$	1	0.00	0.00	F-AGCAGATAGACACACTGGGAAG R-GAGTCTGAATGAATGAATTACTGC	1	3	1	AF223662
TmaM61	176	$(TG)_3(GT)_{17}$	1	0.00	0.00	F-TTGAGGTGTAATCTGTGTG R-GGTAATCGGAGTTGGTGTA	2	2	1	AF223655
TmaM79	154–156	$(GT)_{15}$	2	0.56	0.54	F-CCAATCATGTCCCAAACT R-CAATAGAAGAAGCAGCAG	3	3	2	AF223654

Tests for goodness of fit to Hardy–Weinberg expectations suggested that there were no significant differences between observed and expected values (Raymond & Rousset 1995). The results of cross-species amplification of these markers in three other Sirenian taxa are also provided: Antillean manatee (*Trichechus manatus manatus*), $n = 21$ animals surveyed; Amazonian manatee (*Trichechus inunguis*), $n = 7$; and the dugong (*Dugong dugong*), $n = 3$. '—' indicates no or sub-optimal amplification products in cross-species tests. *N*, number of alleles observed.

ABI PRISM™ 310 Genetic Analyser (Applied Biosystems). Genescan™ 2.1 and Genotyper™ 2.1 Fragment Analysis software (Applied Biosystems) were used to score, bin and output allelic (and genotypic) data.

Fourteen sets of primers amplified fragments of expected size from Florida manatee genomic DNA (Table 1). These markers were screened in 50 manatees collected throughout the Florida peninsula. Eight of the 14 loci were polymorphic in this initial survey, and overall levels of heterozygosity averaged 41%. Low levels of allelic diversity were observed in the Florida manatee. The maximum number of alleles identified was six (TmaE11), and the average number of alleles observed at polymorphic loci was 2.9. This paucity of genetic diversity suggests a founder effect or major population bottleneck of evolutionary significance (see Garcia-Rodriguez *et al*. 1998). In addition, this study reports one of the lowest levels of genetic diversity observed in species-specific microsatellite DNA markers [see Nyakaana & Arctander 1999 (African elephant); Waldick *et al*. 1999 (right whale)].

Cross-species amplification was tested in three Sirenian taxa: the Antillean manatee (*T. m. manatus*), the Amazonian manatee (*T. inunguis*) and the dugong (*Dugong dugong*). Eleven of 14 markers were polymorphic for the Antillean and the Amazonian manatee (Table 1). At least nine markers were polymorphic in the dugong; the polymorphism is likely to be under-estimated due to the small sample size ($n = 3$). This suite of markers appears to be ideal for the identification of population structure and possibly pedigree analysis in all four Sirenian species, and provides a nuclear DNA-based approach to complement existing mitochondrial DNA genetic information for these vulnerable species.

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Microsatellite loci for two European sciurid species (*Marmota marmota, Spermophilus citellus*)

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Two species of European sciurid rodents are of particular interest for behavioural ecology and population genetics: *Marmota marmota* and *Spermophilus citellus*. The Alpine marmot (*M. marmota*) inhabits higher elevations of the European Alps and some isolated mountain massifs. Autochthonous populations occur only in the Alpine core area and in a small area near Berchtesgaden. The distribution of the European ground-squirrel (*S. citellus*) comprises the grassland of the Pannonian plain ranging from eastern Europe to the foothills of the Alps. It is presently listed as endangered (Berner Convention 1999). We isolated six new microsatellite markers for each of the two species (*M. marmota*: L. Kruckenhauser; *S. citellus*: S. Hanslik).

Genomic DNA was extracted from frozen liver (*M. marmota*) or ethanol-stored tissue samples from the tail (*S. citellus*) using a standard phenol–chloroform extraction method (Sambrook *et al*. 1989). Following the protocol of Rassmann *et al*. (1991), partial genomic libraries were established for *M. marmota* and *S. citellus* and around 1400 clones from each species were screened for the presence of microsatellite sequences using a dinucleotide simple sequence polymer probe AC/GT.

Fifty-eight marmot clones showed a positive signal. Twenty-two were sequenced using the SequiTherm EXCEL™ II DNA Sequencing Kit (Epicentre Technologies) with biotinylated primers and the SAAP/CSPD detection system (US Biochemicals, Inc.). Primer pairs were synthesized for 11 loci; six of these microsatellite loci showed unambiguous allelic patterns in *M. marmota* (Table 1). Polymerase chain reaction (PCR) amplifications were performed on a HYBAID Omnigene thermocycler in a volume of 12.5 μL containing 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 150 mM KCl, 0.1% Triton X-100, 0.25 U DynaZyme DNA polymerase (Finnzymes OY), 2 pmol of each primer (forward primer labelled with IRD-800), 200 μmol of each dNTP, 0.25 μL DMSO and 50 ng template DNA. The amplification protocols were as follows: 94 °C for 5 min, then two cycles of 94 °C for 20 s, annealing temperature plus 6 °C for 20 s, 70 °C for 20 s, then 30 cycles of 94 °C for 30 s, annealing temperature for 20 s, 70 °C for 20 s, and finally 72 °C for 2 min. PCR products were separated on 6% denaturating polyacrylamide gels in a Li-Cor automatic sequencer. Analysis of PCR fragments was carried out using RFLPscan (Scanalytics). The six loci were tested in 19 individuals of *M. marmota* from the Austrian allochthonous population Turracher Nockberge. In addition, 10 individuals of *S. citellus* were cross-tested with the same primer sets.

Table 1 Primer sequences (5′ → 3′) of microsatellites from *Marmota marmota* (MS6, MS41, MS45, MS57, MS53, MS56) and *Spermophilus citellus* (ST7, ST10, SB10, SC2, SC4, SX), GenBank accession nos, repeat motifs and annealing temperatures

Locus	Repeat motif	Primer	Accession no.	Annealing temp. (°C)
MS6	$(GT)_{20}$	F: CTGATGGGGTTAAGATTGCC R: CCCCACTGACCCACCTCC	AF259372	53
MS41	$(GT)_{11}$	F: GGTGTATATGGGAATAGGGGG R: GCCTTCAAATCAAAGCAGGTTG	AF259373	53
MS45	$(GT)_{13}$	F: CTGTCTCTTTGTCCCTGCC R: CTCCTTACCATCATCTTTCCG	AF259374	53
MS47	$(GT)_4TC(GT)_3AT(GT)_7GAGG$ $(GA)_4TT(GA)_3AA(GA)_{11}$	F: CCTGATGTAGTCAGTCAG R: TGTGGGAAATGGCACATC	AF259375	50
MS53	$(GT)_{18}$	F: ATTGAGGAGCAGCATCTAGG R: TCAGGGAAAGGCAGACCTG	AF259376	53
MS56	$(CA)_{14}$	F: CAGACTCCCACCAGTGACC R: CCTGATCTATGTAGGTTCCAT	AF259377	53
SB10	$(GA)_{12}(TG)_{18}$	F: TCTGTTTAGTTCATTTGCCATTT R: TCAAGAGAGGTCCTACAGAATGA	AF254435	50
SC2	$(GA)_{31}$	F: CATCATGGCAGAAGATGTGG R: TTGACTGGAAGTGGGACTCTC	AF254438	56
SC4	$(GT)_{20}$	F: AAAAGCGTGCATTGCCTTAC R: CCTCTCAAGACGGGCAGA	AF254437	56
ST7	$(TGG)_7T(GT)_2$ $AT(GT)_7AT(TG)_8$	F: GAATCTTGACTCCTGAGATA R: CCATCTCCTGACATTTAATA	AF254439	50
ST10	$(CA)_{12}$	F: TTGTGATCCTCCAGGGAGTT R: GTGATTTCCAAACCCCATTC	AF254436	52
SX	$(GA)_{25}$	F: TTTTCCTCTCCTGAATGCTTTT R: CAAAGATGTTGTGTCCGACG	AF254440	56

Thirty-six positive ground-squirrel clones were sequenced with the M13-40 forward primer using Sequenase version 2.0 (Amersham Life Sciences). Sequencing products were separated on a 4% denaturing polyacrylamide gel and visualized autoradiographically. Primers were designed with the OLIGO software package (National Biosciences Inc., version 5.0). PCR amplification was carried out on a HYBAID Omnigene thermocycler in 10 μL reaction volume with 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl, 0.1% Triton X-100, 0.2 mg/mL BSA, 200 μM dNTPs, 1 μmol of each primer (0.02 pmol forward primer end-labelled with $\gamma^{32}P$), 50–100 ng template DNA, and 0.5 units Taq DNA polymerase. A 4 min initial denaturation at 94 °C was followed by 30 cycles of 1 min at 94 °C, 1 min at 47–61 °C (depending on the primer combination), 1 min at 72 °C, and a final extension at 72 °C for 45 min. PCR products were separated on a 7% denaturing polyacrylamide gel. Alleles were sized by running a sequencing reaction of M13 next to the amplified microsatellites. Six primer pairs yielded clear amplification products in *S. citellus* (Table 1). The six loci were analysed in 54 ground-squirrel and 10 marmot individuals.

Observed and expected heterozygosities were calculated using GENEPOP (version 1.2; Raymond & Rousset 1995). Altogether 12 microsatellite loci were tested in both species, the results for these are shown in Table 2. Ten loci amplified in both species, two amplified in *M. marmota* only. All loci were polymorphic in at least one of the two species, and up to seven different alleles were observed in one species. Significant deviations from the Hardy–Weinberg expectations as calculated with the program GENEPOP (version 1.2; Raymond & Rousset 1995) were found for the loci SB10 ($P = 0.0038$) and SX ($P = 0.0098$) in the ground-squirrel population and MS56 ($P = 0.0001$) in the marmot population. These deviations might be due to null alleles in the respective populations.

So far, only a small number of microsatellite loci have been identified for *M. marmota* (Klinkicht 1993), and no markers have been isolated for *S. citellus*. The primer sets for 12 loci compiled here should provide sufficient information for genetic investigations not only in *M. marmota* and *S. citellus* but also over a larger species range within the two genera.

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Table 2 Microsatellite loci tested in *Marmota marmota* and *Spermophilus citellus*: number of detected alleles, size range of alleles, expected and observed heterozygosities (H_E, H_O) and number of individuals analysed (*n*)

	Marmota marmota					*Spermophilus citellus*				
Locus	*n*	Number of alleles	Size range	H_E	H_O	*n*	Number of alleles	Size range	H_E	H_O
MS6	19	5	142–164	0.67	0.67	10	—	—	—	—
MS41	19	3	186–190	0.42	0.41	10	3	195–201	0.43	0.55
MS45	19	3	109–113	0.68	0.69	10	2	127–129	0.16	0.17
MS47	19	7	163–191	0.87	0.81	10	—	—	—	—
MS53	19	5	141–149	0.71	0.78	10	4	147–153	0.58	0.46
MS56	19	3	111–115	0.58	0.16	10	3	113–121	0.49	0.62
SC2	10	2	128–130	0.50	0.50	54	1	146	0.00	0.00
SC4	10	2	134–145	0.53	0.00	54	1	102	0.00	0.00
ST7	10	5	135–154	0.60	0.87	54	3	151–156	0.55	0.44
SB10	10	1	154	0.00	0.00	54	4	150–162	0.73	0.65
ST10	10	4	124–130	0.63	0.83	54	3	127–134	0.51	0.62
SX	10	2	142–146	0.50	0.50	54	3	142–146	0.60	0.61

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Microsatellite loci in the Eurasian red squirrel, *Sciurus vulgaris* L.

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Ever since microsatellites were first amplified using the polymerase chain reaction (PCR) and shown to be variable, they have been enthusiastically adopted by population geneticists. Microsatellites quickly became the molecular marker of choice during the 1990s because of the speed and ease with which they can be applied to large samples, and the possibility of their amplification from poor-quality samples collected by non-invasive methods. The level of variability found at microsatellite loci has meant that they can be used to answer phylogenetic questions on many levels (McDonald & Potts 1997). However, the main disadvantage in the use of microsatellites is the frequent need to develop a set of markers for each species under investigation; this limitation will diminish as more markers are isolated for different species. This paper reports the development of five polymorphic microsatellite loci from the genome of *Sciurus vulgaris* L., the Eurasian red squirrel.

The loci were isolated using the enrichment method of Armour *et al*. (1994). Three partial genomic libraries were constructed using DNA extracted from *Sciurus vulgaris* tissue and digested with the enzyme *Mbo*1 (Gibco BRL). SAU linkers were ligated to a size-selected fragment (400–1300 bp), as described in Armour *et al*. (1994), and used to prime a whole-genome PCR reaction. The product of this reaction was further size-selected before hybridization selection was carried out. Each library was constructed using genomic fractions selected by hybridization to a different set of tetra-, tri- and dinucleotide target repeat sequences taken from $(GATA)_n$, $(GACA)_n$, $(CCAT)_n$, $(ACCT)_n$, $(TTGG)_n$, $(GGAA)_n$, $(TTTG)_n$, $(TTTC)_n$, $(GTA)_n$, $(GAT)_n$, $(GCT)_n$, $(CGT)_n$, $(TCC)_n$, $(CAC)_n$, $(GTT)_n$, $(AAG)_n$ and $(GT)_n$.

The hybridization selection reactions were carried out as described by Armour *et al*. (1994). The selected fraction was re-amplified in a whole-genome PCR and ligated directly into the pGEM-T vector (Promega) or the pNoTA/T7 shuttle vector of the Prime PCR Cloner Cloning System (5 Prime → 3 Prime, Inc.). These ligations were used to transform *Epicurian coli* XL2-Blue MRF ultracompetent cells (Stratagene). Positive colonies were cultured and stored as glycerol stocks in microtitre plates; the contents of each plate were replicated onto nylon filters and probed with labelled target oligonucleotide repeat sequences. Positive colonies were identified and sequenced manually using either isolated plasmid DNA or amplified PCR products as template (the PCR products were generated using the primers M13for (Gibco BRL) and M13rev (Promega) which flank the insertion site). Sequencing was carried out using the T7 sequencing mixes and the T7 polymerase enzyme (Pharmacia Biotech) following a protocol based on protocol 11 described in Hoelzel & Green (1998). Primers for all useful repeat sequences were designed (with the aid of the computer program OLIGO™; National Bioscience) and tested for variability on a panel of 10 DNA samples.

PCR amplification of variable loci was optimized using the method described by Cobb & Clarkson (1994). The forward reaction primer in each case was end-labelled with ^{32}P γ-dATP

Table 1 The characteristics of five Eurasian red squirrel microsatellites

Locus name	GenBank accession no.	Repeat structure	Primer sequences (5′ → 3′)	Allele size range (bp)	Number of alleles	H_O (%)	H_E (%)
RSμ1	AF285149	$[GGAT]_{13}$	F 5′-CTGGGTTCACTGACTTCTCC-3′ R 5′-CACTCTCAGAGGCCAAAGTC-3′	172–196	7	71.9	73
RSμ3	AF285150	$[GA]_9[GACA]_9$	F 5′-GCCAAAATCTAGCCCAAGAAG-3′ R 5′-CTCAGGTGTGGGAAAGAAGC-3′	161–173	7	52.2	57.4
RSμ4	AF285151	$[ATCC]_{12}$	F 5′-CAATCCTCCCATCCTGCTGC-3′ R 5′-TAGGCAGTCAGATAGGTGGG-3′	256–284	8	78.1	72.3
RSμ5	AF285152	$[GT]_{10}$	F 5′-CCCAGTCTACATTAAAGGGC-3′ R 5′-GCCTATACACTATAATTGACTG-3′	123–143	7	39.3	45.5
RSμ6	AF285153	$[GTT]_{10}$	F 5′-GGCATAGGGCACGTGAAG-3′ R 5′-GGGCCAATCTCATACCAAG-3′	122–131	4	27.3	36.5

H_O, observed average heterozygosity; H_E, expected average heterozygosity. F, forward primer; R, reverse primer.

using T4 polynucleotide kinase (Gibco BRL). Amplification reactions were carried out on a PTC-200 thermocycler (MJ Research, Inc.) with 25 μL reactions containing dNTPs (0.15 mM for RSμ1, 0.1 mM for RSμ3, 0.2 mM for RSμ4 and 0.05 mM for RSμ5 and 6), 1 mM $MgCl_2$ (1.5 mM for RSμ1), 10 pmol of each primer (5 pmol for RSμ1 and 3) including 1 pmol of labelled primer (2 pmol for RSμ1), 1 unit of 'red hot' Taq DNA polymerase (Advanced Biotechnologies) with Taq buffer (final concentration 0.75 M Tris–HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% w/v Tween; Advanced Biotechnologies) and approximately 0.8 ng of template DNA. The reactions were denatured at 94 °C for 3 min, and then subjected to 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 90 s. A final extension step was carried out for 5 min at 72 °C. The PCR products were visualized by electrophoresis through 6% polyacrylamide gels using Sequi-GenII GT gel rigs (BioRad) and exposure to X-ray film. Allele sizes were determined by comparison to a known sequence ladder.

Five loci, named RSμ1, RSμ3, RSμ4, RSμ5 and RSμ6, were found to be polymorphic in the Eurasian red squirrel (Table 1). These loci were amplified from 163 samples of red squirrel DNA in individuals taken from 11 populations in Belgium and Germany (Todd 2000); the proportion of individuals found to be heterozygous at each locus is also given in Table 1. The study included samples of more than 20 individuals from three large populations, and these were used to test for null alleles. Fisher's exact test was carried out using Biomstat (version 3.2) (Applied Biostatistics Inc.) on the observed and expected number of heterozygotes at each locus in the three populations, and no evidence to indicate the presence of null alleles was found ($P > 0.2$).

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Isolation and characterization of microsatellite loci from the ocellated wrasse *Symphodus ocellatus* (Perciformes: Labridae) and their applicability to related taxa

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Table 1 Characterization of seven *Symphodus ocellatus* microsatellite loci based on five samples (10 individuals each)

Locus	Repeat array	Primer sequences (5′ → 3′)	Annealing temp. (°C)	$MgCl_2$ (mM)	Number of alleles	Size range (bp)**	H_O	H_E
Soc1017PBBE	$(AC)_{20}GC(AC)_2$	*TCC TGT CAG TCT CCC TTC A						
		GTG ATT GAT TAG GCG ATG AG	63	0.8	20 (9–15)	77–123 (101)	0.86 (0.6–1.0)	0.92 (0.87–0.96)
Soc1063PBBE	$(GA)_2(GT)_8$	*CCC TTC TTG TGT CAT TCC						
	$AT(GT)_4$	AAG CCT CAC TTG ATA TGT CC	56	0.8	14 (6–9)	92–134 (98)	0.76 (0.4–1.0)	0.86 (0.83–0.91)
Soc1093PBBE	$(AC)_{26}$	*CCT CCA ATT CCC AAA ACA AC						
		CTG ACC ACT GGC ACA CTC AT	63	0.8	30 (12–15)	96–294 (132)	0.96 (0.9–1.0)	0.95 (0.91–0.97)
Soc1109PBBE	$(GT)_{10}$	*AGG ATT TAG CCT GCC CAG GA						
		TGC GGT GAA TGG CTG TAG GT	57	1.0	13 (7–9)	133–167 (137)	0.84 (0.7–1.0)	0.87 (0.80–0.89)
Soc1198PBBE	$(TG)_5TA(TG)_{13}$	*CTC TTT CTG CCT GCA CTC						
		GAC TTC ATT GGA CAG CAC AC	57	1.2	11 (5–9)	89–113 (109)	0.76 (0.6–0.9)	0.80 (0.67–0.89)
Soc3121PBBE	$(GT)_{18}$	*ACG ACA AGC TGC ACG AAC						
		CCA GTA ATT CTG ACT CCA CCC	56	0.9	28 (12–14)	82–205 (102)	0.90 (0.8–1.0)	0.95 (0.94–0.96)
Soc3200PBBE	$(GT)_{15}$	*AGT GCC AGA TGT ATA TGG G						
		CAT GGA CGC ATT TGT AGC	51	1.0	27 (12–14)	120–188 (134)	0.88 (0.8–1.0)	0.94 (0.91–0.96)

The sequences of cloned fragments have GenBank accession nos AJ278566–AJ278572; H_O, observed heterozygosity; H_E, expected heterozygosity; given are the mean values across the five populations with range in parentheses. *Primer used for end-labelling. **Cloned insert size in parentheses.

The ocellated wrasse, *Symphodus ocellatus*, is a common Mediterranean labrid fish of shallow coastal waters. Its geographical distribution also includes the Black Sea, the Azov Sea and the North-Eastern Atlantic (Whitehead *et al*. 1984). This species is abundant in the Mediterranean and inhabits various biotopes such as shallow rocky areas and seagrass beds (Michel *et al*. 1987; Francour 1997). The ocellated wrasse is a partially sedentary fish, with territorial males, exclusive male parental care and conspicuous male nuptial coloration and courtship (Warner & Lejeune 1985), and thus constitutes an interesting species for investigating various aspects of population genetics and behavioural ecology of marine fishes. Here we report seven microsatellite loci of the labrid fish *S. ocellatus* and their amplification in five related taxa.

Ocellated wrasse microsatellite loci were cloned as described by Estoup *et al*. (1993) and in detailed protocols by A. Estoup and J. Turgeon available at http://www.inapg.inra.fr/dsa/microsat/microsat.htm. The genomic library was constructed with about 10 μg of DNA isolated from muscle tissue of a single ocellated wrasse from a population near Marseille (France). Approximately 1500 colonies were screened for microsatellites using a mixture of six probes $(TC)_{10}$, $(TG)_{10}$, $(CAC)_5CA$, $CT(CCT)_5$, $CT(ATCT)_6$ and $(TGTA)_6TG$, yielding 176 positively hybridizing clones. Plasmid DNA of positive clones was purified using a QIAprep Spin Miniprep Kit™ (Qiagen). Both strands of the wrasse DNA inserts were sequenced using a Thermo sequenase cycle sequencing kit™ (Amersham) and M13 forward and reverse primers end-labelled with fluorescent dye (IRD800™; Li-Cor). Miniprep preparation and sequencing reactions were carried out according to the recommendations of the manufacturers, and sequence reaction products were resolved on an automated DNA sequencer (model 4200™; Li-Cor).

Here we report the seven microsatellite loci (Table 1) for which we so far have successfully designed primer pairs. The genomic DNA for genotyping was prepared either using a phenol–ethanol extraction method or a rapid BIO RAD (Celex 100 resin) extraction protocol as described by Estoup *et al*. (1996). Polymerase chain reaction (PCR) amplifications were carried out in 10 μL volumes using a PTC100™ machine (MJ Research, USA). Each reaction contained 20 ng genomic DNA, 2 pmol of each primer, one of which was end-labelled with an infra-red fluorescent dye (IRD800™), $MgCl_2$ (concentration in Table 1), 0.06 mM of each dNTP, 1 × PCR buffer (Qiagen) and 0.25 U Taq DNA polymerase (Qiagen). Reaction conditions were as follows: an initial denaturation step of 5 min at 95 °C, five cycles consisting of 30 s at 95 °C, 30 s at annealing temperature (see Table 1) and 75 s at 72 °C, 25 cycles consisting of 30 s at 94 °C, 30 s at annealing temperature and 75 s at 72 °C, followed by a final 5 min extension at 72 °C. PCR products were analysed on an automated DNA sequencer (model 4200™), and amplified fragments of cloned alleles were used for size determination at the respective loci. Variability of the loci was tested in five populations of *S. ocellatus* from the French Mediterranean coast (Cap Martin, St Jean Cap Ferrat, Antibes, Cannes and Marseille). Ten individuals from each population were analysed. The number of alleles per locus and the observed and expected heterozygosities are listed in Table 1. All loci were polymorphic, with the number of alleles per locus ranging between 11 and 30 and the observed heterozygosity between 0.76 and 0.96. Additionally, we tested the amplification of these primers in the labrid *Coris julis* and four other species of the genus *Symphodus*: *S. tinca*, *S. roissali*, *S. rostratus* and *S. cinereus* (Table 2). All specimens were sampled along the French Mediterranean coast between Cap Martin and Cannes.

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Table 2 Amplification results of seven *Symphodus ocellatus* microsatellite loci in related taxa

Species	Annealing temp. (°C)	Mg concentration (mM)	*n*	Number of alleles	Size range (bp)
Soc1017PBBE					
S. tinca	63	0.8	5	5	75–85
S. roissali	63	0.8	7	9	83–109
S. rostratus	61	0.8	6	5	93–101
S. cinereus	61	0.8	2	4	81–97
Coris julis	61	0.8	1	2	93–97
Soc1093PBBE					
S. tinca	63	0.8	3	5	114–206
S. roissali	63	0.8	5	1	112
S. rostratus	63	0.8	6	5	86–96
S. cinereus	63	0.8	2	4	106–120
Coris julis	63	0.8	1	1	104
Soc1198PBBE					
S. tinca	57	1.2	5	7	89–105
S. roissali	57	1.2	8	9	95–133
S. rostratus	57	1.2	6	2	85–103
S. cinereus	57	1.2	2	4	93–109
Coris julis	57	1.2	1	1	85
Soc3200PBBE					
S. tinca	61	0.9	5	3	142–148
S. roissali	61	0.9	8	11	117–154
S. rostratus	61	0.9	6	5	122–134
S. cinereus	61	0.9	2	3	132–150
Coris julis	61	0.9	1	—	—
Soc1063PBBE					
S. tinca	56	0.8	5	4	96–102
S. roissali	56	0.8	6	12	98–164
S. rostratus	56	0.8	6	7	94–112
S. cinereus	56	0.8	2	4	92–120
Coris julis	54	0.8	1	2	103–113
Soc1109PBBE					
S. tinca	57	1.0	5	6	141–155
S. roissali	57	1.0	8	12	133–167
S. rostratus	57	1.0	6	4	127–147
S. cinereus	57	1.0	2	3	143–159
Coris julis	57	1.0	1	1	125
Soc3121PBBE					
S. tinca	56	0.9	5	5	91–125
S. roissali	56	0.9	8	13	86–147
S. rostratus	56	0.9	6	2	87–95
S. cinereus	56	0.9	2	4	87–97
Coris julis	56	0.9	1	1	87

n, number of analysed specimens; —, no alleles obtained.

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Characterization of microsatellite loci in the primitive ant *Nothomyrmecia macrops* Clark

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Although a number of microsatellite loci have been isolated for some species of 'primitive ants' in the subfamily Ponerinae [e.g. *Diacamma* (Doums 1999), *Gnamptogenys* (Giraut *et al*. 1999)], the availability of genetic markers for the unique Australian ant *Nothomyrmecia macrops* has been poor. Of 16 allozyme loci studied by Ward & Taylor (1981) only one locus was polymorphic. Colonies appear to have low nestmate relatedness (Ward & Taylor 1981) but these estimates must be interpreted with caution because of limited sample size. *N. macrops* has great significance in evolutionary sociobiology because it possesses a relatively large proportion of ancestral characters (e.g. Taylor 1978). Thus, a more detailed knowledge of the genetics of this ant is desirable and will perhaps shed new light on a number of issues related to the evolution of eusociality in the Hymenoptera. In this paper we describe the isolation of variable microsatellite loci that can be used for precise colony- and population-level genetic analyses in *Nothomyrmecia*, and in the most closely related subfamily, the Myrmeciinae.

Genomic DNA was extracted from five worker pupae as described by Baur *et al*. (1993). The DNA was digested with the restriction enzymes *Sau*3AI and *Rsa*I, and size-selected fragments (300–600 bp) were ligated into the *Bam*HI/*Hin*cII site of the vector pUC19. Electrocompetent *Escherichia coli* JM109 strains were transformed by electroporation using a BioRad genepulser and colonies were hybridized onto Hybond N + (Amersham) nylon membranes. Approximately 9000 recombinant colonies were screened with a radiolabelled $(GA)_{10}$ oligonucleotide probe and 117 positive clones identified. Thirty clones were sequenced either manually using the *f*mol® cycle sequencing kit (Promega) or using the Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer) with an ABI Prism 377 DNA auto-sequencer. Primers were designed for 18 loci using the computer program OLIGO™ (Macintosh version 4.0, National Biosciences Inc.).

DNA for microsatellite analysis was prepared from gasters of single *Nothomyrmecia* workers using a modification of the Chelex® 100 Resin extraction protocol (Walsh *et al*. 1991). Polymerase chain reactions contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton® X-100, 165 μM dNTPs, 0.1 μM of forward primer, 0.03–0.06 μM of forward primer end-labelled with [γ^{33}P]-ATP, 0.4 μM of reverse primer, 0.5 μg/μL bovine serum albumin, 0.4 U of *Taq* DNA polymerase (Promega) and 2 μL of template DNA in a total volume of 10 μL. Amplifications were conducted in a Corbett thermal cycler using the following temperature profile: 2 min at 94 °C followed by 35 cycles of 30 s at 93 °C, 30 s at 50 or 55 °C for annealing (see Table 1) and 30 s at 72 °C, and a final elongation step of 10 min at 72 °C. The amplified products were electrophoresed on 5% polyacrylamide sequencing gels and visualized by autoradiography.

Of the 18 sets of primers, two failed to amplify and one gave a banding pattern that was difficult to interpret. The other 15 loci yielded repeatable and scorable results. A sample of 36 workers from Poochera, South Australia (taken from trees in an area of approximately 200 × 20 m) was used to assess the variability of these markers. We found that all but one of the 15 loci showed considerable polymorphism (Table 1), which is surprising given the small and geographically restricted sample analysed. Each polymorphic locus had between three and 12 alleles. The expected heterozygosity based on allele frequencies ranged from 0.53–0.90 with a mean of 0.70 across all loci. We tested for heterozygote deficiency using the computer program GENEPOP (web version 3.1c) in order to detect the presence of null alleles. Except for the significant excess of homozygotes at locus Nmac 115, no deviations from expected heterozygosities were discovered.

We investigated cross-species amplification in two species of *Myrmecia* and found that a large proportion of the loci could be amplified in *M. forficata* (see Table 1). Despite being highly polymorphic in *Nothomyrmecia*, the loci Nmac 11, 28 and 45 turned out to be monomorphic in *M. pyriformis* (three individuals from each of five colonies examined). The relatively high success rate of cross-species amplification may support the general contention that the two subfamilies Nothomyrmeciinae and Myrmeciinae are closely related (Baroni Urbani *et al*. 1992). In other groups of ants, successful cross-species amplifications have been reported most frequently among genera within the same subfamily (e.g. Doums 1999) suggesting a relatively low level of conservation of microsatellites across ant taxa.

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Table 1 Microsatellite loci and their characteristics developed in the ant *Nothomyrrmecia macrops*. The number of alleles (N_a), frequency of the most common allele (f) and the estimates of observed (H_O) and expected heterozygosity (H_E) are based on a worker sample of 36 individuals collected near Poochera. Amplification success in *Myrmecia forficata* (*Mf* — three individuals examined) is indicated by ± but with the annealing temperature (T_a) set to 50 °C for all runs

Locus	Core repeat	Size (bp)	N_a	f	H_O	H_E	T_a (°C)	Primers (5′–3′)	*Mf*
Nmac 1	$(AG)_{13}$	186–209	8	0.32	0.94	0.82	50	F: CGT TTC CGA TAT TCG AGC AG R: CAG GAT GAC AGC CGG TGA G	+
Nmac 11	$(GA)_3G(GA)_2CG(GA)_{18}(G)_5(GA)_2AA\ (GA)_3$ $(GGA)_2(GA)_3TA(GA)_2$	187–210	12	0.21	0.97	0.90	55	F: ATT ACA ACA TAG ACG GCA AGA T R: AAT CCC TGC TGC GGC TTA G	+
Nmac 13	$(AG)_{15}$	108–126	5	0.65	0.50	0.53	55	F: TGC TCG CCG CTT ATC CTT C R: TAG AAC ACC AGA TGC GTC GT	–
Nmac 14	$(T)_3(CT)_{10}(CCCT)_2(CT)_4TTCA(TC)_2$	151–165	5	0.47	0.49	0.62	50	F: TAT AAG ATT GAG AAT GTA TCG CT R: TGT AAT TCT TAG CTC TCG CAA C	+
Nmac 18	$(TC)_4TTTG\ (TC)_2(N)_9(TC)_{11}(AC)_4$ $TCTT(TC)_2$	282–302	7	0.54	0.78	0.66	50	F: CCA ATT CGT GCG TCC CCA T R: GGC GAG GGT TAT TTC TTA CG	+
Nmac 20	$CC(CCCT)_2(CT)_3A(C)_6TC(CT)_8CA(CT)_2$	198–204	3	0.85	0.31	0.27	55	F: TGG TAA AGC AAA TGT AAA GCC G R: AGA CTG GAA GGT GTG CTC G	–
Nmac 23	$(AG)_{15}$	286–292	4	0.32	0.78	0.74	55	F: TCG GCA AAG TGC GGT TGA GC R: CTC CCA CTG CTG AGT TGG TA	+
Nmac 28	$(C)_9(TC)_{11}TT\ (TC)_5$	156–186	10	0.60	0.64	0.63	55	F: AGA CCG TAT AAA ATT CGT TGA G R: ATA AAT CCT CGA AGG TGG CGA	+
Nmac 39	$(GA)_{14}$	206–222	12	0.21	0.94	0.89	55	F: GGC TCT CCA TTC TGA CGG TG R: GTC CCG AGA CAT GCA CAT AC	–
Nmac 43	$(CT)_{23}$	105–139	11	0.49	0.67	0.73	50	F: GTT CGT GGC AGC AGT CGG R: CTC CGT GCT TTC CAG AAC G	+
Nmac 45	$(GA)_{15}GG(GA)_{10}$	133–173	10	0.33	0.83	0.83	50	F: CGC TTT CAA ACC TGC TTC TG R: GCC ATA CCC TTT TAG AGA TAA C	+
Nmac 47	$(GA)_{24}$	291–321	11	0.22	0.82	0.88	50	F: GAT GTC GTT GGG TTC GTA TC R: GAA ACT TCG GCA GGG ACT C	+
Nmac 53	$(GA)_{18}$	308–328	6	0.42	0.67	0.76	55	F: ACA CAA GGC GAG CCA AAC G R: CCC CCT TTC CTC AAC TAC C	+
Nmac 115	$(CT)_3(AT)_2(CA)_{10}(CT)_{10}TTCT\ (CTTT)_4$	309–313	3	0.56	0.22	0.53	55	F: GCC ATT TAG TAT CGC CGT GTG R: CGG GGG TGA GTG ATT AAG CG	+

GenBank Accession numbers AF264862–264874, 264876.

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Characterization of microsatellite loci in the aflatoxigenic fungi *Aspergillus flavus* and *Aspergillus parasiticus*

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Aspergillus flavus and *Aspergillus parasiticus* are closely related, morphologically similar species belonging to *Aspergillus* section *Flavi*. Both *A. flavus* and *A. parasiticus* have a worldwide impact on agriculture due to their ability to produce aflatoxin. Contamination of crops poses a serious health risk, as aflatoxins are extremely potent hepatocarcinogens (Diener *et al*. 1987). The need to monitor and control aflatoxin levels

Table 1 PCR primer sequences, number of alleles, size range, discriminatory index, and observed heterozygosity for microsatellite loci

Locus	Repeat motif	Primer (5′–3′)	Size Range	No. of alleles		D§		H_O¶		GenBank accession no.
				A.f†	*A.p*‡	*A.f*	*A.p*	*A.f*	*A.p*	
AFPM1	$(CCA)_3(CTA)_4(CCA)_4$	CCCAGTCACGACCATTAC *GGTTCGTAGGTGGATAGAG	117–120	2	1	0.51	0	0.48	0	
AFPM2	$(ACT)_5T(CTC)_4$	CCACGCTCCTCAAATACG *CTGGACGGAGATCACGAC	206–266	7	6	0.85	0.79	0.81	0.74	APU52151
AFPM3	$(AT)_6AAGGGCG(GA)_8$	CACCACCAGTGATGAGGG *CCTTTCGCACTCCGAGAC	199–217	7	4	0.79	0.71	0.75	0.67	APU76621
AFPM4	$(CA)_{13}$	TCTTGCTATACATATCTTCACC *AGCGATACAGTTTTAACACC	179–206	5	2	0.73	0.26	0.70	0.24	AB010432
AFPM5	$(AG)_5AC(AG)_2$	CCATTATGACATGTGGTTAAGAG *TCCTACCCGAGAGAGTCTG	210–338	10	7	0.86	0.88	0.82	0.82	AF098293
AFPM6	$(GT)_6$	CTCAACGCAAGTCAGGTACGC *CGAAAGGCAGTTGTGAAGGC	341–355	4	4	0.62	0.37	0.59	0.35	ASNAMDR
AFPM7	$(AC)_{35}$	CAAATACCAATTACGTCCAACAAGGG *TTGAGGCTGCTGTGGAACGC	215–276	11	9	0.89	0.90	0.85	0.84	AF152374

*Fluorophor-labelled primer.
†*Aspergillus flavus*; ‡*Aspergillus parasiticus*; §numerical index of discriminatory power; ¶observed heterozygosity.

in food and feed means contamination is also a major economic concern in many countries.

Currently the methods used to control aflatoxin contamination are expensive and cannot guarantee the total elimination of toxins. A possible solution to the problem is the use of nontoxigenic isolates of *Aspergillus* to competitively exclude their toxigenic counterparts: a biological control strategy. Knowledge of genetic diversity, dispersal and potential for genetic exchange are essential for predicting the likely success of such a strategy. Small-scale studies of *A. flavus* and *A. parasiticus* populations carried out to date have used random amplified polymorphic DNA (RAPD) markers and DNA sequence data (Geiser *et al*. 1998; Tran-Dinh *et al*. 1999). Studies on a larger scale will require markers that are inexpensive and easy to apply, but also highly discriminatory and reproducible. We, therefore, set out to develop microsatellite markers that could be amplified from the genomes of *A. flavus* and *A. parasiticus*.

In this note, we characterized seven polymorphic microsatellite markers for *A. flavus* and *A. parasiticus*. To our knowledge, no microsatellite markers have been reported for *A. flavus* or *A. parasiticus*. We also examined the ability to amplify these loci from six other species of *Aspergillus*.

Microsatellites were found in GenBank using microsatellite motifs as queries for searches. Searches were performed on submitted sequences from *A. flavus* and *A. parasiticus*, and the two closely related species, *A. oryzae* and *A. sojae*. Six markers were found in this way (AFPM2-AFPM7). GenBank accession numbers are shown in Table 1. One microsatellite marker (AFPM1) was found by hybridizing DNA amplified from *A. flavus* and *A. parasiticus* by RAPD–PCR with chemiluminescent microsatellite probes (Carter *et al*. 1996). Hybridizing bands were identified, reamplified and sequenced.

Primers for polymerase chain reaction (PCR) amplification were designed from sequences flanking the microsatellites using OLIGO™ version 4.0 (National Biosciences) software. One primer from each pair was 5′-labelled with a fluorophor (PE Biosystems) (Table 1). All primer pairs were designed with an annealing temperature of 54 °C and amplified fragment sizes were optimized to allow analysis of all the microsatellite markers in a single lane. PCR amplifications were carried out in 25 μL reaction volumes using a Perkin-Elmer 2400 thermocycler. Reaction mixtures contained 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 200 μM each dNTP, 10 pmol of each primer, and 1 U of AmpliTaq DNA polymerase (Perkin Elmer). Each reaction contained approximately 20–40 ng of DNA, which was isolated using small-scale extraction protocol (Lee & Taylor 1990). Reactions were subjected to an initial denaturing step of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. Electrophoresis was conducted using an ABI 373 XL sequencer with GENESCAN version 3.0 (PE Biosystems) software. Fragment sizes were determined with reference to a TAMRA 500 (PE Biosystems) internal standard.

Microsatellite variability was analysed using 20 isolates of *A. flavus* and 15 isolates of *A. parasiticus* that have previously been found to be genetically diverse (Tran-Dinh *et al*. 1999). All of the microsatellite markers were reliably amplified from each isolate. The number of alleles, range of allele sizes, numerical index of discriminatory power (Hunter 1991) and observed heterozygosities for the two species are detailed in Table 1. All loci were polymorphic, with some showing higher degrees of polymorphism. Greater variation was seen within *A. flavus* than in *A. parasiticus*, which was consistent with our previous analysis using RAPD markers (Tran-Dinh *et al*. 1999). Amplification of the microsatellites was also attempted with DNA from *A. niger*, *A. carbonarius*, *A. tamarii*, *A. nomius*, *A. oryzae* and *A. sojae*. Only *A. oryzae* and *A. sojae* produced clear amplification products. These species are thought to

be domesticated variants of *A. flavus* and *A. parasiticus*, respectively. The microsatellite alleles amplified were consistent with this assumption (results not shown).

In conclusion, given the levels of polymorphism and the ease of amplification and analysis, the microsatellite markers presented here will be very useful for investigating the diversity and population structure of *A. flavus* and *A. parasiticus*.

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Identification of microsatellite loci in the water-rat *Nectomys squamipes* (Rodentia, Sigmodontinae)

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The water-rat, *Nectomys squamipes* (Cricetidae, Sigmodontinae), is a South American semiaquatic rodent that is well adapted to peridomiciliar habitats. This species is a primary host of *Schistosoma mansoni*, the prevalence of which may be as high as 90% in some natural rodent populations (Rey 1993). Because rodent fitness is not reduced by infection (D'Andrea *et al.* 2000), migrating rats, once infected, will carry the parasite and establish new infective foci in sites where secondary parasite hosts (*Biomphalaria* species) are present. These characteristics make the presence of water-rat populations a complicated factor for schistosomiasis control. Several populations of *N. squamipes* studied with random amplified polymorphic DNA (RAPD) showed limited differentiation indicating effects of migration or recent range expansion (Almeida *et al.*, in press). Microsatellites were developed as a tool for studying migration patterns of *N. squamipes* and for evaluating its potential in spreading infection.

Genomic DNA was digested with *Alu*I, and 200–700 bp fragments were excised and purified (QIAquick Gel Extraction, QIAGEN) following separation in low melting agarose gel electrophoresis. Size-selected fragments were ligated to a *Sma*I-digested and dephosphorilated pUC18 vector (Pharmacia) and transferred to *Escherichia coli* DH5α competent cells. Some 18 210 recombinant colonies were transferred to nylon membranes (NEN) following Sambrook *et al.* (1989). Nylon filters were hybridized with [γ^{32}P]-ATP labelled $(GT)_{10}$, $(CT)_{10}$, $(AGG)_7$, $(GAA)_7$ and $(GATA)_5$ oligonucleotides. A total of 11 224 colonies were hybridized with $(GT)_{10}$, 12 984 with $(CT)_{10}$, 8878 with $(AGG)_7$, 5981 with $(GAA)_7$, and 5981 with $(GATA)_5$. One hundred and eleven positive colonies were detected and 53 were sequenced with an ABI PRISM 377 automated sequencer using BigDye terminator labelling (Applied Biosystems). Thirty-three sequenced clones showed microsatellite repeats. Of the 28 well resolved sequences, all had $(CA)_n$ microsatellites motifs except for two, indicating that this was the most abundant in *N. squamipes*.

Eight microsatellites were characterized of which only five were polymorphic (Table 1). Genomic DNA of *N. squamipes* was extracted from blood or liver tissue of several water-rat populations by the standard proteinase-K/phenol–chloroform procedure (Sambrook *et al.* 1989). Polymerase chain reactions (PCR) were carried out in final volumes of 15 μL with ~10–40 ng of genomic DNA, 10 mM Tris-HCl pH 9.0, 50 mM KCl_2, 2.5 mM $MgCl_2$, 7 pmol of fluorescence labelled forward primer, 10 pmol of reverse primer, 300 μM of each dNTP and 1 U of *Taq* DNA polymerase (Pharmacia). PCR amplifications were performed using a thermal cycler (GeneAmp PCR System 9700 – PE Applied Biosystems) under the following conditions: an initial denaturation at 94 °C for 5 min followed by 30 cycles (except for *Nec15* and *Nec18* with 34 cycles) of 15 s at 94 °C, 30 s at T_a °C (Table 1), 30 s at 72 °C and a final extension period of 4 min at 72 °C. Fragment analyses were conducted with an ABI PRISM 377 with standard loading and electrophoresis conditions. Alleles were sized relatively to an internal size standard (ROX GS 500; Applied Biosystems) and analysed with GENESCAN version 2.1 (Applied Biosystems).

A maximum of 110 *N. squamipes* individuals was analysed for each locus (Table 2). Five microsatellites were polymorphic (Table 2) with the number of alleles ranging from 12–26. Linkage disequilibrium between all pairs of loci was not detected ($P > 0.5$ Fisher's exact test) when tested using GENEPOP version

Table 1 Motifs and primer sequences of eight microsatellite loci of *Nectomys squamipes*. F, Forward primer; R, Reverse primer. T_a °C, annealing temperature

Locus	Repeat motif	Primer sequences (5′ → 3′)	T_a °C	GenBank accession no.
Nec12	$(CA)_4T(CA)_{19}$	F: CTCCCTTCCCTCAATTTGCTGAGT	61	AF283417
		R: ACATGTGCAAAGCATGAAAATGGA		
Nec14	$(CA)_{24}$	F: CAGGCGATTTACACAAAAGAAT	57	AF283420
		R: CACTGAGCCATCTATCCAGTTC		AF283419
Nec15	$(AC)_{24}T(CA)_6$	F: AGGAAATGCTTATCTGGAGGAG	58	AF283422
		R: GACTCCTGATGTTGAACTGACC		AF283421
Nec18	$(CA)_{34}$	F: CTCTTTGAGGCCACTTCATTAA	58	AF283426
		R: GAACTAACATTTGCATCCTCCAG		AF283424
Nec28	$(CA)_{19}$	F: AGGAGAAAACCTGTATGCCATG	59	AF283428
		R: GTTTCTTCTTGCTGACCATGAGG		

Table 2 Genetic variation of eight microsatellite loci in *Nectomys squamipes*. *N*, number of examined animals; *A*, number of alleles per loci; Freq., frequency of the most common allele; H_O, observed heterozygosity; H_E, expected heterozygosity

Locus	*N*	*A*	Freq.	H_E	H_O	Allele range (bp)
Nec12	110	26	0.15	0.93	0.72*	206–242
Nec14	110	16	0.16	0.90	0.73***	204–236
Nec15	100	19	0.17	0.90	0.68***	171–213
Nec18	109	21	0.11	0.93	0.80**	128–170
Nec28	110	12	0.24	0.85	0.82	133–155

*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$. *P*-values obtained with Fisher's exact test for difference between H_E and H_O considering the null hypothesis of heterozygote deficiency.

3.2 (Raymond & Rousset 1995). Expected heterozygozity was significantly higher than observed heterozygosity for all but one locus (Table 2). Although this was probably a result of the Wahlund effect (Hartl & Clark 1997), and since samples were collected in eight different localities, the existence of null alleles cannot be ruled out until a more detailed population study can be performed.

The five polymorphic microsatellites loci, the first known for *Nectomys*, will be useful for assessing genetic variability within and among water-rat populations as well as for detecting differentiation and migration.

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A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*

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The pool frog, *Rana lessonae*, is broadly distributed in central Europe and often forms hybridogenetic, hemiclonal hybrids with the lake frog, *Rana ridibunda* (Blankenhorn 1977). These hybrids, known as *Rana* klepton *esculenta*, sexually parasitize either one or the other of the parental species, the most common form of which is the L-E system (*R. lessonae* LL × *R. esculenta* LR) (Graf & Polls-Pelaz 1989). In this system, hybrids transmit a clonal *R. ridibunda* haplotype by mating with a syntopic *R. lessonae*, while hybrid by hybrid crosses result in inviable offspring (Graf & Müller 1979; Uzzell *et al*.

1980). Hybrid lineages, therefore, represent frozen lineages that are assumed to be subject to an accumulation of deleterious mutations; mutations that are expressed when a hybrid by hybrid cross occurs and are suppressed when backcrosses with the parental species occur (Uzzell *et al*. 1980). The obvious lack of fitness benefits for *R. lessonae* individuals involved in LL × LR pairings make investigations of mate choice and sexual selection in this complex of great interest (Abt & Reyer 1993; Reyer *et al*. 1999). As well, pure *R. esculenta* populations have been detected, while theoretical investigations show that such pure populations cannot persist in isolation (Som *et al*. 2000). Even when a few *R. lessonae* are present, these generally are involved in hybrid matings due to the predominance of hybrids, which suggests that immigration by *R. lessonae* into such populations is required for population maintenance (Som *et al*. 2000; Hellriegel & Reyer in press). In these cases, management of pure or almost pure hybrid populations also requires identifying and managing *R. lessonae* source ponds.

With these and other applications in mind, we identified and characterized a suite of CA repeat microsatellite loci derived from *R. lessonae*. We constructed a highly enriched subgenomic library following standard protocols (Tenzer *et al*. 1999). A brief outline follows: genomic DNA isolated from a single male *R. lessonae* was digested to completion with *Tsp*509I (New England Biolabs) and the 500–1000 bp size fraction was isolated from LM-MP agarose (Boehringer Mannheim) using freezer phenol extraction. This size fraction was ligated to TSPADSHORT/TSPADLONG linkers (Tenzer *et al*. 1999) and amplified using TSPADSHORT and the polymerase chain reaction (PCR) as follows; total reaction volume was 25 μL and included 100 ng DNA, 1 U *Taq* polymerase (Quantum-Appligene), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% TritonX100, 0.2 mg BSA (Quantum-Appligene), 100 μM of each dNTP (Promega), and 1 μM of TSPADSHORT. PCR was performed on a Techne Genius thermocycler (Techne Ltd) using the following thermotreatment: 2 min at 72 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °. A total of 32 PCRs were carried out, pooled, cleaned and concentrated to minimize the likelihood of redundant products being detected during screening for positive clones (B. Gautschi *et al*. submitted). PCR products were hybridized to biotinylated CA(20) probes bonded to streptavidin-coated magnetic beads (Dynabeads M-280 Streptavadin, DYNAL, France) and amplified again. These final PCR products were cloned following the Original TA Cloning® Kit (Invitrogen) protocol. White colonies were dot-blotted onto nylon membranes (Hybond™-N^+, Amersham Pharmacia) and screened for CA repeats using the ECL 3′-oligolabelling and detection system (Amersham Pharmacia) and a 40mer CA oligonucleotide. All positive clones were sequenced using M13 forward and reverse primers, following the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit protocol, version 2.0 (PE Biosystems) and using the ABI 377 automated sequencing system (PE Biosystems). Primer design was carried out using primer 3 software (Rozen & Skaletsky 1998) and oligonucleotides were synthesized by Microsynth GmbH (Switzerland). Initial tests for amplification and polymorphism were done at 55 °C and electrophoresed on 8%, nondenaturing, 14.5 cm by 17 cm acrylamide gels at 80 V overnight. Those primers amplifying polymorphic products using five test templates (Table 1) were used for subsequent analyses reported below.

PCR amplification of frog DNA isolated from a sample of *R. lessonae* and *R. esculenta* adults captured and toe-clipped at a pond near Hellberg, north of Zürich, Switzerland was performed as follows. Reactions were 10 μL total volume and contained 50–100 ng template DNA, 0.5 U *Taq* polymerase (Quantum-Appligene), buffer components and dNTPs as listed above, and 0.5 μM of both forward and reverse primer. All PCR was performed using the following conditions: 3 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 57 or 58 °C, and 30 s at 72 °C, followed by a final step of 2 min at 72 °C. Products were electrophoresed on Spreadex™ gels, either EL-300 or EL-500 (Elchrom Scientific AG, Switzerland), depending on the size of the alleles generated. All electrophoresis was performed using the SEA 2000™ advanced submerged gel electrophoresis apparatus (Elchrom Scientific AG, Switzerland) at 100 V for 60–120 min, depending on allele size, then scored against the M3 Marker ladder (Elchrom Scientific AG, Switzerland) and a 20-bp ladder (Bio-Rad). Expected and observed counts for homozygotes/heterozygotes were determined using GENEPOP, version 3x (Raymond & Rousset 1995) and tested for significant deviations using Chi-square analysis (null hypothesis rejected at $P < 0.05$).

All 10 loci were variable in *R. lessonae* and as well in *R. esculenta* (data not shown). Locus RlCA1b5 amplified an allele 137 bp in length only in *R. esculenta* individuals and is most likely the clonally transmitted *R. ridibunda* allele (data not shown). Loci RlCA1b17, RlCA1b20, RlCA1b27, RlCA18, RlCA19 and RlCA31 all appear to amplify only a single allele in a sample of *R. esculenta* tested in two other populations not reported here. Only *R. lessonae* frogs were used to test for homozygote excess, for obvious reasons. Loci RlCA5, RlCA1b17, RlCA1b20 and RlCA2a49 all exhibited homozygote excess ($P \geq 0.05$), which may indicate the presence of at least one null allele at each of these loci. Considering the bizarre nature of the LE complex, these homozygote excesses may instead be indicative of a departure from Hardy–Weinberg due to a violation of the assumption of random mating.

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Table 1 10 CA repeat microsatellite loci developed for *Rana lessonae*. All data based on PCR analysis of 25 *R. lessonae* individuals. T_a, annealing temperature; H_O, observed number of homozygotes; H_E, unbiased average heterozygosity estimate (Nei 1978). Both size and repeat motif are based on that detected in the original sequenced clone (GenBank Accession nos: AF286384–93)

Locus	Primer Sequences (5′–3′)	Repeat motif	T_a (°C)	No. alleles	Size (bp)	H_O	H_E
RlCA1	AAATGCAAGCGTCCCAATAC GGACGCAGTTTCTGGATTTG	$(CA)_{16}$	58	10	110	0.20	0.832
RlCA5	CTTCCACTTTGCCCATCAAG ATGTGTCGGCAGCTATGTTC	$(CA)_{17}$	58	6	250	0.52	0.678
RlCA18	CTCTGCTCCCTCAGCTATGC AAAAAGTGGTCCTTTCATTTTGAG	$(CA)_{22}$	57	5	177	0.48	0.573
RlCA19	GTCTGTCCGTGTGCAGAGAG CAAGTGATTGAGAGCCTCAGC	$(CA)_{15}$	57	2	129	0.52	0.490
RlCA1b5	CCCAGTGACAGTGAGTACCG CCCAACTGGAGGACCAAAAG	$(CA)_{17}$	58	3*	145	0.56	0.476
RlCA1b17	TAAACCTTAAAAGTGGTTATAAAAACC GTAAGTGTTAGGGATGCTGAGG	$(A)_8(CAAA)_2(CA)_{16}$	57	8	134	0.44	0.742
RlCA1b20	GGGCAGGTATTGTACTCAATATCAC CAACACAAGGACTCCACTGC	$(CA)_8(C)_{13}$	57	4	87	0.72	0.506
RlCA2a49	TGTCCACATTAAGGAACTTTTGC TTCAGAGATCAGGGGTCTCC	$(C)_8(A)_2(CA)_{15}CG(CA)_4$	57	5	200	0.48	0.710
RlCA1b27	GTAAGTGTTAGGGATGCTGAGG TAAACCTTAAAAGTGGTTATAAAAAGG	$(CA)_{15}(CAAA)_3(A)_5$	58	6	134	0.48	0.644
RlCA31	GAAGCTTAAACCACTTGACCAAC TCCCTTTTTCAGGTCTTTGG	$(C)_4A(C)_5GACAAA$ $CATA(CA)_6TA(CA)_5$	58	3	98	0.44	0.640

*Third allele detected at this locus only amplifies in *R. esculenta* and is not included in enumerations of H_O, and calculations of H_E and homozygote excess (see text for last).

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Characterization of microsatellite and minisatellite loci in Atlantic salmon (*Salmo salar* L.) and cross-species amplification in other salmonids

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The Atlantic salmon (*Salmo salar*) is a salmonid fish species which naturally inhabits cool rivers and oceans of the Northern hemisphere. It is of considerable economic importance, both for recreational fishing and as a major aquaculture species. Novel polymorphic genetic markers are in continual demand to extend familial and population genetic studies in this species. We report here on the identification of 'higher order' (tri- and tetranucleotide) Atlantic salmon microsatellites.

A number of different size-selected Atlantic salmon genomic DNA libraries were constructed, employing a microsatellite enrichment methodology (comprehensively described by Kijas *et al*. 1994). This protocol uses biotinylated microsatellite motif

Table 1 Repeat motif, PCR primer sequences, optimal annealing temperature (T_a), $MgCl_2$ concentration for amplification and preliminary population characteristics (based on 21 individuals) for 20 polymorphic Atlantic salmon microsatellite and minisatellite loci

Locus	Repeat motif of original clone†	Primer Sequence (5′–3′)‡	PCR Conditions		Allele size range (bp)	No. of alleles	Heterozygosity§			EMBL Accession no.
			T_a (°C)	$MgCl_2$ (mM)			H_O	H_E	H–W	
Ssa401UOS	$(GACA)_{38}$	* f: ACTGGTTGTTGCAGAGTTTGATGC	64	1.5	230–340	20	0.86	0.92	ns	AJ402718
		r: AAACATACCTGATTCCCGAACCAG								
Ssa402/1UOS¶	$(GA)_{55}$	* f: GCTTTGGCAATGCATGTGGTAAT	64	0.9	206–246	10	0.95	0.84	ns	AJ402719
/2UOS		r: CCTATCCCTGTTGTTGCTGAC			154–172	5	0.57	0.70	ns	
Ssa403UOS	$(GACA)_{28}(N)_{39}(GT)_{56}$	* f: CTTTAGAAGACGGCTCACCCTGTA	62.5	1.0	152–252	18	1.00	0.94	ns	AJ402720
		r: GCTACTTCGTACTGACTGCCTCA								
Ssa404UOS	$(GACA)_{27}$	* f: ATGCAGTGTAAGAGGGGTAAAAAC	59	0.9	194–314	20	0.95	0.94	ns	AJ402721
		r: CTCTGCTCTCCTCTGACTCTC								
Ssa405UOS	$(GACA)_{34}$	* f: CTGAGTGGGAATGGACCAGACA	62	1.0	302–405	16	0.95	0.95	ns	AJ402722
		r: ACTCGGGAGGCCCAGACTTGAT								
Ssa406UOS	$(GA)_{18}C(GGAC)_5A(GACA)_4$	* f: ACCAACCTGCACATGTCTTCTATG	62	1.0	322–520	12	0.71	0.82	ns	AJ402723
		r: GCTGCCGCCTGTTGTCTCTTT								
Ssa407UOS	$(GACA)_{37}$	* f: TGTGTAGGCAGGTGTGGAC	61	1.0	176–304	15	0.95	0.93	ns	AJ402724
		r: CACTGCTGTTACTTTGGTGATTC								
Ssa408UOS	$(GACA)_{27}$	* f: AATGGATTACGGGTACGTTAGACA	62	1.5	248–340	16	0.90	0.93	ns	AJ402725
		r: CTCTTGTGCAGGTTCTTCATCTGT								
Ssa410UOS	$(GACA)_{22}$	* f: GGAAAATAATCAATGCTGCTGGTT	58	1.0	198–324	25	0.90	0.97	ns	AJ402727
		r: CTACAATCTGGACTATCTTCTTCA								
Ssa411UOS	$(CT)_{70}$ inc. interspersed $(GT)_1$	* f: TCCGCACAGACCAGAAGAACG	62	1.0	290–294	2	0.20	0.18	ns	AJ402728
		r: AGGGGAGACCGCGAGTGAGA								
Ssa412UOS	$(GA)_7GG(GA)_{10}GG(GA)_{20}$	* f: GTGGAGATACACAGCACTTA	62	1.0	246–252	3	0.56	0.51	ns	AJ402729
		r: CACCCCTCCGTTTTATCAC								
Ssa413/1UOS¶	$(ATT)_2G(TTA)_4(GTA)_3(N)_{65}(ATT)_7$	* f: GTAGACGCCATCGGTATTGTG	64	0.9	234	1	0.00	0.00		AJ402730
/2UOS		r: CGTGATGCCGCTGTAGACTTG			214–234	5	0.71	0.75	ns	
Ssa416UOS	$[90\ bp\ MS]_5$	* f: TGACCAACAACAAACGCACAT	63	0.9	214–400	6	0.67	0.66	ns	AJ402733
		r: CCCACCCATTAACACAACTAT								
Ssa417UOS	$(GATA)_{114}$	* f: AGACAGGTCCAGACAAGCACTCA	60	1.0	265–424	24	0.95	0.96	ns	AJ402734
		r: ATCAAATCCACTGGGGTTATACTG								
Ssa418UOS	$(GATA)_{59}$	* f: CACACCTCAACCTGGACACT	64	1.0	328–570+	22	1.00	0.96	ns	AJ402735
		r: GACATCAACAACCTCAAGACTG								
Ssa419UOS	$[86\ bp\ MS]_3$	* f: GGTCGTATCGCGTTTCAGGA	64	1.0	314–406	3	0.19	0.18	ns	AJ402736
		r: TGCTGCAATAAAGAGATGCTTGTT								
Ssa420/1UOS¶	$(CA)_5T(GACA)_{21}$	* f: GCAGGAGAGTCGCTACAG	63	1.0	164–700	22	0.48	0.96	***	AJ402737
/2UOS		r: GATCTATGCCCACAAACAG			142	1	0.00	0.00		
Ssa421UOS	$(GACA)_{24}(GAGACA)_{10}(GA)_4$	f: CAGGGTCTGTGGTGGACTGTTC	60	1.3	282–370	18	0.95	0.94	ns	AJ402738
		* r: CGTTTGCACATTGTGAGGTGTC								
Ssa422UOS	$(GA)_3(GT)_2(GA)_6GGG(GA)_{20}$	f: TTATGGGCGTCCACCTCTGACA	60	1.1	194–220	10	0.86	0.87	ns	AJ402739
		* r: CACCCCAGCCTCCTCAACCTTC								

†N, any nucleotide; [25 bp MS], minisatellite with 25 bp repeat motif.

‡f & r are forward and reverse primers, respectively; *indicates isotopically labelled primer.

§H_O, observed heterozygosity; H_E, expected heterozygosity (Nei's unbiased); H–W, test for conformance to Hardy–Weinberg equilibrium (ns, not significant, $P > 0.05$; ***$P < 0.001$). Pseudo-exact tests performed using GENEPOP v3.1 population genetics software (Raymond & Rousset 1995).

¶Two loci detected — presumed duplicate pair reflecting the tetraploid origin of the salmonid genome (Ohno 1970).

Table 2 Cross amplification of 19 Atlantic salmon derived primer sets in seven other salmonid species, as determined by agarose gel electrophoresis. + or (+) indicates detection of a discrete fragment of appropriate size at 1 °C or 5 °C below Atlantic salmon optimum annealing temperature, respectively; – indicates absence or smeared product. Additionally, P indicates polymorphism confirmed by isotopic screening (*Salmo trutta* and *Oncorhynchus mykiss* individuals only); see text for details

Primer set	*Salmo trutta*	*Oncorhynchus mykiss*	*Oncorhynchus clarki*	*Oncorhynchus nerka*	*Salvelinus alpinus*	*Coregonus lavaretus*	*Thymallus thymallus*
Ssa401UOS	(+)	–	–	–	–	–	–
Ssa402UOS	+ P	+ P	+	+	+	+	+
Ssa403UOS	+ P	+ P	+	–	+	+	–
Ssa404UOS	+	–	–	–	+	–	–
Ssa405UOS	+	+	+	–	+	–	–
Ssa406UOS	+ P	+	+	+	+	+	+
Ssa407UOS	+ P	+ P	+	+	+	–	+
Ssa408UOS	+ P	+ P	+	+	+	+	(+)
Ssa410UOS	+ P	+ P	+	+	+	+	–
Ssa411UOS	–	–	–	–	–	–	–
Ssa412UOS	+	+	+	+	+	+	–
Ssa413UOS	+ P	+ P	+	(+)	+	+	(+)
Ssa416UOS	+ P	+	+	–	+	–	–
Ssa417UOS	+ P	+ P	+	+	+	+	+
Ssa418UOS	+ P*	+ P*	+	+	+	+	+
Ssa419UOS	+ P	–	+	(+)	+	–	–
Ssa420UOS	+ P	–	+	(+)	+	+	–
Ssa421UOS	–	–	–	–	–	–	–
Ssa422UOS	+ P	(+)	+	–	+	+	+

*Two loci detected.

sequences bound to streptavidin-coated magnetic particles as the basis for enrichment. The libraries were constructed using dephosphorylated pBluescript II KS(–) phagemid vector (*Bam*HI or *Eco*RV digested), Epicurian Coli XL2-Blue ultracompetent host cells (Stratagene) and size selected (≈200–500 bp) restriction-digested Atlantic salmon genomic DNA. One library was constructed using *Mbo*I digested DNA fragments which was enriched for $(GACA)_n$ sequences. Additional libraries were made using blunt-ended restriction digested DNA (pooled from separate *Alu*I, *Hae*III and *Rsa*I digests). These libraries were potentially enriched for $(GACA)_n$, $(GATA)_n$, $(TAA)_n$, and $(AAGG)_n$ motifs. Microsatellite containing recombinant clones were identified by ordered array screening of the libraries (Armour *et al*. 1994). Recombinant DNA was fixed onto Hybond-N membrane (Amersham) and hybridized with isotopically [γ^{32}P]-ATP end-labelled target oligonucleotide [$(TAA)_6$, $(GACA)_4$, $(GATA)_4$ or $(AAGG)_4$]. Hybridization was performed overnight in 6× SSC (20× SSC is 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0), 0.1% SDS (sodium dodecyl sulphate), 42 °C with subsequent washes to a stringency of 5× SSC, 0.1% SDS, 42 °C for 30 min. Following autoradiography, clones exhibiting strong signal were sequenced (ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit and ABI 377 mediated automated detection) and, where possible, appropriate polymerase chain reaction (PCR) primers were designed (assisted by Primer Select software, DNASTAR Inc.).

Characterization of primer sets involved both nonisotopic and isotopic screening. Atlantic salmon DNA was extracted from liver tissue by a rapid phenol-based method (Taggart *et al*. 1992). Common components in both assays (10 μL final volume) were: 50 ng template DNA, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 0.9–1.5 mM $MgCl_2$, 130 μM each nucleotide and 0.5 U *Taq* DNA polymerase (Promega). For nonisotopic PCRs 1 μM of each primer was included. For isotopic assays 0.1 μM of each primer was added with 10% of one primer being end-labelled with [γ^{32}P]-ATP (4500 Ci/mMol). Cycling parameters, using a Hybaid TouchDown thermocycler, were: 96 °C for 3 min, four cycles of 95 °C for 50 s, *xx* °C annealing for 50 s, 72 °C for 50 s and *n* cycles of 94 °C for 50 s, *xx* °C annealing for 50 s, 72 °C for 50 s, where *xx* is locus specific annealing temperature (Table 1) and $n = 28$ for nonisotopic or 25 for isotopic reactions. Non-isotopic products were resolved on ethidium bromide stained 1.4% agarose gels while isotopic products were separated on 50 cm long denaturing polyacrylamide gels (SequaGel XR; National Diagnostics) followed by autoradiography. Allele sizes were determined relative to pBluescript II KS(–) sequence reactions run on each gel.

Level of variability at each identified locus was assessed in 21 wild adult salmon. Polymorphic loci were also screened in two Atlantic salmon families (each consisting of two parents +46 progeny). Cross-species amplification was assessed in seven other salmonid species (Table 2). Two individuals of each species were screened (nonisotopically) using two different annealing temperatures (1 °C and 4 °C below optimum for Atlantic salmon) and 1.5 mM $MgCl_2$. Additionally four brown trout (*Salmo trutta*) and two rainbow trout (*Oncorhynchus mykiss*) were screened for polymorphism using identical isotopic conditions to those employed for Atlantic salmon.

Of 164 clones sequenced, 144 had identifiable repeat motifs. While most (87%) were the expected target repeats both

dinucleotide microsatellites (5%) and minisatellites (8%) were also identified. Clones containing $(AAGG)_n$ showed few consecutive repeat units (2–3) and these were invariably found within larger minisatellite motifs. Identified $(TAA)_n$ microsatellites comprised relatively low numbers of repeats (n = 4–15) while both $(GACA)_n$ and $(GATA)_n$ repeats were much larger (n = 10–100+ ; Table 1). Forty-two primer sets could be designed that flanked micro- or minisatellite sequences. Twenty-two sets gave discrete products on nonisotopic testing with Atlantic salmon samples and were further optimized for isotopic screening.

Of 25 loci amplified, 20 were detected as being polymorphic (Table 1). Inheritance studies confirmed disomic segregation of alleles at each locus. Length mutations were observed for two loci (Ssa404UOS, one allele; Ssa417UOS, three alleles; out of 184 progeny alleles assayed). Presence of a high frequency null allele at Ssa420UOS, suggested from population data (Table 1), was confirmed in both pedigrees screened. Furthermore, joint segregation statistics identified four significant linkage associations (P < 0.01): Ssa402/1UOS with Ssa403UOS; Ssa402/2UOS with Ssa404UOS; Ssa407UOS with Ssa422UOS; and Ssa408UOS with Ssa413UOS. Many of the primer sets are potentially informative for other salmonid species (Table 2).

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Isolation and characterization of microsatellite loci in the orchid *Ophrys araneola* (Orchidaceae) and a test of cross-species amplification

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The orchid genus *Ophrys* shows a highly specialized pollination system in which flowers deceive male hymenopterans by imitating females. Pollination occurs when males attempt to mate with the labellum of the flowers. This interaction, known as sexual deception, is assumed to be highly specific

Table 1 Characteristics of seven microsatellite loci of *Ophrys araneola*. Data are based on two populations, one from Switzerland (CH), and one from France (FR). †labelled primer; ‡labelled dNTPs; T_a, locus specific annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; *significant heterozygote deficiency (P < 0.05). Repeat motifs and PCR-product lengths are derived from the sequenced clone

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Size (bp)	Total no. of alleles	Population (no. of individuals)	H_O	H_E	Accession number
OaCT1	F: TCGTGCTACATAGGAAGGCAAATC†	$(CT)_{20}$	50	168	7	CH (24)	0.375	0.370	AF277788
	R: AGTCTCCAAACGGCACCCAG					FR (19)	0.684	0.634	
OaCT2	F: GCCAACCCCTTGGAGAAAGC†	$(CT)_{31}TT(CT)_5$	58	201	15	CH (23)	0.700	0.793*	AF277789
	R: CAAGCTCGCTCCTTTAACTCGC					FR (19)	0.789	0.910	
OaCT3	F: ATAGAGGCGGTCTCCTTCAAGTCG†	$(CT)_{19}$	58	171	7	CH (24)	0.750	0.714	AF277790
	R: CAGTGACGAACTCATGCTCTCCAG					FR (17)	0.529	0.745	
OaCT4	F: CACGTCGGTGCCTCATTTAC†	$(CT)_{16}AT(CT)_5(ATCT)_5$	50	158	14	CH (24)	0.917	0.872	AF277791
	R: TGAGTCGATATGAATAACCTGCC					FR (19)	0.737	0.872	
OaCT5‡	F: AGCATTGGAGGCATATCCGAC	$(CT)_{28}AA(CT)_8AA(CT)_4$	49	164	16	CH (24)	0.833	0.772	AF277792
	R: CGTGCTTTGTGATTTTTGGCG					FR (19)	0.789	0.899*	
OaCT6	F: GGTTTGTGGTTGTTGTTTGCG†	$(CT)_{28}$	58	186	14	CH (23)	0.478	0.769*	AF277793
	R: AAGCTCCTCCAATGGAACCTTC					FR (19)	0.368	0.765*	
OaCT7	F: GCACTGAGGTTGTATGCTGAGAGG†	$(CT)_{25}$	58	195	22	CH (24)	0.667	0.775	AF277794
	R: GCTCGGATTGTGATTCCAAGC					FR (19)	0.895	0.940	

Species	Locus						
	OaCT1	OaCT2	OaCT3	OaCT4	OaCT5	OaCT6	OaCT7
Ophrys aveyronensis	+	+	+	+	+	+	+
Ophrys fuciflora	+	+	–	+	+	+	+
Ophrys insectifera	+	+	–	+	+	–	+
Ophrys lutea	–	–	–	–	–	–	–
Ophrys sphegodes	–	+	+	+	+	+	+
Ophrys tenthredinifera	–	+	–	–	–	+	+

Table 2 Cross-species amplification with *Ophrys araneola* microsatellite primers. +, successful amplification (qualities 1–3 of Smulders *et al*. 1997); –, no amplification (qualities 4 and 5 of Smulders *et al*. (1997)

due to the imitation of sexual pheromones (Schiestl *et al*. 1999). To study the influence of this specialized pollination system on genetic population structure and to estimate gene flow among morphologically similar, coflowering *Ophrys* species with presumably different pollinators, variable and codominant genetic markers are needed. We, therefore, isolated and characterized microsatellite loci from *Ophrys araneola* and tested their variability in two *O. araneola* populations. Furthermore, we assessed whether these loci can be amplified in other *Ophrys* species.

Genomic DNA was extracted from leaf material stored in silica gel using the CTAB protocol (Doyle & Doyle 1990). Microsatellite loci were isolated and identified from a partial genomic library enriched for GA/CT repeats, following Tenzer *et al*. (1999). Enriched DNA was ligated into pGEM®-T vector and cloned using JM109 high efficiency competent cells (Promega). The 672 colonies with inserts were blotted onto nylon filters (Hybond-N$^+$, Amersham Pharmacia Biotech) and screened for GA/CT repeats using the ECL 3′-oligolabelling and detection systems (Amersham Pharmacia Biotech). Plasmid DNA of 74 positive clones was purified with the GFX™ *Micro* Plasmid Prep Kit (Amersham Pharmacia Biotech). Cycle-sequencing reactions were performed with BigDye terminator chemistry (PE Biosystems) using the universal primers pUC/M13 forward and pUC/M13 reverse, and run on an ABI Prism 310 Genetic Analyser. Microsatellite motifs were found in 51 clones. Primers annealing to flanking regions were designed for 21 loci using MacVector™ 6.0 (Oxford Molecular LTD). Polymerase chain reactions (PCRs) were performed in 10 μL reaction volumes containing 4.5 μL ddH_2O, 10 mM Tris-HCl, 50 mM KCl, 0.4 μM of each forward and reverse primer, 200 μM of each dNTP, 0.5 U AmpliTaq Gold® DNA Polymerase (PE Biosystems), 1.5 mM $MgCl_2$ and 10–50 ng of DNA. PCRs were run on a Perkin-Elmer GeneAmp PCR System 9700 thermocycler. An initial denaturation step (95 °C, 10 min) was followed by 35 cycles of 30 s at 95 °C, 30 s at the locus specific annealing temperature (see Table 1), 30 s at 72 °C; a final extension step for 5 min at 72 °C was performed at the end. Products were visualized on an ABI PRISM 310 Genetic Analyser (PE Biosystems) using either labelled primers or labelled nucleotides (Table 1). Allele sizes were scored against the internal GeneScan-500 (ROX) size standard (PE Biosystems) and individuals were genotyped using GeneScan Analysis® 3.1 and Genotyper® 2.1 software (PE Biosystems). Seven out of 21 primer pairs amplified fragments of the expected size.

Levels of variability detected at these seven loci are high, with numbers of alleles ranging from 7–22 and observed heterozygosities ranging from 37–92% (Table 1). Using GENEPOP 3.1c (Rousset & Raymond 1995) we found a significant heterozygote deficiency ($P < 0.01$) for loci OaCT2 and OaCT6 in the Swiss *O. araneola* population, and for OaCT5 and OaCT6 in the French population. Loci OaCT1 and OaCT7 exhibit significant linkage disequilibrium across both populations ($P < 0.01$).

We tested for cross-species amplification of *O. araneola* primers with six other *Ophrys* species of increasing phylogenetic distance to *O. araneola* (M. Soliva *et al*., unpublished results), using two individuals per species. PCR conditions were the same as described above. Qualities of PCR products were classified according to Smulders *et al*. (1997). Amplification products of qualities 1–3 were regarded as successful cross-species amplifications, whereas products of qualities 4 and 5 were treated as failure. Microsatellite loci were successfully cross-amplified with *Ophrys aveyronensis*, partially with *O. sphegodes*, *O. fuciflora*, *O. insectifera*, and *O. tenthredinifera* and failed to cross-amplify with the more distantly related *O. lutea* (Table 2).

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Microsatellite DNA loci suitable for parentage analysis in the yellow-pine chipmunk (*Tamias amoenus*)

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The yellow-pine chipmunk (*Tamias amoenus*) exhibits female-biased sexual size dimorphism (Schulte-Hostedde & Millar 2000), and an understanding of the evolution and/or maintenance of this dimorphism requires the determination of individual reproductive success. DNA-based genetic markers are necessary for assigning parentage to quantify reproductive success in promiscuous mating systems, such as chipmunks (Callahan 1981). Here, we: (i) characterize primers for 11 microsatellite loci suitable for parentage studies of yellow-pine chipmunks; and (ii) assess variation in loci derived from Columbian ground squirrels (*Spermophilus columbianus*) which produce amplification products in the least chipmunk (*Tamias minimus*) (Stevens *et al*. 1997).

We extracted DNA from the kidney of a yellow-pine chipmunk taken from the Kananaskis Valley, Alberta and constructed a plasmid library consisting of 250–400 bp fragments using the method described by Dawson *et al*. (1997). Briefly, approximately 10 µg of DNA was digested and fragments containing 250–400 bp were purified from an agarose gel and cloned into a plasmid vector. The library was transformed into XL1-Blue (Stratagene) competent cells and plate lifts made using Hybond-N (Amersham-Pharmacia) nylon membranes. Approximately 50 000 colonies were screened using two dinucleotide polymers [$(TG)_n$ and $(TC)_n$ (Amersham-Pharmacia) labelled with ^{32}P-dCTP] and 165 positive clones were identified. Twenty-five clones, each containing a single insert, were sequenced by MOBIX Central Facility, McMaster University, using dye-terminator chemistry on an ABI 373 A Stretch DNA sequencer. Primers to amplify regions containing repeats were designed from 17 clones using PRIMER (version 0.5; Lincoln *et al*. 1991); however, only 11 of these primer pairs were sufficiently variable for parentage studies (i.e. ≥3 alleles). To assess variability of these 11 loci, we used DNA from ear tissue collected from 76 chipmunks (43 adults, 33 juveniles) in the Kananaskis Valley in 1999. DNA was extracted using QIAGEN® QIAmp tissue kits. Polymerase chain reaction (PCR) was performed on the samples generally following Dawson *et al*. (1997) on a 480 Perkin-Elmer DNA Thermal Cycler, with the following changes: after the

Table 1 Primer sequences, repeat motif, PCR product size for clone, annealing temperature (T_a), number of alleles among 43 adults surveyd, observed (H_O) and expected (H_E) heterozygosity, and GenBank accession nos for microsatellite loci of the yellow-pine chipmunk. F and R are the forward and reverse primer, respectively

Primer	Primer sequence	Repeat	Clone size	T_a	No. of Alleles	H_O	H_E	GenBank
EuAmMS 26	**F** 5′ ACA GGA ACA GCA GAT TGT TGT 3′ **R** 5′ CAC TGT TTG CCT GTG AAG AG 3′	$(CA)_{20}$	181 bp	55 °C	4	0.605	0.551	AF255957
EuAmMS 35	**F** 5′ ATC CGT TTA GTC TGT TAT GTC TCA 3′ **R** 5′ TTT AAT CTA AAG GAC AAC AAT TGC 3′	$(TG)_{12}$	139 bp	55 °C	5	0.674	0.657	AF255958
EuAmMS 37	**F** 5′ CCT GGG AGA AAA TAC TTG GAT G 3′ **R** 5′ AGA AAT GAG GGC AGG GAT AAT T 3′	$(GA)_{17}$	134 bp	55 °C	3	0.488	0.506	AF255959
EuAmMS 41	**F** 5′ ATT CAG GCT CCA GAA AAA CAA A 3′ **R** 5′ TCT GCC CCA GAG ATA TTG ATC T 3′	$(GT)_{16}$	143 bp	54 °C	5	0.721	0.715	AF255960
EuAmMS 86	**F** 5′ AAA GAA TGT GCA GCA AAC CTG 3′ **R** 5′ TTC AAT CCT TTC TAG TGC TCT TCC 5′	$(AC)_{21}$	159 bp	55 °C	5	0.465	0.533	AF255961
EuAmMS 94	**F** 5′ TGG CTC AGT TTT TCA GTT TTT 3′ **R** 5′ ATC TCA AAG CCA TCA AGA GTT T 5′	$(GT)_{14}$	104 bp	51 °C	4	0.279	0.282	AF255962
EuAmMS 108	**F** 5′ TCC CAA CAA CCT CTC TTG ATG 3′ **R** 3′ AAC TTG AAA ATT TTC TTC TGG GC 3′	$(GT)_{10}$	182 bp	53 °C	4	0.651	0.634	AF255963
EuAmMS 114	**F** 5′ CTC AGT CTC CCC AAA CAT TG 3′ **R** 5′ TAG TTC AGT GGT AGG GCA TTC 3′	$(CT)_{21}$	159 bp	53 °C	8	0.860	0.745*	AF255964
EuAmMS 138	**F** 5′ AAT GTA TGC TAG AGT GCC CAC C 3′ **R** 5′ TTT TCT AGA GAC ACA AAA ATT TAG CA 3′	$(AC)_{19}$	128 bp	54 °C	5	0.581	0.694	AF255965
EuAmMS 142	**F** 5′ CTG TGG CGG TCT TAT CTG TAT G 3′ **R** 5′ CCA GTT ACA GCC AGA ACC ACT T 3′	$(CT)_{14}(CA)_{14}$	120 bp	53 °C	4	0.814	0.698	AF255966
EuAmMS 163	**F** 5′ GCC CAT CAA TAG TTG AAT GGA TA 3′ **R** 5′ CCT GGA AAT GCC ATA ATT TTA TTC 3′	$(TC)_6G(TC)_5G(TC)_9(AC)_{20}$	169 bp	60 °C	9	0.710	0.642	AF255967

*indicates a significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

initial denaturing step at 94 °C for 3 min, 32 PCR cycles were performed consisting of 45 s at 94 °C, 45 s at the appropriate annealing temperature, and 45 s at 72 °C. Amplification products were resolved on polyacrylamide gels, as described in Dawson *et al*. (1997) except gels were run at 70 W. PCR reactions (1 μL volume) consisted of the following reagents; 2.5 mM of $MgCl_2$ (MBI Fermentas), PCR buffer [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween (MBI Fermentas)] 1 μg/μL BSA (Amersham-Pharmacia), 200 μM dNTP's, 0.25 U *Taq* DNA polymerase (MBI Fermentas), 0.2 pmol of the forward primer labelled with [γ-^{33}P]-ATP (Amersham-Pharmacia), 0.3 pmol of the unlabelled forward primer, and 0.5 pmol unlabelled reverse primer. Table 1 describes the primer sequence, size of clone product, annealing temperature, and number of observed alleles for each locus. We determined whether there were deviations from Hardy–Weinberg equilibrium for each locus from adult chipmunks using GENEPOP (Raymond & Rousset 1995). Only EuAmMS 114 was found to deviate from Hardy–Weinberg expectation due to heterozygote excess (Table 1).

To assess the utility of these microsatellite loci for parentage analysis, we used the likelihood-based approach and simulation procedures of CERVUS 1.0 (Marshall *et al*. 1998). Using this program, we were able to assign maternity to all 33 juveniles (100%) with 80% confidence, 18 (54.5%) of these with 95% confidence. Using known maternity data, we were able to assign paternity to 30 juveniles (90.9%) with 80% confidence, 20 (60.6%) of these with 95% confidence. The microsatellite loci presented here provide adequate information to assess parentage in yellow-pine chipmunks.

We also attempted to amplify samples of yellow-pine chipmunk DNA using four primers which amplify DNA from Columbian ground squirrels and least chipmunks (Loci: GS3, GS17, GS20, and GS34) (Stevens *et al*. 1997). Only two alleles were observed among samples from 22 chipmunks for GS22. At a low-stringency annealing temperature (50 °C) we found only GS20 to amplify, producing one allele. These primers were not considered to be appropriate for further parentage analysis.

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Polymorphic di-nucleotide microsatellite loci isolated from the humpback whale, *Megaptera novaeangliae*

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The study of cetaceans by genetic methods is moving increasingly towards the estimation of kinship among individuals using genetic data. Microsatellite loci are ideal for this kind of study given their high mutation rates and co-dominant inheritance. However, a large number of loci need to be genotyped in order to ensure reliable estimation of kinship. Even for relatively small sample sizes, reliable identification of parent–offspring pairs is likely to require more than 17 loci genotyped in each individual (Palsbøll 1999). Towards this end, we isolated an additional nine polymorphic microsatellite loci from genomic DNA of the humpback whale, *Megaptera novaeangliae*, which are presented here.

The loci originate from the same partial genomic library from which we previously presented tri- and tetra-nucleotide microsatellite loci (Palsbøll *et al*. 1997). In this paper, we present additional di-nucleotide loci identified among the positive clones in the above-mentioned genomic library. The isolation and sequencing of clones containing inserts has been described previously (Palsbøll *et al*. 1997). The data presented here are based upon genotypes obtained from up to 353 individual humpback whales, 65 individual fin whales (*Balaenoptera physalus*), 169 individual minke whales (*B. acutorostrata*) and 92 individual blue whales (*B. musculus*).

Total-cell DNA was extracted from skin biopsies by standard phenol and chloroform extractions (Sambrook *et al*. 1989) and the DNA re-suspended in 1 × TE (Sambrook *et al*. 1989). The nucleotide sequence at each locus was amplified by polymerase chain reaction (PCR) (Mullis & Faloona 1987) using 10 μL reaction volumes, each containing 10 ng of genomic DNA, 67 mM Tris–HCl, pH 8.8, 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 0.2 mM dNTPs, 1 mM unlabelled oligo-nucleotide primer, 40 μM end-labelled oligo-nucleotide

Table 1 Summary of the experimental conditions for amplification of the microsatellite loci

Locus	Oligo designation*	Oligonuceotide primer sequence (5′ → 3′)	GenBank accession no.	Thermocycling profile			
				Annealing temperature (°C)	Cycling times†	Number of cycles	Thermocycler
GT023	GT023R	CAT TTC CTA CCC ACC TGT CAT	AF309690	62	15/15/15	28	MJR PCT100‡
	GT023F	GTT CCC AGG CTC TGC ACT CTG					
GT101	GT101R	CTT TCT CCT AGT GCT CCC CGC	AF309691	60	30/30/30	30	RoboCycler§
	GT101F	CTG TGC TGG TAT ATG CTA TCC					
GT195	GT195R	TGA GAA AGA TGA CTA TGA CTC	AF309692	54	15/15/15	30	MJR PCT100
	GT195F	TGA AGT AAC AGT TAA TAT ACC					
GT211	GT211R2	CAT CTG TGC TTC CAC AAG CCC	AF309693	60	30/30/30	28	RoboCycler
	GT211F2	GGC ACA AGT CAG TAA GGT AGG					
GT271	GT271F	GCT CAC ACT GGT AAT CTG TGG	AF309694	62	15/15/15	28	MJR PCT100
	GT271R	CCC TAG GAA GGA TAG ACA TAG					
GT307	GT307F	ATA TAG TTA TAT CTG TTG CTC	AF309695	49	15/15/15	33	MJR PCT100
	GT307R	TTA GCG AGT CAT ATT ATA AAG					
GT310	GT310R	TAA CTT GTG GAA GAT GCC AAC	AF309696	62	15/15/15	28	MJR PCT100
	GT310F	GAA TAC TCC CAG TAG TTT CTC					
GT509	GT509F	CAG CTG CAA AAC CTT GAC ATT	AF309697	58	15/15/15	28	MJR PCT100
	GT509R	GTA AAA TGT TTC CAG TGC ATC					
GT575	GT575F	TAT AAG TGA ATA CAA AGA CCC	AF309698	60	20/45/60	30	MJR PCT100
	GT575R	ACC ATC AAC TGG AAG TCT TTC					

*The upper oligonucleotide primer was end-labelled. †Times are given in seconds, starting with time at denaturing temperature (94 °C), then time at annealing temperature, followed by time at extension temperature (72 °C). ‡MJ Research model PCT100. §Stratagene RoboCycler model 96.

primer, and 0.4 units of *Taq* DNA polymerase (Life Technologies Inc.). The end-labelled oligo-nucleotide primer was labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Sambrook *et al*. 1989). The thermo-cycling profiles and GenBank accession numbers are listed in Table 1.

The amplification products were separated by electrophoresis through a denaturing 5% polyacrylamide gel. After electrophoresis, the gel was fixed in 5% ethanol: 5% acetic acid for 40 min, followed by a 15 min rinse in tap water. The fixed polyacrylamide gel was dried at 80 °C for 45 min and autoradiography performed with Kodak BioMax™ film for 5–48 h depending on the intensity of radioactive signal. The size of the amplification products was estimated from λM13 sequences and multiple positive control samples (of known genotype) included in each amplification and detection.

Our Gulf of Maine sample contained 73 known mother and calf pairs in which we detected no indications of null alleles (i.e. a mother and calf both homozygous but for different alleles; Pemberton *et al*. 1995) at the loci analysed in these samples. Neither did we detect any significant deviation between the observed and expected levels of heterozygosity for any of the remaining locus and species combinations (Table 2).

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Table 2 Levels of genetic variation estimated in selected baleen whale species

Locus	Species	*n**	Number of alleles	Size range (bp)	H_O†	H_E‡
GT023	*M. novaeangliae*	353	8	114–128	0.82	0.80
	B. acutorostrata	91	9	100–116	0.86	0.81
	B. musculus	92	8	122–136	0.85	0.83
	B. physalus	65	7	112–138	0.66	0.64
GT101	*M. novaeangliae*	4	2	92–94	—	—
	B. acutorostrata	—				
	B. musculus	92	9	85–101	0.65	0.67
	B. physalus	4	5	94–112	—	—
GT195	*M. novaeangliae*	353	5	151–163	0.65	0.65
	B. acutorostrata	3	2	162–166	—	—
	B. musculus	4	2	146–148	—	—
	B. physalus	65	8	158–176	0.74	0.70
GT211	*M. novaeangliae*	353	7	196–208	0.80	0.82
	B. acutorostrata	21	7	185–203	0.85	0.75
	B. musculus	—				
	B. physalus	73	6	193–213	0.60	0.55
GT271	*M. novaeangliae*	353	10	97–123	0.57	0.59
	B. acutorostrata	2	3	101–104	—	—
	B. musculus	4	3	101–105	—	—
	B. physalus	65	6	112–128	0.45	0.43
GT307	*M. novaeangliae*	353	7	127–139	0.67	0.68
	B. acutorostrata	2	3	135–141	—	—
	B. musculus	4	3	127–133	—	—
	B. physalus	65	7	121–139	0.70	0.64
GT310	*M. novaeangliae*	4	2	102–106	—	—
	B. acutorostrata	21	6	112–122	0.60	0.70
	B. musculus	4	3	110–116	—	—
	B. physalus	65	2	104–130	0.54	0.50
GT509	*M. novaeangliae*	6	1	195	—	—
	B. acutorostrata	169	11	195–217	0.81	0.81
	B. musculus	—				
	B. physalus	—				
GT575	*M. novaeangliae*	5	6	140–154	—	—
	B. acutorostrata	21	5	195–211	0.80	0.85
	B. musculus	—				
	B. physalus	5	5	140–154	—	—

*Number of individual whales genotyped. Estimated †observed and ‡expected heterozygosity. *Megaptera novaeangliae* was sampled in the Gulf of Maine, *Balaenoptera acutorostrata* across the North Atlantic, *B. musculus* in the Gulf of S. Lawrence as well as off West Greenland, and *B. physalus* in the Sea of Cortez (a small population with low levels of genetic variation, Bérubé *et al*. 1998).

Novel chloroplast microsatellites reveal cytoplasmic variation in *Arabidopsis thaliana*

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The analysis of levels and patterns of cytoplasmic variation in plants is now widely recognized as providing important and complementary information to that obtained using nuclear markers. In particular, the uniparentally inherited, non-recombining chloroplast genome has been utilized in many studies in plant population and evolutionary genetics (Soltis *et al*. 1992; Ennos *et al*. 1999). Until recently, however, the typically low substitution rates associated with the chloroplast genome meant that detecting sufficient levels of polymorphism

Table 1 *Arabidopsis* chloroplast microsatellite primers

Locus	Repeat	Location	Primers (5′–3′)	Alleles	Range (bp)
ATCP112	$(A)_{15}$	*trn*H(GUG)/*psb*A intergenic	ATCCGCCCCTACGCTACTAT AGGTGGAATTTGCTACCTTTTT	5	96–100
ATCP7905	$(A)_{13}$	*trn*S(GCU)/*trn*G(UCC) intergenic	CGAACCCTCGGTACGATTAA TGGAGAAGGTTCTTTTTCAAGC	2	140–141
ATCP28673	$(T)_{13}$	*ycf*6/*psb*M intergenic	GCGTTCCTTTCATTTAAGACG TGCACTCTTCATTCTCGTTCC	4	140–145
ATCP30287	$(A)_{13}$	*trn*D(GUC)/*trn*Y(GUA) intergenic	CCCTATACCCTGAAATTTGACC CAGCTCGGCCCAATAATTAG	3	100–102
ATCP46615	$(A)_{14}$	*trn*T(UGU)/*trn*L(UAA) intergenic	AATTTTTTTCCATTGCACATTG TCAGAAATAGTCGAACGGTCG	3	111–113
ATCP66701	$(T)_{16}$	*trn*P(UGG)/*psa*J intergenic	TCCACATCCTCCTTCTTTTTT CATTTGAAAACGTAAAGGCC	5	145–150
ATCP70189	$(A)_{13}$	*rps*12/*clp*P intergenic	CGGGTTGATGGATCATTACC GCAATGCACAAAAAAAGCCT	6	124–132

Table 2 Cross-species amplification in *Brassica* species using *Arabidopsis* chloroplast microsatellite primers

	Brassica species					
Locus	*B. oleracea*	*B. rapa*	*B. napus*	*B. nigra*	*B. carinata*	*B. juncea*
ATCP112	✘	✘	✘	✘	✘	✘
ATCP7905	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔
ATCP28673	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔
ATCP30287	✔	✔	✔	†	†	✔
ATCP46615	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔
ATCP66701	✔	✔	✔	✔	✔	✔
ATCP70189	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔

✔✔ — Strong amplification; ✔ — Weak amplification; ✘ — Poor or no amplification.
†Primer ATCP30287 amplified two bands in both *B. nigra* and *B. carinata*.

was the main drawback to the analysis of cytoplasmic variation, particularly below the species level. The discovery of polymorphic mononucleotide repeats in the chloroplast genomes of plants analogous to nuclear microsatellites, or simple sequence repeats, has provided a new approach to detecting cytoplasmic variation that had previously gone undetected using traditional restriction fragment length polymorphism (RFLP) studies. These chloroplast microsatellites have been used for the high-resolution analysis of cytoplasmic diversity in both crop species and natural plant populations (Provan *et al*. 1999b, 2000).

This report describes the development of chloroplast microsatellite markers in the weedy crucifer *Arabidopsis thaliana* (Brassicaceae). Despite *Arabidopsis* being the model organism for studies into the physiology, genetics and development of higher plants, very little work has been carried out on the analysis of natural populations of the species. Indeed, to date there have been no published studies investigating levels of cytoplasmic variation in *Arabidopsis* and only a limited number assessing levels of nuclear diversity in natural populations (Vander Zwan *et al*. 2000 and references therein).

The complete chloroplast sequence of *A. thaliana* (EMBL accession number AP000423) was searched for mononucleotide repeats of $n = 8$ or greater using the FINDPATTERNS program (Genetics Computer Group). A total of 231 repeats were found and primers were designed to amplify seven mononucleotide repeat loci in noncoding regions using the PRIMER program (Genetics Computer Group; Table 1). Primers were tested on 22 *A. thaliana* accessions from 11 populations in Europe and the USA, as well as on six *Brassica* species (see Table 2). Polymerase chain reaction (PCR) was carried out in a total volume of 10 μL containing 50 ng genomic DNA, 10 pmol ^{32}P end-labelled forward primer, 10 pmol reverse primer, 1× PCR reaction buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 15 mM $MgCl_2$, 0.05 U *Taq* polymerase (Gibco BRL). Reactions were carried out on a Techne GENIUS thermal cycler using the following parameters: initial denaturation at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s; final extension at 72 °C for 5 min. After addition of 10 μL loading buffer (95% formamide), products were resolved on 6% denaturing polyacrylamide gels containing 1× TBE buffer and 8 M urea at 80 W constant power for 2 h. Gels were transferred onto 3 mM blotting paper (Whatman) and exposed to X-ray film overnight at –70 °C.

All seven loci were polymorphic in the sample studied, with between two and six alleles detected per locus (Table 1).

Combining the alleles from the seven linked loci gave 11 haplotypes in the 22 individuals. Intrapopulation variation was detected in several populations, highlighting the resolving power of the chloroplast microsatellite technique even in highly inbreeding species such as *Arabidopsis*, which is believed to have a selfing rate of ≈99%. Although no previous cytoplasmic studies have been carried out in *Arabidopsis*, it is unlikely that such levels of variation would be detected with RFLPs. This has been observed in chloroplast microsatellite studies of other inbreeding species, e.g. barley (Provan *et al*. 1999a), where diversity levels were far in excess of those revealed by chloroplast RFLPs.

Due to high levels of conservation of both sequence and gene organization in plant chloroplast genomes, primers designed to amplify chloroplast microsatellites in one species have been shown to amplify polymorphic products in related species, even at the intergeneric level (Provan *et al*. 2000). Consequently, the primers developed for *Arabidopsis* were tested on several *Brassica* species (Table 2). Although primer pair ATCP112 did not amplify in the *Brassica* species and primers ATCP30287 and ATCP66701 gave poor and/or nonspecific amplification, the other four primer pairs amplified a single, strong product in all six species tested. This suggests that these primers may have considerable value in studying cytoplasmic variation within the Brassicaceae.

In summary, these chloroplast microsatellite primers offer new opportunities to study levels and patterns of cytoplasmic variation within and between *Arabidopsis* natural populations and ecotypes. A comparison of chloroplast and nuclear microsatellite markers will provide new insights into the relative roles of seed and pollen movement in shaping the genetic structure of natural populations. Furthermore, their utility across the Brassicaceae means that this ability to discriminate seed and pollen movement will be of value in assessing modes of potential transgene escape in genetically modified oilseed rape (*Brassica napus*) and in possible hybrids between *B. napus* and its wild relatives.

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Characterization of 14 tetranucleotide microsatellite loci derived from sockeye salmon

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The use of microsatellites for research and conservation of Pacific salmon (*Oncorhynchus* spp.) is increasing. Examples of applications include gene mapping (e.g. Lindner *et al*. 2000), pedigree analysis (e.g. Estoup *et al*. 1998), and population assignment (e.g. Olsen *et al*. 2000). Through these studies researchers have become more discriminating in their choice of microsatellites to meet specific project objectives and to improve genotyping efficiency. Examples of selection criteria include locus polymorphism, presence of null alleles, allele size range, polymerase chain reaction (PCR) annealing temperature, and PCR amplification quality. New microsatellites are needed to improve the selection of loci for each species of Pacific salmon. In particular, there is need for tetranucleotide microsatellites. This class of microsatellites does not generally exhibit the complicated shadow banding ('stutter') observed in many dinucleotide microsatellites, and they provide sufficient range in polymorphism for various applications (Jarne & Lagoda 1996). Here we report the development of primers for 14 novel tetranucleotide microsatellites in sockeye salmon (*O. nerka*).

Sequences for 27 novel DNA fragments (~350–550 bp) containing TAGA tetranucleotide microsatellites were identified by Genetic Identification Services (GIS, Chatsworth CA) using an enrichment protocol similar to Edwards *et al*. (1996). Genomic DNA from a single sockeye salmon was partially restricted with a cocktail of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B 1, *Pvu*II, *Stu*I, *Sca*I, *Eco*R V). Fragments in the size range of 300–750 bp were adapted and subjected to magnetic bead capture (CPG Inc., Lincoln Park, NJ), using $(TAGA)_8$ biotinylated capture molecules (Integrated DNA Technology, Coralville, IA). Captured molecules were amplified and

Table 1 Estimates of polymorphism for 14 novel sockeye salmon microsatellites. *n*, A, H_O, H_E refer to sample size, allele number, observed and expected heterozygosity, respectively. An asterisk denotes a significant difference ($P < 0.05$) between H_O and H_E. The forward primer is labelled for each locus and the annealing temperature is 56 °C for all primer pairs

Locus	Repeat sequence of cloned allele	Primer sequence (5′–3′) (F, forward, R, reverse)	*n*	Size range (bp)	A	H_E	H_O	GenBank no.
One100	$(TAGA)_{18}N_{14}(TAGA)_{18}$	F: CAATGCACTGTGATAGGAGG R: AGGGGAAGAAGAAGTTTTGG	89	246–378	22	0.92	0.80*	AF274516
One101	$(ATCT)_{26}N_{12}(ATCT)_{13}$	F: AAATGACTGAAATGTTGAGAGC R: TGGATGGATTGATGAATGG	89	182–344	28	0.94	0.92	AF274517
One102	$(ATCT)_{10}$	F: CATGGAGAAAAGACCAATCA R: TCACTGCCCTACAACAGAAG	89	207–275	15	0.86	0.85	AF274518
One103	$(ATCT)_{27}N_{16}(ATCT)_{11}$	F: AATGTTGAGAGCTATTTCAATCC R: GATTGATGAATGGGTGGG	89	167–447	29	0.93	0.94	AF274519
One104	$(ATCT)_{15}N_4(ATCT)_{10}$	F: ATCTTTATGGTGGCAAGTCC R: ATCTGGTACTTCCCTGATGC	89	167–239	19	0.91	0.92	AF274520
One105	$(TAGA)_9$	F: TCTTTAAGAATATGAGCCCTGG R: GCTCAAATAAACTTAAACCTGTCC	89	127–151	6	0.44	0.42	AF274521
One106	$(ATCT)_9N_4(ATCT)_{10}N_8(GTCT)_{10}$	F: TACCCTGCAAGACAGTGAGA R: GCTGTTTAGGAAGGAGGGTT	89	111–259	30	0.90	0.82	AF274522
One108	$(ATCT)_{21}$	F: TGCAGAGCCATACTAAACCA R: AAGAATTGAGAGATGCAGGG	89	184–244	16	0.90	0.91	AF274523
One109	$(TAGA)_9$	F: AGGGAGAGAAGAGAGGGAGA R: CCTCAGAAGTAGCATCAGCTC	89	127–175	13	0.88	0.90	AF274525
One110	$(TAGA)_{21}$	F: CCTCCATTTCAATCTCATCC R: ACAGAGAACAGTGAGGGAGC	89	235–287	13	0.89	0.87	AF274526
One111	$(TAGA)_{21}$	F: ATGACCAAGGAGCTTCTGC R: TATCCAGGTACTCCACTGGC	89	194–322	30	0.88	0.86	AF274527
One112	$(ATCT)_{28}$	F: GTGACCCAGACTCAGAGGAC R: CACAACCCATCACATGAAAC	88	127–241	27	0.90	0.87	AF274528
One114	$(TAGA)_{12}N_4(TAGA)_{12}$	F: TCATTAATCTAGGCTTGTCAGC R: TGCAGGTAAGACAAGGTATCC	89	211–295	22	0.93	0.89	AF274530
One115	$(ATCT)_{24}$	F: CGCTATACATTTTCCATTTTCC R: TTTTTAAGTGGGAGAACTTGC	86	173–237	16	0.92	0.89	AF274531

restricted with *Hin*dIII to remove the adapters. The resulting fragments were ligated into the *Hin*dIII site of pUC19. Recombinant molecules were electroporated into *Escherichia coli* DH5alpha. Recombinant clones were selected at random for sequencing. Sequencing was preformed in an MJ Research PTC-200 thermocycler using ABI Prism *Taq* DyeDeoxy™ terminator chemistry. The sequences were visualized on an ABI 373 DNA sequencer.

Primer pairs for 27 sequences were designed using the program Primer 3 (Rozen & Skaletsky 1996). Unlabelled primers, purchased from Operon Inc. (Alameda, CA), were tested for amplification effectiveness in four sockeye salmon using agarose gel electrophoresis. PCR was carried out in 10 μL volumes 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 units AmpliTaq DNA polymerase (Perkin-Elmer Corp, Foster City, CA), 0.15 μM each primer, and 100 ng DNA template] using an MJ Research PTC-225 thermocycler. DNA amplifications involved the following profile: 92 °C (5 min); 25 cycles of 92 °C (30 s) + 56 °C (30 s) + 72 °C (30 s); 72 °C (30 min). The PCR product was electrophoresed for 2 h at 100 V in a 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light (312 nm). Four primer pairs failed to yield distinct PCR products. Fluorescein-labelled forward primers were purchased for the remaining 23 sequences from Perkin-Elmer Corp. (Foster City, CA). Fourteen of these 23 primer pairs yielded high-quality amplification product as determined using an ABI 377–96 DNA sequencer in GeneScan mode (ABI 1996a) to detect the labelled primers in a 4.5% denaturing polyacrylamide gel (Table 1).

Estimates of polymorphism were obtained for the 14 loci by genotyping 89 sockeye salmon using the ABI 377–96. Allele scoring was performed with Genotyper software, version 2.0 (ABI 1996b). The number of alleles per locus ranged from 6 to 30 and averaged 20 (Table 1). The expected heterozygosity ranged from 0.44 to 0.94 and averaged 0.87. The observed and expected heterozygosity differed significantly ($P < 0.05$) at *One100*. The allelic size averaged 103 bp and ranged from 24 bp (*One105*) *to* 280 bp (*One103*).

At least some of the microsatellites amplified in six related species of *Oncorhynchus* and only one locus (*One100*) failed to amplify in all species (Table 2). In most instances (27) the amplified fragment length was within the size range identified

Table 2 Results of cross-species testing of sockeye salmon microsatellite primers. Two individuals were tested for each species

	Locus (*One*)													
Species	100	101	102	103	104	105	106	108	109	110	111	112	114	115
Oncorhynchus gorbuscha	–	+	+	+	–	–	–	–	–	+	+	+	–	+↓
O. keta	–	+↓	+	+↓	–	+	+	+	–	+	+	+	+	–
O. tshawytscha	–	+↓	+	+↓	–	–	+	–	+↓	+↓	+	–	+	–
O. kisutch	–	+	–	+↓	+↓	–	+	–	+↓	+↓	+↓	+↑	–	–
O. mykiss	–	+↓	+	+↓	+↓	–	–	+	+↓	+↓	+	+	+↓	–
O. clarki	–	+↓	+	+↓	–	–	–	+↓	+↓	+↓	+	+	–	–

+, amplified at designed annealing temperature; –, did not amplify; ↓, amplified fragment smaller than smallest allele in sockeye; ↑, amplified fragment larger than largest allele in sockeye.

in sockeye salmon. In 23 instances the amplified fragment was smaller than the smallest allele in sockeye salmon and in one instance the amplified fragment was larger than the largest allele in sockeye salmon (*One112*). These microsatellites should prove useful for a number of conservation genetic applications in sockeye salmon and, to a lesser degree, the other species examined here.

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Microsatellite characterization in central stoneroller *Campostoma anomalum* (Pisces: Cyprinidae)

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The central stoneroller (*Campostoma anomalum*) is a small cyprinid fish that is native to streams and rivers of central and eastern North America. It can be found in a range of anthropogenically modified habitats, ranging from nearly pristine to highly polluted waters (Zimmerman *et al*. 1980), and has intermediate sensitivity to habitat degradation relative to other fishes in the region (Zimmerman *et al*. 1980; Gillespie & Guttman 1989). The species is the focus of intensive study by the United States Environmental Protection Agency due to its biological and distributional characteristics. An important aspect of this research is to understand the fine-scale genetic structure of the species across its native range, and to determine how this 'genetic landscape' relates to underlying environmental processes. To date, genetic analyses have focused on multi-locus fingerprints generated by the random amplified polymorphic DNA (RAPD) method to delineate levels of similarity among and within populations (Silbiger *et al*. 1998). Because allelic counts are highly sensitive to recent changes in population size, highly polymorphic microsatellite DNA markers should provide genetic information that is highly complementary to the RAPD data and may reveal finer levels of population structuring. Here, we report a suite of highly polymorphic microsatellite markers developed for the central stoneroller.

Table 1 Locus name, primer sequences, annealing temperature (T_A), repeat motif of cloned allele, product size based on sequenced allele (bp), number of individual fish tested (n), number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) and GenBank accession number for the cloned sequences for 17 microsatellite primers developed for *Campostoma anomalum*

Locus	Primer sequence (5′ → 3′)	T_A (°C)	Repeat motif	Size (bp)	n	N_A	H_O	H_E	Accession number
Ca1	AAGACGATGCTGGATGTTTAC CTATAGCTTATCCCGGCAGTA	58	$(CA)_{24}$	112	10	6	0.60	0.78	AF277573
Ca2	ACCTTTCCTTTCGTGTCGAGA GGACCCAGCGAGCACCT	64	$(CA)_{19}$	100	11	10	0.80	0.90	AF277574
Ca3	GGACAGTGAGGGACGCAGAC TCTAGCCCCCAAATTTTACGG	55	$(TAGA)_{14}$	243	13	10	0.70	0.80	AF277575
Ca4	CGGTATCGGTGCATCCCTAAA AACAGCGCGAGCGTCATTC	55	$(CA)_{12}$	157	13	5	0.92	0.84	AF277576
Ca5	TTGAGTGGATGGTGCTTGTA GCATTGCCAAAAGTTACCTAA	51	$(TAGA)_{15}$	149	13	8	0.67	0.83	AF277577
Ca6	CAGGTCTTGCCCACGTCTGAG CACCTGTGGAACCGGCTTGA	59	$(CA)_{14}CG(GA)_6$	204	13	5	0.42	0.72	AF277578
Ca7	ACACGGGCTCAGAGCTAGTC CAAATGTCAGGAGTTCTCCGA	59	$(CA)_{15}$	103	13	7	0.54	0.78	AF277579
Ca8	ACGCAGACATATTTTAGATG AATAATACAACTCGCTCTCA	53	$(TAGA)_{20}$	183	13	12	0.61	0.89	AF277580
Ca9	ATCAAGCCTGCCATGCAC ATCACTGTAGACTGCGACCAG	57	$(CA)_{15}$	118	13	7	0.50	0.67	AF277581
Ca10	CTGCACGGGTTTTAATATCTT AATGATGTCATCGCCATGTA	57	$(TAGA)_{16}$	243	13	7	0.42	0.78	AF277582
Ca11	TCCCTCACTGTGCCCTACA GGCGTAGCAATCATTATACCT	57	$(TAGA)_7$	203	9	6	0.66	0.81	AF277583
Ca12	GTGAAGCATGGCATAGCACA CAGGAAAGTGCCAGCATACAC	57	$(TAGA)_{10}(CAGA)_4(TAGA)_2$	238	13	6	0.38	0.70	AF277584
Ca13	GATCATTGATCCGCATGTCTC CTCCCTGACAGCAGCGACC	57	$(CA)_{16}$	163	11	4	0.36	0.61	AF277585
Ca14	GCGGAATAGCAGTCAATA GTTAAACTGTTCCTGTTACGGT	54	$(CA)_{13}$	90	13	9	0.90	0.87	AF277586
Ca15	TGATTTTATATCTTCGAGGAA AAACCCAACCGTTAGTCTAAT	57	$(CA)_{23}$	213	10	8	0.81	0.85	AF277587
Ca16	CGCGACCAGTTGTGAC GACGAGCGTATTCAGATTACA	54	$(TAGA)_7$	213	13	3	0.25	0.55	AF277588
Ca17	GTTTGAAGTGGGATTAACT GTTGTGTATACCTGGTTAAAG	51	$(TAGA)_8$	131	9	3	0.11	0.30	AF277589

A partial genomic library was constructed using the strategy described by Kandpal *et al*. (1994). After digestion of *C. anomalum* genomic DNA with *Sau*3AI restriction enzyme, DNA fragments ranging from 400 to 1500 bp were ligated to *Sau*3AI linkers. After removal of excess linkers by electrophoretic fractionation, fragments were amplified by polymerase chain reaction (PCR) using a primer complimentary to the *Sau*3AI linker. The whole genomic library was enriched for sequences containing CA repeats by hybridization to a biotinylated CA probe. Hybridized molecules were captured using VECTREX avidin D matrix (Vector Laboratories, Burlingame, California, USA). The microsatellite-enriched library was amplified by PCR and ligated into the *p*CR2.1 vector (Invitrogen, San Diego, California, USA). The transformed colonies were screened with a $(CA)_{21}$ oligonucleotide probe conjugated to alkaline phosphatase (Lifecodes Corp. Stamford, California, USA). Additional microsatellite loci containing TAGA and CA motifs were identified from microsatellite-enriched libraries produced by Genetic Identification Services Inc. (Chatsworth, California, USA) by using a microsatellite enrichment procedure similar to the one described above. A total of 130 clones were sequenced using BigDye terminator sequencing chemistry (PE Biosystems, Foster City, California, USA) on an ABI 310 Genetic Analyser (PE Biosystems). Di- and tetra-nucleotide repeat motifs were identified in 67 of the sequences. PCR primer pairs flanking repetitive regions were designed for 27 microsatellite loci using Oligo 6.21 (Molecular Biology Insights Inc., Cascade, Colorado, USA). Total genomic DNA was extracted (DNeasy, Qiagen Inc., Valencia, California, USA) from muscle tissue of 13 samples collected from throughout the *C. anomalum* species range. Between 9 and 13 *C. anomalum* samples and between 1 and 3 samples from five other species of cyprinids were genotyped for each microsatellite marker to confirm amplification and estimate the level of polymorphism. Each 15 μL PCR raction included 25 ng of template, 250 μM dNTP, 3 pmol of each primer, 2.5 mM $MgCl_2$, 0.3 units Taq DNA polymerase (Perkin Elmer), 10 mM Tris–HCl, pH 8.3, and 50 mM KCl.

Table 2 Cross-specific amplification of microsatellite loci isolated from *Campostoma anomalum*

Locus	Bluntnose minnows *Pimephales notatus* (n = 3)	Fathead minnows *Pimephales promelas* (n = 3)	Blacknose dace *Rhinichthys atratulus* (n = 3)	Creek chubs *Semotilus atromaculatus* (n = 3)	Zebra fish *Danio rerio* (n = 1)
Ca1	4	4	4	3	1
Ca3	1	—	3	—	1
Ca6	4	3	—	—	—
Ca7	—	—	3	2	—
Ca9	4	—	—	—	—
Ca11	—	—	2	4	—
Ca12	3	3	6	5	1
Ca13	—	—	—	—	1
Ca14	—	—	2	—	—
Ca16	4	5	—	—	—
Ca17	3	—	—	—	—

Fragments were resolved on 5% polyacrylamide gels. The data indicate the number of alleles counted from n genotyped individuals. '—', no or unreadable amplification.

Cycling was performed with a PE Biosystems Geneamp 9600 thermal cycler under the following conditions: 30 s at 94 °C; 27 cycles of 1 min at 92 °C, 1 min at 51–64 °C, depending on the specific primer set (Table 1), and 1.5 min at 72 °C; followed by 7 min at 72 °C. PCR products were separated on denaturing 5% polyacrylamide gels and visualized with Vistra Green (Amersham Life Science, Amersham, Buckingamshire, UK) fluorescent dye using a FluorImager 595 fluorescent scanner (Molecular Dynamics, Sunnyvale, California, USA).

A total of 17 microsatellite markers were identified as highly polymorphic for *C. anomalum* (Table 1). The other 10 primer sets either failed to produce a reliable PCR product or were not polymorphic for the samples assayed. Two of the 17 primer sets that were informative for *C. anomalum* produced a PCR product of similar size for all of the other five cyprinid species tested (Table 2). In addition, nine primer sets produced a PCR product of similar size in at least one of the other cyprinid species. The extensive polymorphisms identified for these markers within *C. anomalum*, and their apparent applicability to other species, indicate that they will have utility for future population studies.

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Dinucleotide microsatellite loci for *Andrena vaga* and other andrenid bees from non-enriched and CT-enriched libraries

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Andrena is a large genus (>1000 species) of bees with a primarily Holarctic distribution (Michener 1979). Analysis of *Andrena* population genetics has been hampered by their limited allozyme variability (Ayasse *et al*. 1990). Microsatellites potentially make up this shortfall, although there are few loci described for this group of bees (Paxton *et al*. 1996).

We developed microsatellites for the andrenid bee *Andrena vaga* Panzer 1799 using both non-enriched and enriched partial genomic libraries. DNA for cloning was isolated from the thorax of one male bee using phenol/chloroform, digested to completion with *Sau*3AI, resolved on an agarose gel, and fragments between 200 and 800 bp were isolated from the gel

Table 1 Description of 19 microsatellite loci for *Andrena vaga* and heterozygosities for eight females

Locus	Sequence (5′ → 3′)	Repeat sequence	Fragment size (and range) (bp)	Annealing temp. (°C)	H_E	H_O	Number of alleles	GenBank accession no.
vaga01	F: GTGCCAAGTCAGTTAGTGTGC R: GAAACACGTAGCGAACACG	$(CT)_{20}$	186 (184–194)	65	0.76	0.63	4	G64722
vaga02	F: CTTCTCCAAGCCGAATCTTCC R: GATCGGCCTGGGAAATTCC	$(CT)_{27}$	232 (210–240)	65	0.91	0.88	8	G64906
vaga03	F: GATTCGGGAACGACACTCG R: CGTTTATAGCGATGATGTCCG	$(CT)_{17}$	107 (105–123)	65	0.82	0.63	7	G64907
vaga04	F: TTCTACGTTAGTCCGCAGG R: CTTAGTCCGTTAAGGAGCAAC	$(CT)_{17}$	228 (226–238)	60	0.88	0.75	7	G64908
vaga05	F: GGAAGGTTGAGTGGAAATTG R: TGTCCGAAGTGAAGAGAACG	$(CT)_{16}$	318 (316–326)	56	0.88	0.75	6	G64909
vaga06	F: GCTTTGGTTCCTCGTGTCG R: CCACTGAAACTCATCTAGGTACACG	$(CT)_{15}$	194 (192–218)	60	0.24	0	2	G64910
vaga08	F: GATCCGAAAAGTTGAAGGTG R: CTACGTGACTTTCCTGTCCTC	$(CT)_{29}$	171 (165–187)	56	0.88	0.75	7	G64911
vaga09	F: CCGTTGTAATCGAATGAACC R: GATGGAGGAAAGGGGAGA	$(CT)_{21}$	117 (105–131)	56	0.91	0.63	8	G64912
vaga12	F: GGAATTCGTCGACGAAAGG R: CGATGGGTGTAGGTGGGAT	$(CT)_{21}$	178 (164–194)	63	0.86	0.88	7	G64913
vaga13	F: CTTAGTCCGTTAAGGAGCAAC R: GGAACGAAAGTCTTCTCTTCTC	$(CT)_{18}$	153 (145–163)	56	0.89	1.00	8	G64914
vaga14	F: CTGCCACCTCTGTACATGG R: CGTGTGAGCTAGAGTTCCATC	$(CT)_{16}$	218 (216–224)	65	0.65	0.50	4	G64915
vaga18	F: CGACTTTGCTACAGCGATTC R: CGACTTGGATAGGCAGGG	$(CT)_{20}$	135 (123–155)	60	0.68	0.25	6	G64916
vaga19	F: GGGTAACGAGAGAAGGGG R: GAGGAGTCGTGTTACGTGC	$(CT)_{23}$	160 (146–162)	56	0.82	0.38	6	G64923
vaga20	F: GATCTTCTTACCTCCCCCC R: CTTCCTTTTGCTCCCTCTTG	$(CT)_{17}$	183 (179–191)	60	0.79	0.63	7	G64917
vaga21	F: CCTTGTTACGCGTGCATAG R: TCGGAAACTGTACGTCGTC	$(CT)_{19}$	149 (149–161)	60	0.80	0.38	5	G64918
vaga23	F: CTGTGTGGAAAGGTGATAACG R: GAAGGGAACAGTAATGGACAAG	$(CT)_{33}$	219 (203–233)	56	0.87	0.38	7	G64919
vaga25	F: GTCGCTACACACTCGTTATCTTG R: CATGGATTCCAACGAATTCTC	$(CT)_{18}$	126 (126–128)	56	0.23	0	2	G64920
vaga26	F: CGAGGGCAATCGACAGTG R: GCCGTTGAATTCACGTAGG	$(CT)_{20}$	166 (156–170)	60	0.77	0.50	5	G64921
vaga27	F: GACGGACTCGGATACACCC R: CGAGTTGCCGCTAACTTTC	$(CT)_{14}$	100 (98–106)	65	0.69	0.63	4	G64922

using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol.

For the non-enriched library, the 200–800 bp fragments were ligated into plasmid vector pUC18/*Bam*HI (Amersham/Pharmacia). Highly competent *E. coli* (INVαF′ One Shot, Invitrogen) were transformed with plasmids, and resultant colonies were simultaneously screened for microsatellites using digoxigenin (DIG)-end labelled $(GA)_{10}$ and $(CA)_{10}$ exactly as described by Estoup & Turgeon (1996).

We used filter hybridization (Armour *et al*. 1994) to generate a CT-enriched library, following methods described by Segelbacher *et al*. (2000) (see also Piertney *et al*. 1998). A 1 μg aliquot of the *A. vaga* 200–800 bp fragments was ligated to a SauL linker molecule, denatured and hybridized to a 1 cm^2 piece of Hybond N^+ membrane (Amersham/Pharmacia) to which synthetic $(GA)_n$ polymers had previously been bound (Schlötterer 1998, pp. 241–244). After overnight hybridization at 65 °C in 2 × SSC and 0.1% SDS, nylon membranes were given three washes of 2 × SSC and 0.1% SDS, and then the enriched fragments were stripped from the membrane by heating to 95 °C for 5 min in water. The enriched fraction was precipitated and complementary strands were reformed in a polymerase chain reaction (PCR) (30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C) using the SauL-A oligonucleotide as a primer (Schlötterer 1998). Linkers were removed from the fragments by digestion with *Sau*3AI, and the fragments, now enriched for CT/GA sequences, were subsequently ligated into a plasmid, cloned and screened exactly as described above for the non-enriched library.

From the non-enriched library, four of 732 screened colonies were positive (0.5%), and, from the enriched library, 154 of 435 screened colonies were positive (35.4%), demonstrating the utility of the enrichment protocol. Plasmid DNA was extracted from positive colonies, inserts were cycle-sequenced using Big Dye Terminator chemistry (Perkin Elmer), and fragments were resolved on an ABI Prism 377 automated sequencer.

Table 2 Cross-species amplification of 19 pairs of *Andrena vaga* microsatellite primers with two individuals each of six other bees. Where a single PCR product was obtained, the number of alleles resolved is provided

	Locus (vaga)																		
Species	01	02	03	04	05	06	08	09	12	13	14	18	19	20	21	23	25	26	27
Andrenidae																			
Andrena agilissima	—	—	—	1	2	1	—	2	—	1	1	—	1	1	—	1	—	1	3
Andrena scotica	2	—	1	2	1	1	2	1	3	2	—	—	2	2	1	2	1	2	1
Andrena ferox	1	—	1	1	1	1	3	1	3	1	—	—	1	1	1	2	1	1	1
Halictidae																			
Lasioglossum malachurum	—	—	—	1	1	—	1	2	—	—	—	—	1	—	—	—	—	—	—
Apidae																			
Scaptotrigona postica	—	—	1	—	1	—	1	1	—	—	—	—	1	—	—	—	—	—	—
Anthophoridae																			
Nomada lathburiana	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—

—, a multiple band, a smear, or no product was detected.

The four colony plasmids from the non-enriched library each contained a unique $(CT)_n$ repeat, whilst 45 of 49 colony plasmids from the enriched library each contained a unique $(CT)_n$ repeat. PCR primers were designed on sequences flanking 22 perfect dinucleotide repeats using the software package Amplify, version 1.2 (www.wisc.edu/genetics/CATG/Amplify).

DNA for PCR was extracted from thoracic tissue using a high-salt protocol (Paxton et al. 1996). PCR amplifications were performed in 10 μL reaction volumes using an MJ Research PTC-100 thermal cycler. Individual mixes consisted of 10 ng template DNA, 4 pmol of each primer, 75 μm of each dCTP, dGTP and dTTP, 6 μm dATP, 0.125 μCi [α33P]-dATP, 1.5 mm MgCl2, 10 mm Tris–HCl, pH 8.8, 50 mm KCl, 0.1% Triton X-100, 200 μm spermidine and 0.4 units of thermostable DNA polymerase (Finnzymes). Samples were processed through one denaturing step of 3 min at 94 °C followed by 25 cycles consisting of 45 s at 94 °C, 30 s at the annealing temperature specified in Table 1, and 45 s at 72 °C, with a final elongation step of 10 min at 72 °C.

Nineteen of 22 primer pairs gave an amplification product using *A. vaga* as template DNA, many with numerous alleles per locus (Table 1). Primers were also successful in amplifying DNA extracts of other andrenid bees, although less successful in amplifying DNA from phylogenetically distant taxa, namely anthophorid, apid and halictid bees (Table 2). These loci should prove useful in the analysis of the population genetic structure of many andrenid bees.

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Isolation and characterization of microsatellite loci in the dice snake (*Natrix tessellata*)

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Table 1 *Natrix tessellata* microsatellite primer sequences, annealing temperatures (T_A), population-specific allelic diversity (A), total number of alleles detected (A_{total}) and observed (H_O) and expected (H_E) heterozygosities in samples from populations from Lake Lugano (Switzerland) (n = 10) and Lake Garda (Italy) (n = 19). Repeat motifs are derived from the sequenced clones (GenBank accession numbers AF269184–AF269191)

Locus	Primer sequence (5′ → 3′)	T_A (°C)	Size range (bp)	Repeat motif	Population	A	A_{total}	H_O	H_E
μNt1	GGAGTAGCCATTATTGCCAAAG	60	129–137	$(CA)_{15}$	Lugano	2	4	0.000	0.340
	GCTCCGACCACACTTTAAGC*				Garda	3		0.158	0.301
μNt2	TGGCACCATTTCAGTTTCTG	60	172–226	$(CA)_{21}$	Lugano	7	10	0.600	0.735
	GGGACCTCATCGAAACATTG*				Garda	8		0.369	0.712
μNt3	GGCAGGCTATTGGAGAAATG	63	142–156	$(AC)_{16}$	Lugano	5	6	0.000	0.680
	GGCAAAACTCCAGGTGCTAC*				Garda	4		0.053	0.652
μNt5	TGCTTTTCGGATTTGACATTC	58	116–126	$(CA)_2GA(CA)_3GA(CA)_4GA(CA)_{15}$	Lugano	5	6	0.700	0.595
	CTGCATTTGAAGCGTGGTAG*				Garda	5		0.632	0.474
μNt6	TGCTGGCATGTGAAATCAAG	62	187–209	$(CT)_6CA(CT)_{14}(GT)_{13}TT(GT)_4$	Lugano	6	11	0.600	0.780
	GGGGCTGTTTTCTGTCAATC*				Garda	10		0.474	0.819
μNt7	TTTGAAAGGAGAATGAATCGTG	58	171–185	$(AC)_{17}$	Lugano	6	6	0.900	0.800
	CGCGAGGAATCAGAATGAAC*				Garda	5		0.632	0.632
μNt8	GGGGTATCGTCCTTCCAGAC*	58	152–166	$(AC)_{15}$	Lugano	5	5	0.700	0.545
	GCCAAGTGTTTCTTCAAGTGG				Garda	3		0.632	0.571
μNt10	AATTACAGTAGGTAGGTAGTTAGGGAGG	64†	291–301	$(GA)_{27}$	Lugano	3	6	0.400	0.515
	CTGTGCCAGCAGAAACACC*				Garda	5		0.368	0.501

*Fluorescent-labelled primer.

†For locus μNt10, a hot-start protocol was used. The PCR conditions are as described before but with HotStarTaq™ DNA polymerase and buffer (Tris–Cl $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$; pH 8.7 (20 °C), Qiagen) and an initial denaturing step of 95 °C for 15 min.

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The dice snake, *Natrix tessellata* (Laurenti) 1768, has a large geographical distribution, ranging from Italy in the west to China in the east (Hecht 1930). While population sizes are often large in suitable habitats, they are small in many range-marginal populations, such as along the rivers Mosel, Lahn and Nahe in Germany (Gruschwitz 1985), where populations are in danger of becoming extinct as a consequence of either stochastic catastrophic events or genetic erosion. On the other hand, allochthonous populations in Switzerland that result from introductions of a small number of founding individuals may be very large (Mebert 1993). To assess the levels of genetic variation within large natural populations, declining range-marginal populations and allochthonous populations, suitable molecular markers are necessary. Allozymes or mitochondrial DNA are not suitable for this purpose because levels of genetic variation in small populations are typically very low, or because relatively large amounts of fresh blood or tissue are necessary. Microsatellites, on the other hand, are often variable even in small and endangered populations and can be easily amplified from minute amounts of DNA recovered from blood samples, shed skin or faeces.

We constructed a partial genomic library enriched for CA and GA repeats using a slight modification of the procedures described by Tenzer *et al*. (1999) and Gautschi *et al*. (2000). Briefly, total genomic DNA was isolated from blood samples using a standard phenol–chloroform extraction protocol (Sambrook *et al*. 1989). DNA was digested with *Tsp*509I (New England Biolabs), 200–700 bp fragments were isolated and ligated to TSPADSHORT/TSPADLONG linker sequences (Tenzer *et al*. 1999). DNA linker molecules were amplified according to Gautschi *et al*. (2000) using TSPADSHORT as the polymerase chain reaction (PCR) primer, and PCR products were hybridized to biotinylated $(CA)_{13}$ and $(GA)_{13}$ probes attached to streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin, Dynal, France) (see Tenzer *et al*. 1999 for details). Enriched fragments were again amplified and products were cloned using the Original TA Cloning® Kit (Invitrogen BV) following the manufacturer's instructions. After dot-blotting of recombinant colonies onto Nylon membranes (Hybond N^+, Amersham Pharmacia), oligonucleotide probes labelled using the ECL3′-oligolabelling and detection system (Amersham Pharmacia) were used to screen for inserts containing CA and GA repeats. The hybridization was carried out in accordance with the manufacturer's instructions. Plasmids from positive clones were sequenced as described in Gautschi *et al*. (2000) and the sequences submitted to GenBank (Table 1). Primer design was carried out using primer 3 software (Rozen & Skaletsky 1998), oligonucleotides were synthesized by Microsynth GmbH (Switzerland), and one primer for each pair was labelled with fluorescent dye (see Table 1).

PCR amplification for polymorphism assessment was performed in a 10 μL reaction volume containing 10 ng of genomic DNA, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0), 150 μM of each dNTP (Amersham Pharmacia), 0.5 μM each of forward and reverse primer and 0.5 units of Taq DNA polymerase (Amersham Pharmacia). We used the following

thermotreatment on a PTC-100™ Programmable Thermal Controller (MJ Research Inc.): 25–30 cycles at 95 °C for 30 s, the locus-specific annealing temperature (Table 1) for 30 s, and 72 °C for 30 s. Before the first cycle, a prolonged denaturation step (95 °C for 5 min) was included, and the last cycle was followed by an extra 8 min extension. The amplified products were diluted with double-distilled water containing GENESCAN-350 (TAMRA) Size Standard (PE Biosystems) and genotyped on an ABI Prism 310 Genetic Analyser using GeneScanAnalysis® Software version 2.1 and Genotyper® version 2.1 software (PE Biosystems). Observed and expected heterozygosities for each locus were calculated using Popgene version 1.32 (Yeh & Boyle 1997).

All eight microsatellite loci reported here were variable in *Natrix tessellata* and detected between four and 11 alleles in the two populations studied. Likelihood ratio tests indicated significant deviations from Hardy–Weinberg equilibrium (HWE) at loci μNt1 and μNt3, suggesting that null alleles may be present at these loci. Genotype frequencies at all other loci conformed to HWE. These microsatellites will therefore provide a valuable tool for the analysis of genetic variation in natural and allochthonous populations of the dice snake and help to devise appropriate conservation management strategies for small and endangered populations.

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Isolation and characterization of microsatellite loci in the bearded vulture (*Gypaetus barbatus*) and cross-amplification in three Old World vulture species

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During the last one hundred years the bearded vulture, *Gypaetus barbatus*, has suffered extreme population declines in Europe primarily because of hunting, but changes also in agriculture, and especially in grazing practices, have resulted in poor food conditions for carrion feeders. Small populations have survived in the Pyrenees, on Corsica and Crete. The population in the Alps was completely extinct by the beginning of the 20th century. To re-establish a self-sustaining population in the Alps, over 90 juvenile bearded vultures have been released since 1986, all originating from a captive population. The amount of genetic variability in the captive and released populations, genealogical relationships between individuals, and the degree of gene flow among wild populations in the past (represented by over 200 Museum specimens) and at present are important criteria in the development of a genetic management strategy. We describe the development of 14 microsatellite primers for conservation genetic analyses of the bearded vulture. We designed the primers with special emphasis on their later use for ancient DNA (aDNA) and tested their suitability for use in other Old World vulture species.

We constructed a genomic library enriched for CA and GA repeats using a modification of the method described in Tenzer *et al*. (1999). Digestion of total genomic DNA with *Tsp*509I (New England Biolabs), isolation of 200–700 bp fragments and ligation to TSPADSHORT/TSPADLONG linker sequences were carried out according to Tenzer *et al*. (1999). The ligation produces blunt-ended molecules that were amplified in 40 25 μL reactions containing 10 mM Tris-HCl, pH 9.0; 50 mM KCl, 1.5 mM $MgCl_2$; 0.1% TritonX 100; 0.2 mg/mL BSA, 150 μM of each dNTP, 1 μM of TSPADSHORT (primer), and 1.25 U of *Taq* DNA polymerase (Appligene oncor). The thermal profile on a PTC100™ Programmable Thermal Controller (MJ Research) was 30 cycles of 93 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. An initial 5 min extension step at 72 °C allowed the DNA polymerase to synthesize the nick between genomic DNA and linker sequences. Polymerase chain reaction (PCR) products were hybridized to biotinylated $(CA)_{13}$ and $(GA)_{13}$ probes that were immobilized onto Dynabeads M-280 Streptavidin (DYNAL, France). Hybridization was carried out

Table 1 Genetic characteristics of 14 bearded vulture microsatellite loci. Data on numbers of alleles and heterozygosities are based on genotypes of 30 bearded vulture individuals. T_a, locus-specific annealing temperature; H_O, observed heterozygosity (direct count); H_E, unbiased expected heterozygosity (Nei 1978). No significant departures from Hardy–Weinberg equilibrium were detected using likelihood ratio tests. The characteristics of repeat motifs and sizes are based on the sequenced clones (GenBank Accession nos: AF270729–AF270742)

Locus	Primer Sequences (5′–3′)	Repeat motif	T_a (°C)	No. of alleles	Size (bp)	H_O	H_E
BV 1	ATACTTTGGCTGCATGAAGTGC† GGTCTCACTCCTTGTGTCCC	$(CA)_{14}$	58	3	101	0.400	0.371
BV 2	CAGCATGTTATTTTGGCTGC† TTGCTAAACCGGTTAGAAGTTG	$(CA)_{11}$	58	4	136	0.633	0.705
BV 5	GTTCTGAGGGTAGAGGGACTG† GCTGAGCAGCTTCAGAAAGTC	$(CA)_{17}$	62	6	197	0.733	0.708
BV 6	AATCTGCATCCCAGTTCTGC† CCGGAGACTCTCAGAACTTAAC	$(CA)_{11}$	60	7	115	0.633	0.586
BV 7	TGAACTCCTGGAGACTTCCC† CTCCTTGTAGCGTTGCCTTC	$(CA)_{15}$	55*	5	247	0.467	0.502
BV 8	TGGCATGCTGCTATGAGAAC† GTGCTTTGCATGCTTTTACTC	$(CA)_{11}$	60	2	113	0.067	0.066
BV 9	ATCTAGGGACATCGAGGAGC† ACAGGGATGCAGGTAAGCC	$(TA)_6\,(CA)_{11}$	60	2	219	0.567	0.463
BV 11	TGTTTGCAAGCTGGAGACC† AAAAGCCTTGGGGTAAGCAC	$(CA)_{22}$	62	11	181	0.867	0.884
BV 12	TCAGGTTTTGACGACCTTCC† GTGGTAACGGAGGAACAAGC	$(CA)_{15}$	62	11	245	0.867	0.857
BV 13	AAAACAGAGTTTTCACATTTTCATAAG TTCAGGAAACAGAAGCATGAAC†	$(CA)_{16}$	50	10	184	0.900	0.900
BV 14	GGCAGTGTGGAGCCTACATC† CTCCAGGGTCCTTGTTTGC	$(CA)_{16}$	60	6	179	0.733	0.730
BV 16	CCCCTCACCTCACAGTCAC† GGAGTGATTTTCATTGTCTTGC	$(GA)_3(CA)_3A_{13}(GA)_{13}$ $AACC(GA)_8$	62	13	221	0.867	0.825
BV 17	TGATGTGCAGATGCGTGAC† GGACTCTGATGAAGCCAAGC	$(CA)_{11}$	58	2	199	0.267	0.325
BV 20	GAACAGCACTGAACGTGAGC† GTTTCTCCTGACAGTGAAATAACTC	$(CA)_{13}$	58	3	141	0.267	0.320

*For this PCR, a hotstart protocol is needed. PCR conditions are as described before but with HotStarTaq™ DNA Polymerase and buffer (Tris-Cl $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$; pH 8.7, Qiagen) and an initial denaturing step of 95 °C for 15 min.
†Fluorescent labelled primer.

as described in Tenzer *et al*. (1999). Retained fragments were amplified in a second PCR as above without the initial extension step. PCR products were digested with *Eco*RI (Amersham Pharmacia) in preparation for ligation with dephosphorylated pUC18 (precut *Eco*RI/BAP, Amersham Pharmacia). Following transformation of JM109 High Efficiency competent cells (Promega), plating onto selective agar media and dot-blotting colonies onto Nylon-Membrane (Hybond™-N+, Amersham Pharmacia), the library was screened for inserts containing CA and GA repeats using oligonucleotide probes labelled with ECL3′-oligolabelling and detection system (Amersham Pharmacia). Hybridization was carried out in accordance with the manufacturer's instructions.

Plasmids from positive clones were sequenced using M13 forward and reverse primers and the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequences were analysed on an ABI Prism 310 Genetic Analyser and edited with Sequence Navigator Software (PE Biosystems). Primers were designed using Primer 3 software (Rozen & Skaletsky 1998). Where possible, we considered only primers that did not bind to a template thymine or cytosine residue at the 3′ end because these nucleotides are most likely to be degraded in aDNA (Pääbo 1989). One primer for each pair was labelled with fluorescent dye (Table 1).

To assay variation among individuals, amplifications were performed in 10 μL volumes containing 10 ng of genomic DNA, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 150 μM per dNTP, 0.5 μM of each primer, 0.5 U of *Taq* DNA Polymerase (Amersham Pharmacia), and the following thermotreatment: 25–30 cycles of 30 s at 95 °C, 30 s at locus specific annealing temperature (Table 1) and 30 s at 72 °C. An initial denaturation step (95 °C for 5 min) was included and the last cycle was followed by an 8-min extension. Amplified products were diluted and mixed with formamide containing GENESCAN-350(ROX) Size Standard (PE Biosystems) and the genotype was determined on an ABI Prism 310 Genetic Analyser using GeneScan Analysis® software version 2.1 and Genotyper® software version 2.1 (PE Biosystems). We screened 30 bearded vulture individuals from the captive population (Table 1), 15 Egyptian vultures, *Neophron percnopterus*, 15 black vultures, *Aegypius monachus*, from Spain, and 10 griffon vultures, *Gyps fulvus*, from France (Table 2).

Table 2 Results of the cross-species amplification. Summarized are locus specific annealing temperature (T_a), PCR product size range and number of alleles for each of the species tested. When amplification did not result in a clear allelic pattern, received fragment sizes are listed below

	Egyptian Vulture (n = 15)			Griffon Vulture (n = 10)			Black Vulture (n = 15)		
Locus	T_a (°C)	Product size (bp)	Number of alleles	T_a (°C)	Product size (bp)	Number of alleles	T_a (°C)	Product size (bp)	Number of alleles
BV 1	50	100	1	50	91	1	58	91	1
BV 2	55	123	1	55*	119	1	55	118	1
BV 5	58*	190	1	58*	177–183	4	58	189–201	4
BV 6	58	95–117	5†	58	118–120	2	55*	127–161	4‡
BV 7	No amplification			No amplification			No amplification		
BV 8	58	106	1	58	106	1	58	103	1
BV 9	58*	224–228	3	58	207	1	58	205	1
BV 11	58	148–150	2	58	152–162	4	58	164–180	7
BV 12	58	235–239	3	58*	243–279	7§	58	227	1
BV 13	50	170–176	4	50	172–178	3	50	174–176	2
BV 14	55	161–163	2	55	162–164	2	55	158	1
BV 16	58	179–185	4	58	184–188	3	55	160–170	4
BV 17	55*	185	1	55*	185–187	2	55	185–187	2
BV 20	55	133–135	2	55	132–138	4	55	136–140	3

*For this PCR, a hotstart protocol is needed. PCR conditions are as described before but with HotStarTaq™ DNA Polymerase and buffer (Tris-Cl $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$; pH 8.7, Qiagen) and an initial denaturing step of 95 °C for 15 min.
†Amplification products are (in bp): 95, 97, 105, 115, 117.
‡Amplification products are (in bp): 127, 133, 139, 161.
§Amplification products are (in bp): 243, 245, 259, 263, 265, 267, 279.

We received an individual genetic fingerprint for all analysed captive birds [probability of identity for sibs (PIsibs) = 7.8×10^{-5}; see Taberlet & Luikart 1999)], showing that apart from the conservation genetic analysis mentioned above, these microsatellites will provide an important tool in the long term monitoring of the released population in the Alps.

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Microsatellites for Barren Brome (*Anisantha sterilis*)

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Barren Brome *(Anisantha sterilis*: synonym *Bromus sterilis)* is a diploid grass weed of cereals which naturally occurs in field margins and waste ground. It is an inbreeding annual which can invade and compete with cereal crops especially when cereals are grown repeatedly with minimum cultivation (Cussans *et al*. 1994). Investigations into the genetic diversity within *A. sterilis* may provide indications of its ability to respond to future control measures. Here, we describe the identification of polymorphic microsatellites in *A. sterilis* for population genetic studies.

A small-insert genomic library, enriched for microsatellites, was developed using a modified procedure of Edwards *et al*. (1996). Genomic DNA (2 µg) was digested with *Rsa*I and *Ssp*I. An *Mlu*I adapter [21-mer: (5′-CTCTTGCTTACGCGTGGAC-TA-3′) and phosphorylated 25-mer: (5′-pTAGTCCACGCGTA-AGCAAGAGCACA-3′)] was ligated to the ends of the DNA

fragments. Five identical reactions (25 μL each) were prepared: 2 μL ligated DNA, 1× polymerase chain reaction (PCR) buffer (10 mM Tris-HCl, pH 8.5, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatine), 200 μM each dNTP, 300 ng 21-mer and 1 U *Taq* polymerase (GibcoBRL). Amplification proceeded for 20 cycles (94 °C for 20 s, 60 °C for 1 min and 72 °C for 3 min) using a Perkin Elmer 9600 Thermal Cycler. Reactions were pooled, purified by phenol–chloroform extraction, concentrated by ethanol precipitation and resuspended in 25 μL sterile distilled water (SDW).

Oligonucleotides [$(CA)_{15}$, $(CT)_{15}$] were cross-linked by UV irradiation to separate 0.7 cm^2 nylon membranes (Hybond N^+, Amersham), then used to hybridize the amplified DNA in one tube, at 45 °C overnight. Filters were washed four times in 2× SSC (20× SSC: 3 M NaCl, 0.3 M Na Citrate, pH 7) at 65 °C for 5 min and three times in 1× SSC at 65 °C for 5 min. Eluted DNA was ethanol precipitated and resuspended in 25 μL SDW. Five identical reactions (25 μL) were prepared: 2 μL DNA, 1× PCR buffer, 400 μM each dNTP, 200 ng 21-mer and 2 U *Taq* polymerase. Amplification proceeded for 25 cycles (94 °C for 30 s, 60 °C for 1 min and 72 °C for 3 min). Reactions were pooled, purified, concentrated and resuspended as before. DNA was digested with *Mlu*I and fragments were selected using a Size Sep™ 400 Spun Column (Pharmacia). Fragments were cloned into pJV1 vector and transformed into Epicurian coli® Competent Cells (XL1-Blue MRF' Kan Supercompetent Cells, Stratagene). Plasmid DNA was extracted using Wizard™ Plus Minipreps DNA Purification System (Promega) and sequenced using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit. Sequences were separated on the ABI Prism 377 DNA Sequencer. Primers were designed using PRIMER version 0.5 (Whitehead Institute for Biomedical Research, Massachusetts) and synthesized by Genosys Inc.

Table 1 Microsatellite library characterization

Clones sequenced	97
Microsatellites >18 base pairs	56
Of these: CA/GT repeats	41
CT/GA repeats	6
CT/GA & CA/GT repeats	6
Duplicated sequences	3
Primers designed	17
Monomorphic	2
Unscorable	3
Multilocus	2
No product	1
Informative	9

Leaf samples were collected from 20 *A. sterilis* plants at an Oxfordshire farm and six *A. diandra* plants from farms across England. Genomic DNA was extracted using the Nucleon® Phytopure Extraction Kit (Amersham). DNA was also extracted from one seed of *A. rigida* (Australia). For genotyping, the forward primer was end-labelled with [γ^{33}P]-ATP (Amersham). Microsatellite amplification was performed in 12.5 μL reactions: 5 ng DNA, 1× PCR buffer, 200 μM each dNTP, 25 ng forward primer, 25 ng reverse primer, 0.5 U *Taq* polymerase. Amplification proceeded for 35 cycles (94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min) and one cycle of 72 °C for 10 min. PCR products were separated on 6% polyacrylamide denaturing gels using M13 control sequence as a size marker and exposed to Kodak Biomax MR-1 film overnight.

Table 2 Primer sequences and characteristics of *Anisantha sterilis* microsatellites

Locus	Primer sequences (5'–3')	Repeat motif	Size range (bp)	No. of alleles	Gene diversity
AS115 *†	**F:** GTTGCTGCTGCCAGGCTGA **R:** TTAACAAAACAGGCAACACA	$(GT)_{19}$	117–131	3	0.585
AS124 *	**F:** GAATGTAGATAAAAACTGGTGT **R:** GCACTCACTTCATAAATTCAA	$(CA)_5AG(CA)_6AGCG(CA)_{20}$	182–184	2	0.320
AS133 †	**F:** ATGGACAACCATGGCGTGAGA **R:** TGATAGAAGTAATACGAGGCG	$(TG)_{15}C(GT)_2(TG)_3A(GT)_4$	204–210	3	0.580
AS139	**F:** AAACACCAAAAATAATTAAGG **R:** GCCCATCCAACATGTGCCAG	$(TG)_{23}$	174–188	3	0.485
AS147 *	**F:** ATTTTAGCTGATGTGCTTTTG **R:** ACTGTGGTGATCGTACCCGTG	$(CA)_{28}AA(CA)_{10}GT(CA)_{11}$	194–210	5	0.780
AS152 *	**F:** AAGGTTCAAAGTGTAAGGACG **R:** AGGAGAAGAAGAACGAGAGAA	$(GT)_3(TG)_2C(TG)_2C(TG)_8C(GT)_{14}$	178–187	4	0.685
AS184 *	**F:** CGGAATGTTGTCAGAATAGTT **R:** ACGAACCGTGGAACTTGTTAC	$(AC)_3(AT)_7AA(GT)_{16}(A)_5$	144–170	4	0.700
AS211 *†	**F:** TTCTATGTAATCATGGCTTGC **R:** TCCAAGGACCGACCGATCTC	$(CA)_{12}$	126–140	3	0.545
AS219 *†	**F:** CAGGAATTTGTCAGGTTAAG **R:** AGCTATAAAAGTAACCACATCA	$(TG)_{15}$	146–150	3	0.535

*Polymorphic in *A. diandra*; †cross-amplification in *A. rigida*.
GenBank accession nos: AF285620–AF285628.

The results of sequencing and characterizing 97 library clones are shown in Table 1. Nine primer pairs amplified polymorphic markers producing 2–5 alleles per locus and gene diversity ($D = 1-\Sigma p_i^2$) (Nei 1973) values from 0.32 to 0.78 (Table 2). No heterozygotes were observed. Cross-amplification was investigated by testing the primers on the related species *A. diandra* and *A. rigida*. All nine loci were amplified from *A. diandra* and seven were polymorphic with between two and seven alleles per locus. For *A. rigida*, four loci were amplified (Table 2).

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Development and characterization of microsatellite loci from lynx (*Lynx canadensis*), and their use in other felids

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On 24 March 2000, the United States Fish and Wildlife Service declared the Canadian lynx (*Lynx canadensis*) to be threatened throughout the contiguous United States (United States Fish and Wildlife Service Website: http://www.fws.gov/). Lynx conservation programmes have been attempted in Colorado (Kloor 1999) and are currently in development throughout the contiguous United States. Because an understanding of the genetics of wildlife populations may assist in their conservation, we set out to identify microsatellite markers that might facilitate population genetic studies of Canadian lynx.

Muscle tissue chips from a single lynx were frozen in liquid nitrogen and ground to powder. High molecular weight genomic DNA was then isolated by phenol extraction (Sambrook *et al*. 1989) and digested to completion with *Sau*3A. Fragments of 200–800 bp (size-selected as in Davis & Strobeck 1998) were cloned into *Bam*HI-linearized M13 mp18 RF, and transformed into *Escherichia coli* strain *DH5ãF'IQ* (Gibco BRL) made competent using the SEM (18 °C) method of Inoue *et al*. (1990). The library was plated in 0.7% top agarose (containing X-gal and IPTG) at a density of 1000–2000 plaques per 150 mm Petri plate.

Approximately 2600 recombinant clones were screened with a biotinylated $(GT)_{12}$ oligonucleotide probe, and clones containing putative microsatellites identified using a non-radioactive detection kit (BluGene®, Gibco BRL). Forty-one insert-containing clones screened positive in the primary platings. These plaques were picked, regrown and replated at low density in a secondary hybridization/detection screen. Inserts from 24 confirmed positive clones were polymerase chain reaction (PCR) amplified using universal M13 forward and reverse primers. PCR products were then electrophoresed in 1.0% agarose (TAE) and gel-purified using the glass powder binding method of Vogelstein & Gillespie (1979). These purified products were cycle-sequenced using a dRhodamine Terminator sequencing kit (with Amplitaq DNA polymerase FS, ABI Prism, PE Applied Biosystems) and an ABI Prism 377 DNA sequencer.

Primer pairs were designed for 10 microsatellite loci using OLIGO 4.0 (National Biosciences Inc.) and tested on lynx genomic DNA extracted from muscle samples (Alberta Fish and Wildlife). Six of these loci (Table 1) gave strong, clean PCR products. Furthermore, multiplexing allows the amplification of these six loci in three 15 μL reactions: **Lc 106, Lc 110 and Lc 118** = 0.16 μM each primer, 160 μM dNTPs, 2 mM $MgCl_2$, 0.36 U *Taq* DNA Polymerase (prepared as in Engelke *et al*. 1990) and approximately 50 ng genomic DNA [extracted using QIAamp™ spin columns (QIAGEN)] in PCR buffer (50 mM KC1, 10 mM Tris-HC1, pH8.8, 0.1% Triton-X 100, 0.16 mg/mL BSA); **Lc 111 and Lc 120** = 0.16 μM each primer, 160 μM dNTPs, 2mM $MgC1_2$, 1.44 U *Taq* DNA Polymerase and 50 ng template DNA in PCR buffer; **Lc 109** = 0.16 μM each primer, 120 μM dNTPs, 2 mM $MgC1_2$, 0.3 U *Taq* DNA Polymerase and 50 ng template DNA in PCR buffer. All cycling reactions were performed as in Davis & Strobeck (1998) and their products analysed on an ABI 377 Sequencer with Genescan and Genotyper software (Applied Biosystems).

Twenty-nine lynx tissue samples were genotyped to estimate the variability of each locus (these samples do not represent a population as they were collected from a variety of sites in Alberta over approximately 15 years). Complete genotypes were obtained with a single exception: for one individual, locus Lc 109 could not be amplified. Size ranges and variability are given in Table 1. Mean number of alleles was 6.17, and there was no significant difference between observed and expected heterozygosity.

Six additional felid species were also tested: cougar (*Felis concolour*); bobcat (*Felis rufus*); African lion (*Panthera leo*); Siberian tiger (*Panthera tigris*); Asian leopard cat (*Felis bengalensis*); and domestic cat (*Felis catus*, breed unknown). Table 2 summarizes the size range and number of alleles observed in each species. Variability in cougars does not exceed

Table 1 Size range of PCR products, variability, repeat motif and primer sequences for each locus. Expected heterozygosity (H_E) was calculated using the formula ($1-\Sigma P_i^2$)

Locus	Size range (bp)	No. of alleles	H_O	H_E	Repeat motif	Primer sequences (5′–3′)	Accession no.
Lc 106	96–108	7	0.793	0.786	$(T)_3(GT)_{17}$	F: TCTCCACAATAAGGTTAGC R: FAM–GGGATCTTAAATGTTCTCA	AF288054
Lc 109	172–182	6	0.893	0.801	$(GT)_{18}$	F: AAGTGGCAAGATTACATTC R: TET–AACATCCTTTTATTCATTG	AF288055
Lc 110	91–103	7	0.828	0.815	$(T)_3(GT)_{14}$	F: CCTTTGTCACTCACCA R: TET–CGGGGATCTTCTGCTC	AF288056
Lc 111	140–154	6	0.586	0.619	$(GT)_{17}$	F: GAGGATCATTGTGCAT R: FAM–ATCCACTCACCCTCTA	AF288057
Lc 118	133–145	7	0.759	0.766	$(T)_4(GT)_{22}(T)_2$	F: TGGGGTGGGAACTCTC R: TET–AGTGCCCCAGATTTTT	AF288058
Lc 120	196–204	4	0.577	0.551	$(T)_3(GT)_{11}(GA)_{13}$	F: TGAGCCTGAGCATACATT R: HEX–GTTTGTGAGTTGGAGCC	AF288059

FAM, TET and HEX are fluorescent dye labels (Gibco BRL).

Table 2 Survey of amplification potential in six felid species. Size ranges are given in base pairs. Number of unique alleles observed/number of alleles scored is provided in brackets. '–' indicates no PCR product, while ' + ' represents a multiple banding pattern

Locus	Cougar *Felis concolour*	Bobcat *Felis rufus*	African Lion *Panthera leo*	Siberian Tiger *Panthera tigris*	Asian Leopard Cat *Felis bengalensis*	Domestic Cat *Felis catus*
Lc 106	87–97 (2/16)	87–89 (2/6)	89 (1/2)	88–98 (2/2)	93–99 (2/2)	+
Lc 109	191–199 (2/18)	163–169 (3/6)	171 (1/2)	163 (1/2)	169 (1/2)	167–177 (2/2)
Lc 110	102–104 (2/12)	80 (1/6)	122–134 (2/2)	124 (1/2)	88–90 (2/2)	96 (1/2)
Lc 111	–	136–142* (3/6)	–	–	146 (1/2)	140 (1/2)
Lc 118	112–113 (2/16)	129–137 (3/6)	–	–	111 (1/2)	114–116 (2/2)
Lc 120	209 (1/20)	212–220 (3/6)	205–207 (2/2)	202–208 (2/2)	201–203 (2/2)	+

*Lc 111 may include an additional Bobcat allele at 194 bp.

that observed using domestic cat loci (data not shown), and patchy results for this species strongly suggest the existence of null alleles. However, the level of variation observed in lynx, and the ability of these primers to amplify microsatellites across a range of felid species, suggests they may be useful in a variety of population genetic studies.

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Variation at tri- and tetranucleotide repeat microsatellite loci in the fruit bat genus *Cynopterus* (Chiroptera: Pteropodidae)

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Table 1 Primer sequences and characteristics of nine tri- and tetranucleotide repeat microsatellite loci used in the genetic analysis of *Cynopterus sphinx* from Pune, India (18°32′ N, 73°51′ E). Repeat numbers refer to cloned alleles and plus signs denote sequence interruptions between tracts of ≥2 repeat units. T_a, annealing temperature; n, number of bats genotyped per locus; N_A, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity. Loci CSP-4, CSP-6, and CSP-7 segregated subsets of alleles that differed by 2 bp rather than 4 bp. It is not known whether this was due to interruptions within the array of tetranucleotide repeats or insertions/deletions in flanking sequences

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size range	n	N_A	H_O	H_E	GenBank accession no.
CSP-1	F: GGGGAAACAAAGGAAAAGT R: AGAAAAGTGAGACCTGACAGAG	$(ATC)_{2+4+5+3}$	55	191–218	431	9	0.73	0.71	AF289705
CSP-2	F: CCCGATGATGGATTTCTAC R: CTGGGCTGTAATAAGTGCTC	$(ATC)_{3+13+2}$	57	113–134	431	7	0.78	0.74	AF289706
CSP-3	F: AACACCACCACCACCACTA R: TGTGGCAACAACTCAGACA	$(ATC)_8$	57	95–107	431	5	0.37	0.38	AF289707
CSP-4	F: GAGAGGACTCCGTTCTTTTAGA R: ATGGATGGGTGACAGATGA	$(CATC)_{12}$	57	139–163	431	10	0.79	0.78	AF289708
CSP-5	F: CATTTGTGGTAACTTGTGATG R: ACAGCAGTGAAACTTCCTCT	$(ATGG)_8(ACGG)_4$	55	110–170	431	12	0.76	0.73	AF289709
CSP-6	F: TGAGGAGTGTTCCCGAGTA R: AAAAATCCCAACGCACAG	$(CATC)_{10}$	55	127–219	431	14	0.81	0.85	AF289710
CSP-7	F: CCACAAGAAACCCAATACTAAC R: CTTCCTAGCCCCACAATC	$(TATC)_{3+8}$	57	231–265	431	17	0.82	0.82	AF289711
CSP-8	F: CCAGGTGTTATGGGTTGA R: TGAGGTGTTGGGAGTTTG	$(TAGA)_{3+3+5+11}$	57	150–202	420	14	0.75	0.74	AF289712
CSP-9	F: GGTCCCTCTGCTCTTCAG R: AGCATGGGGAATATAGTCAAG	$(TAGA)_{3+7}$	57	278–298	431	5	0.49	0.47	AF289713

Species in the fruit bat genus *Cynopterus* (Chiroptera: Pteropodidae) are widely distributed across the Indomalayan region (Corbet & Hill 1992). The two most geographically widespread members of the genus are the short-nosed fruit bat (*Cynopterus sphinx*) and the lesser dog-faced fruit bat (*C. brachyotis*). There is considerable uncertainty surrounding the taxonomic relationship between *C. sphinx* and *C. brachyotis*, and the status of the many named forms within *C. sphinx* (Storz & Kunz 1999). The availability of polymorphic microsatellite markers for cynopterine fruit bats would greatly aid efforts to elucidate species boundaries and genetic correlates of morphological variation within species. The primary motivation for developing microsatellite markers for *C. sphinx* was to investigate the influence of polygynous mating and harem social organization on population genetic structure (Storz *et al*. 2000a,b). Efforts are also underway to investigate comparative levels of geographical differentiation in body size and microsatellites in Indian populations of *C. sphinx* (see Storz *et al*. 2000c).

Genomic DNA was isolated from wing-membrane biopsy samples of *C. sphinx* using QIAamp extraction columns (Qiagen). Microsatellite loci were isolated from three genomic libraries enriched for tri- and tetranucleotide repeat motifs following the methods of Jones *et al*. (2000). Following partial digestion with a combination of seven blunt-end restriction endonucleases, size-selected genomic fragments (350–650 bp) were ligated to 20 bp oligonucleotide adapters that contained a *Hin*dIII restriction site. Genomic fragments were subjected to magnetic bead capture using the following 5′-biotinylated oligonucleotides: ATG_8, $CATC_8$, and $TAGA_8$ (Integrated DNA Technologies). Captured fragments were Polymerase chain reaction (PCR)-amplified using primers complementary to the adapter sequences. The resultant products were ligated into the *Hin*dIII restriction site of the plasmid pUC19. Recombinant plasmids were transfected into *Escherichia coli* strain DH5α by electroporation. Colonies were screened according to the protocol of Jones *et al*. (2000). Following PCR amplification, a total of 27 clones in the size range 350–650 bp were sequenced using Prism Cycle Sequencing kits and labelled dNTP's (Applied Biosystems). Sequences were resolved on an ABI 373 automated sequencer (Applied Biosystems).

All clone sequences contained at least one microsatellite locus. Primers were designed for a total of 21 microsatellite loci using the program Designer PCR version 1.03 (Research Genetics). Primer pairs were tested by amplifying DNA from eight individual *C. sphinx* sampled from various localities in peninsular India. Sixteen primer pairs amplified variable PCR products, as revealed by electrophoresis in 3.5% agarose gels followed by ethidium-bromide staining. Nine primer pairs that yielded the most consistent results were selected for further testing, and the forward primer of each pair was fluorescently labelled with 6-FAM, TET, or HEX (Applied Biosystems). PCR was performed using 20 μM of each primer, 5 mM dNTP's, 25 mM $MgCL_2$, 0.012 U of AmpliTaq DNA polymerase (Applied Biosystems), 10× PCR buffer (100 mM Tris-HCl buffer, pH 8.3, 500 mM KCl), ddH_2O, and 10 ng of template DNA in a total reaction volume of 15 μL. Thermal cycling was performed in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55–57 °C (Table 1) for 45 s, and extension at 72 °C for

Table 2 Summary statistics for five tri- and tetranucleotide repeat microsatellite markers used in the genetic analysis of *Cynopterus sphinx* (from localities <18° N latitude) and *C. brachyotis* in peninsular India. *n*, number of bats genotyped per locus; N_A, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity. In both species, locus CSP-7 segregated multiple alleles with lengths that differed by 2 bp, even though the cloned allele was a $(TATC)_n$ repeat

	Cynopterus sphinx (southern localities)					*Cynopterus brachyotis*				
Locus	Allele size range	*n*	N_A*	H_O	H_E	Allele size range	*n*	N_A	H_O	H_E
CSP-1	191–224	189	12	0.79	0.86	176–227	111	12	0.61	0.69
CSP-2	119–134	189	6(7)	0.74	0.71	101	20	1	0	0
CSP-5	130–190	189	11(16)	0.82	0.81	110–166	111	11	0.29	0.34
CSP-7	227–285	189	21	0.78	0.84	229–263	111	17	0.72	0.86
CSP-9	286–302	189	5(6)	0.55	0.60	270–282	111	4	0.45	0.49

*Numbers in parentheses refer to numbers of alleles observed in the complete sample of *C. sphinx*, from Pune and the southern localities (*n* = 620 bats).

50 s (with a final extension at 72° for 2 min 30 s). Allele sizes were quantified using an ABI Prism 377 automated sequencer and analysed using GENESCAN software (PE Applied Biosystems).

To assess levels of variation in *C. sphinx* and *C. brachyotis*, microsatellite genotypes were obtained for a total of 731 bats (620 *C. sphinx* and 111 *C. brachyotis*). A total of 431 adults and juveniles of *C. sphinx* from a single population in Pune, India (Storz *et al*. 2000b) were genotyped at all nine loci (Table 1). A total of 185 known mother–offspring pairs were examined, and no genotypic mismatches were observed at any locus.

Using a subset of five microsatellite loci, an additional 189 *C. sphinx* that were sampled from localities in south-western India (see Storz *et al*. 2000c), and 111 *C. brachyotis* that were sampled from high-elevation wet forest sites in the Western Ghats were genotyped. In the total sample of *C. sphinx* (*n* = 620), mean number of alleles per locus was 12.4 (range = 6–21; Table 2). Although preliminary screening of 20 individuals indicated that CSP-2 was monomorphic in *C. brachyotis*, the remaining four loci segregated 4–17 alleles. Relative to *C. sphinx*, homologous loci in *C. brachyotis* segregated alleles that were generally shorter in length (Table 2). These markers should open up many new opportunities for studying the population biology and phylogeography of Old World fruit bats.

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Isolation and characterization of microsatellite DNA loci in Japanese flounder *Paralichthys olivaceus* (Pleuronectiformes, Pleuronectoidei, Paralichthyidae)

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Japanese flounder *Paralichthys olivaceus* is an important species consisting coastal fisheries resources in Japan, and is of high commercial value. Interest has been directed toward resource enhancement, and accordingly, millions of *P. olivaceus* are released into Japanese coastal fisheries grounds every year (Furusawa 1997), yet little is known about reproductive success of the stocked fish. To promote effective stocking management, it is necessary to monitor the fate of stocked fish and their relatedness apart from naturally reproduced fish. Microsatellite DNA loci are expected to provide an invaluable tool for this purpose because of the power and ability of microsatellite markers in regard to resolution for genetic relatedness among individuals (Blouin *et al*. 1996) and parentage determination (O'Reilly *et al*. 1998). Here, we describe the characterization of microsatellites isolated from *P. olivaceus* that will be useful to address the stocking effects.

The method described by Sekino *et al*. (2000) was used for cloning *P. olivaceus* microsatellites. In brief, genomic DNA was fragmented by sonication. Sonicated fragments were blunted by mung bean nuclease (Takara, Shiga, Japan), and the fragments ranging from 300–500 bp were recovered. The fragments were ligated into *Srf*I site of pCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA, USA), and recombinant plasmid vector was transformed into XL2-Blue MRF′ ultracompetent cells (Stratagene). Single-stranded DNA was prepared, and selective second-strand DNA synthesis was employed using $(CA)_{12}$ oligonucleotide and cloned *pfu* DNA polymerase (Stratagene). The resultant double-strand DNA was transformed into XL2-Blue MRF′ cells again and these transformants were referred to a $(CA)_n$-enriched library. From the library, 80 clones were randomly chosen, and plasmid DNAs were purified using GFX *Micro* Plasmid prep kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The DNA sequences were determined in both directions using Thermo Sequenase™ cycle sequencing kit (Amersham Pharmacia Biotech) in combination with KS and T3 primers and subjected to an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech). Of the 80 clones, 59 contained one or more repeat sequences. We designed 27 polymerase chain reaction (PCR) primer pairs using a Premier software package (Premier Biosoft International, Palo Alto, CA, USA). To examine microsatellite polymorphisms, PCR was employed. PCR amplification was carried out in a 20 μL reaction volume, which included 20 pmols of each primer set (one primer in each pair was 5′ end-labelled with Cy5), 100 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5 U of Ampli Taq Gold™ (Perkin Elmer, Foster City, CA, USA), and approximately 50 ng of template DNA using PC-960G gradient thermal cycler (Corbett Research, Mortlake, NSW, Australia). PCR amplification cycles were as follows: 12 min at 95 °C, 35–40 cycles of 30 s at 94 °C, 1 min at a primer-specific temperature, 1 min at 72 °C, and final elongation for 5 min at 72 °C. Analyses of PCR products were performed using ALFexpress sequencer in combination with an Allelelinks software package (Amersham Pharmacia Biotech).

All 27 microsatellite loci were successfully amplified, out of which we finally chose 16 primer sets (the remaining 11 having been rejected because their polymorphisms were low, and/or they produced unexpected PCR products in an initial sample of *P. olivaceus*) and assessed further microsatellite polymorphisms in a natural *P. olivaceus* population collected from the Japanese coast of the Japan Sea.

As shown in Table 1, the number of alleles ranged from 4–40, and the observed and expected heterozygosity ranged from 0.43–0.99 and 0.43–0.97, respectively. All but one of the 16 loci conformed to Hardy–Weinberg's (HW) equilibrium in the Markov-chain method (parameters used; 100 000 Markov-chain steps; 10 000 dememorization steps), using an Arlequin verion 1.1 software package (Schneider *et al*. 1997). At the *Po31* locus, the observed genotype frequencies showed significant departure from HW expectations ($P < 0.05$) with a large discrepancy between the observed and expected heterozygosity (0.34 and 0.91, respectively). This may be explained by sampling errors due to limited sample size or substructuring of the samples, however, this seems unlikely because the observed genotype frequencies in all other 15 loci were consistent with the expectations. We believe that the presence of null alleles (Pemberton *et al*. 1995) may be a valid explanation causing these results. Further investigation of this topic is necessary. Microsatellite DNA loci described in the present study possess hypervariability, suggesting that these loci will be useful for genetic monitoring of stocked *P. olivaceus* in furthering our understanding of stocking effects.

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Table 1 Core repeat and primer sequences, PCR amplification conditions, and results of variability of the 16 microsatellite loci in a *Paralichthys olivaceus* population. H_O is observed and H_E is expected heterozygosity

Locus	Core repeat sequence (5′–3′)	Primer sequence (5′–3′)	Anneal. (°C)	Sample size	No. of alleles	Size range† (bp)	H_O	H_E	P‡	GenBank accession no.§
Po1	$(TG)_3T_2(TG)_8$	F-GCCTTTTGTCAGCCATTAACAGAGC R-CTGAGGCCAGACATGACATTACCTT	55	67	20	160–216	0.68	0.71	0.84	AB046745
Po13	$(TG)_3GA(CA)_{13}$	F-CGGCCTAAACCTGGACATCCTCTCTA R-CGGGACAACGGAGGTTTGACTGAC	58	69	23	206–276	0.78	0.92	0.18	AB046746
Po20	$(CACG)_4(CA)_4CG(CA)_{18}C(GT)_3$	F-TGCTCCTTCACCTGCACGGCCTCAAA R-TGCACCCTGACCTGTCACTGGGGATT	58	69	40	239–379	0.99	0.97	1.00	AB046748
Po25A	$(GATG)_2A_2CA(GATG)_{10}$	F-TGAGGAGTCAGGTTTCAGGCCACT R-TCGCAGGAACACCCAGAGTACAGA	55	68	12	201–253	0.76	0.76	0.26	AB046749
Po26	$(CA)_6CGCACGGA(CA)_7$	F-ACACTGGGCCCTCTGTTAAACAC R-AGAGGAGAAAGGGCACCGAGATA	55	67	5	141–159	0.73	0.65	0.72	AB046750
Po31	$(CA)_4(GA)_2(CA)_{11}$	F-AGGGTTAATTATAGAGGACGCAG R-CTGAAACAACAACTCAGAAGACG	57	69	25	129–193	0.43	0.91	0.00*	AB046751
Po33	$(TG)_5T_2(TG)_{10}$	F-GTTGGTTTAACTGATTCATCTGCAG R-TTACATATCCCACAATGCTTCACTC	55	69	10	257–290	0.74	0.68	0.82	AB046752
Po35	$(CA)_7$	F-TGGTTCTAGTGTTTGTCTGGTGA R-CCTACAGCACAGATATGACCTTT	54	69	15	283–333	0.81	0.78	1.00	AB046753
Po42	$(CA)_5(TA)_{13}(CA)_3$	F-CGAGCGCTGTTTCAACTACGGTCATT R-ATGATGATCTAACCGTCCGGCTCCAT	55	69	23	164–224	0.88	0.91	0.67	AB046754
Po48	$(CACG)_4(CA)_5$	F-GCCTCCAGAAACATTTATGGGG R-TGTCTTGCCTCTGGTCCTTCTT	55	64	6	126–142	0.44	0.43	0.69	AB046755
Po52	$(CA)_2CG(CA)_6\ GA(CA)_5$	F-TCAGACAGAGGAGCGGGGTTGTTGC R-GCTGTACCCAGGGTTCCGCTGAAGA	58	64	4	155–163	0.46	0.50	0.62	AB046756
Po56	$(AC)_{20}$	F-TCGAGCGTAAACAAACCAGCTAACA R-GCTGAAAATCGCTTTAGCTTCCCAT	55	69	26	139–205	0.94	0.94	0.62	AB046757
Po58	$(CA)_{11}(GA)_2GC(GA)_9$	F-GCCCCTCACTGAGACTGTGACA R-CAAGGTATGTGCATGAGCAGGC	52	69	27	101–159	0.84	0.90	0.52	AB046758
Po83	$(CA)_5AG(CG)_2(TG)_3(CG)_2(CA)_{15}$	F-TGCGGTCATCATGTCTTTAAAATA R-AGCAAATGTTTGCTTTTGGATACA	57	68	32	227–313	0.91	0.93	0.18	AB046759
Po89	$TA_3(CA)_7$	F-ATCAGAAGTCATCCATGCACTGGCAC R-AGCTACTTATCCACAGGTGTCGACGG	60	69	20	252–327	0.86	0.90	0.44	AB046760
Po91	$(CA)_{18}$	F-AGGTTTCAAGGTGTTCATTGCGAGTC R-TAAAGGAAGTGCCTCACTGTGGAGAA	55	69	34	146–246	0.96	0.94	0.97	AB046761
				mean	20.1	—	0.76	0.80	—	

†Size is indicated as number of the base pairs of PCR products.

‡P is the exact P-value estimated by a test anologous to Fisher's exact test described by Schneider *et al*. (1997). Significant departure of the observed genotype frequencies from H-W expectations was determined by adding $*P < 0.05$.

§The nucleotide sequence data will appear in the DDJB/EMBL/GenBank nucleotide databases with the accession numbers.

Polymorphic microsatellite loci for primitively eusocial Stenogastrine wasps

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Social wasps of the subfamily Stenogastrinae live in South-east Asia and comprise about 50 described species in six genera (Turillazzi 1996). Most species in this tropical subfamily of Vespidae have a colony with a small number of individuals and a simple temporal division of labour which makes them a suitable group for studying the origin of sociality in wasps (Turillazzi 1991; Strassmann *et al*. 1994).

Microsatellite loci are useful tools for studying Stenogastrine population structure, relatedness within colonies and brood, and for determining males (Queller & Strassmann 1993). All of these are crucial factors for understanding Stenogastrine societies. In this paper, we describe 33 microsatellite loci isolated from *Eustenogaster fraterna* that are likely to be useful in this and many other species of the subfamily Stenogastrinae.

We made a partical genomic library of *E. fraterna* following published protocols (Strassmann *et al*. 1996), but used a positive-selection plasmid (pZErO-2.1, Invitrogen) which eliminated the need for plasmid dephosphorylation. We cut genomic DNA with *Sau*3AI then ligated the fragments ranging from 300–900 bp into pZErO-2.1 plasmids. We transformed TOP10F′ cells to obtain a 15 000 clone library which was plated into nylon. Probing library replicates with oligoncleotides of all 10 trinucleotide motifs yielded 361 positives. Southern blots of plasmid DNA confirmed 121, 70 of which were sequenced. We designed polymerase chain reaction (PCR) primers for 31 clones containing five or more trinucleotide repeats, and two clones consisting of long dinucleotide repeats (Table 1).

We evaluated these primers for heterozygosity on 24 individuals from six species following standard protocols (Strassmann *et al*. 1996). We extracted genomic DNA first and then performed PCR in a 10-μL volume with final concentrations of 50–200 ng genomic DNA, 250 nM of each primer, 100 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 1.55 mM $MgCl_2$, 1.875 μCi ^{35}S-dATP and 0.25 U *Taq* DNA polymerase under an oil overlay. We ran 40 cycles of 30 s at 92 °C denaturing, 30 s primer annealing (temperature varied depending on each primer and species) and 45 s extension at 72 °C, followed by 5 extra minutes at 72 °C to allow for the complete extension of all PCR fragments using a PTC-100 thermocycler (MJ Research). PCR products were run on 6% denaturing acrylamide gels, and an M13 sequence was used as a size standard.

Of the 33 primer pairs we designed, 29 yielded scorable microsatellite alleles. The number of alleles and expected heterozygosity of each variable locus in different species are detailed in Table 2. Out of 33 microsatellite loci, 27 were polymorphic in *E. fraterna* though heterozygosity varied from 0.20–0.81. Eighteen and 15 loci were polymorphic in *E. calytodoma* and *Eustenogaster* sp., respectively. Six loci were polymorphic in both *Parischnogaster jacobsoni* and *P. alternata*. Ten loci were polymorphic in *Liostenogaster flavolineata*. These results were congruent with the finding that polymorphisms of microsatellite loci decrease with increasing phylogenetic distance cross species (Ezenwa *et al*. 1998; Zhu *et al*. 2000).

We analysed the relationship between repeat length and heterozygosity. Repeat length was represented by two measures: (i) the number of longest perfect, uninterrupted repeats; and (ii) total number of repeats including ones with base pair imperfections. We found significantly positive correlations between heterozygosity and repeat length (both perfect $P = 0.038$ and total $P = 0.011$, Spearman correlation). We had more than two microsatellite loci in *E. fraterna* for three repeat motifs, AAG, AAT and GAC. If we split the analysis for different motif types, we found no significant relationships between repeat length (either perfect or total) and heterozygosity for these three repeat motifs. Both correlations for the GAC motif were insignificant. Small sample size leads to a lack of power and might obscure any relationship between numbers of repeats and heterozygosity for these motifs.

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Table 1 Characteristics of 28 microsatellite loci in social wasp *Eustenogaster fraterna*

Locus	Size (bp)	T_a (°C)	Repeat motif	PO	Primers sequences (5′–3′)	Accession no.
EF79TCT	176	51	$(AAG)_{13}$	F	TCGCTGTTCGACCATCG	AF225508
				R	AATTCTTACCGCCGAATGG	
EF80CTT	173	50	$(AAG)_{15}$	F	TGTCTTTCGCCTAACCG	AF225495
				R	CCTCCTGCCTGTTTCTTG	
EF91CTT	161	53	$(AAG)_{7}$	F	GACCGTTCCAACTGGCA	AF225510
				R	CGACGTGTGAAATAAAGCAGGAG	
EF92CTT	173	54	$(AAG)_{9}$	F	TCTACCGCCAACAGTCCCA	AF225511
				R	CGAACGAGAAAAGTCCAAGCA	
EF97AAG	183	49	$(AAG)_{8}AAT(AAG)_{2}$ $AAT(AAG)_{2}$	F	GGGTTCCTTTATTAGTCCAAC	AF225487
				R	TTCCTGGAGCATCCGTAAGC	
EF98AAG	246	57	$(AAG)_{9}$	F	ATTGAGATGCAGAGAGCGTCGG	AF225488
				R	AACAGGAGCACGGAGAAGAGGAAG	
EF99AAG	153	51	$(AAG)_{6}$	F	CTGTCGTTCGTTTCGTTCTTCC	AF225489
				R	AGTAGCGAGCAGATGATGATGATG	
EF103AAG	258	53	$(AAG)_{7}$	F	TCCCTTCTCCTTCTCTTCGC	AF225479
				R	CCTCCTTACTCCTTCTGGAC	
EF104AAG	125	52	$(AAG)_{14}$	F	CGACCAGTGGCGTTTCA	AF225502
				R	CCCTTACCGTTGAGACCCTG	
EF107AGA	217	55	$(AAG)_{9}$	F	AAGCAAGGACGCACAGG	AF225497
				R	ATCGACCGATGCACCGA	
EF109AAG	178	53	$(AAG)_{7}AGA(AAG)_{5}$	F	CGCCTACAGAGTTCCTTG	AF225490
				R	CGTCCTCGTTCATGGATTG	
EF131CAT	121	51	$(CAT)_{4}CGT(CAT)_{2}$	F	TCATCTTCGTTGTCCTCG	AF225480
				R	AAGCGGTTCTCTCGATG	
EF183AAG	124	49	$(AAG)_{8}GAG(AAG)_{4}$	F	GCTCTTTGGGAATTTCTCG	AF225498
				R	CGTTTCTCTTCGTCTTCG	
EF184AAC	117	51	$(AAC)_{12}AAA(AAC)_{2}$	F	GCTCACATTTTTTCCCAGTCCC	AF225481
				R	AATCTGCGTGCGTTGTTCTTG	
EF189TAA	206	48	$(AAT)_{14}$	F	CGGATCTCGTAACGACTGATA	AF225503
				R	GGAGCAAGTTGAAGGTACAA	
EF197TTC	141	50	$(AAG)_{8}$	F	ACTCGGAAGCAACCTCG	AF225504
				R	TGGAAAAGGCGGTAGAG	
EF201TCT	175	50	$(AAG)_{8}$	F	GCGTGCCTCGAACATTA	AF225499
				R	TGGAAAAGGCGGTAGAG	
EF204TTC	187	54	$(AAG)_{6}ACG(AAG)_{2}$	F	GCGTTGTCCAGTCGTTTAACA	AF225505
				R	TCGGCACGAAGACGATG	
EF211CTT	214	53	$(AAG)_{7}$	F	AGGCTCTTCAGACGCTG	AF225492
				R	TGGTGTAATCCGTGAGTGAG	
EF213AAT	238	50	$(AAT)_{10}$	F	GCGATTTGAAGAAGCATTTAGTCG	AF225483
				R	CAGGAAGTATATTAAGTGAAGCGTG	
EF217GA	237	52	$(AG)_{4}AA(AG)_{19}$	F	GAAACTTTGCTCGCACACTG	AF225484
				R	TCTATTCAGGGGAGGAAAAGC	
EF229AAG	175	52	$(AAG)_{14}TAG(AAG)_{3}$	F	TGTAGGAAGAACGAAGGGTG	AF225506
				R	GAGTGATTGATGGTCCGAGA	
EF238AAT	212	48	$(AAT)_{12}$	F	GGATCACCGTGTAAAGACG	AF225500
				R	CGATTTTTCTCGTTCGACGAAG	
EF280GCA	252	55	$(CAG)_{3}CAA(CAG)_{5}$	F	CGCAGTCATCGCTTTTCA	AF225493
				R	CCTAACCTACCCCAATCG	
EF290CCT	121	54	$(GAG)_{8}AG(GAG)_{2}$	F	TCTTGCGTTCGTTCGGA	AF225501
				R	GCAGAGCGGAAAAAAGGG	
EF293CAG	197	55	$(GAC)_{2}AAC(GAC)_{6}$	F	CGCAGTCATCGCTTTTCA	AF225494
				R	GCATCGGCAACAGGAAA	
EF299TGC	184	55	$(GAC)_{9}$	F	AGCTATCTCGGCTGTCG	AF225507
				R	CTCCATCCATCCATCCA	
EF318CAG	144	58	$(GAC)_{15}$	F	GTTTATCGCTCGTTGCTATCGG	AF225486
				R	CTCCTATCCATCGCCCTTTCTC	

T_a (°C), Annealing temperature; PO, primer orientation; F, forward primer; R, reverse primer.

Table 2 Expected heterozygosities and number of alleles in different wasp species

	Expected heterozygosity (number of alleles)					
Locus/Species	*Eustenogaster fraterna*	*E. calyptodoma*	*E.* sp.	*Parischnogaster jacobsoni*	*P. alternata*	*Liostenogaster flavolineata*
EF79TCT	0.620 (3)	0.610 (3)	0.560 (3)	+	0.000 (1)	0.000 (1)
EF80CTT	0.809 (7)	0.000 (1)	0.000 (1)	+	+	+
EF91CTT	0.521 (3)	0.000 (1)	0.000 (1)	0.500 (2)	0.500 (2)	0.500 (2)
EF92CTT	0.198 (2)	0.720 (4)	0.480 (2)	0.625 (3)	0.000 (1)	0.720 (4)
EF97AAG	0.620 (3)	0.716 (4)	0.625 (3)	+	+	0.000 (1)
EF98AAG	0.711 (5)	0.375 (2)	0.480 (2)	0.000 (1)	0.500 (2)	+
EF99AAG	0.579 (3)	0.000 (1)	0.000 (1)	0.630 (2)	0.480 (2)	+
EF103AAG	0.710 (5)	0.444 (2)	0.444 (2)	0.000 (1)	0.000 (1)	+
EF104AAG	0.791 (6)	0.722 (4)	0.560 (3)	+	+	+
EF107AGA	0.716 (5)	0.500 (2)	0.560 (3)	+	+	0.500 (2)
EF109AAG	0.661 (3)	0.444 (2)	0.444 (2)	+	0. 000 (1)	0.610 (3)
EF131CAT	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	+	0.500 (2)
EF183AAG	0.615 (3)	+	+	+	+	+
EF184AAC	0.684 (5)	0.480 (2)	0.720 (4)	+	+	0.611 (3)
EF189TAA	0.806 (6)	0.625 (3)	0.625 (3)	+	+	+
EF197TTC	0.444 (2)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.375 (2)
EF201TCT	0.463 (2)	0.375 (2)	0.000 (1)	0.445 (2)	0.000 (1)	0.000 (1)
EF204TTC	0.639 (2)	0.375 (2)	0.000 (1)	+	+	0.500 (2)
EF211CTT	0.444 (2)	0.000 (1)	+	+	+	+
EF213AAT	0.645 (3)	0.000 (1)	0.000 (1)	+	+	+
EF217GA	0.805 (7)	0.833 (6)	0.500 (2)	+	+	+
EF229AAG	0.667 (4)	0.444 (2)	0.000 (1)	0.000 (1)	+	0.000 (1)
EF238AAT	0.531 (3)	+	+	+	+	+
EF280GCA	0.716 (4)	0.667 (4)	0.500 (2)	0.480 (2)	0.500 (2)	0.444 (2)
EF290CCT	0.791 (6)	+	+	+	+	+
EF293CAG	0.755 (6)	0.722 (3)	0.500 (2)	0.000 (1)	0.444 (2)	0.000 (1)
EF299TGC	0.678 (4)	0.500 (2)	0.500 (2)	0.480 (2)	0.500 (2)	0.500 (2)
EF318CAG	0.597 (4)	0.667 (3)	0.625 (2)	+	+	+
Number of polymorphic loci	27	18	15	6	6	10

+, no PCR product.

Microsatellites from the compact genome of the green spotted pufferfish (*Tetraodon nigroviridis*)

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The green spotted pufferfish (*Tetraodon nigroviridis*) is an euryhaline species native to rivers and estuaries of South-East Asia (Kottelat *et al*. 1993). Beside the Japanese pufferfish (*Fugu rubripes*) (Brenner *et al*. 1993), *T. nigroviridis* is also becoming a model for cytogenetic and genomic studies (Grutzner *et al*. 1999; Roest Crollius *et al*. 2000a), because of its small genome (350 Mb). Green spotted pufferfish has not been bred in captivity and little is known about its biology, especially reproduction. Most researchers rely on individuals collected from the wild, but very little is known about natural populations of the species.

Microsatellites have been successfully applied to assess genetic diversity, population structure and individual relatedness in animals and plants. To our knowledge, no microsatellite has been characterized in *T. nigroviridis*. This paper describes the isolation and characterization of seven microsatellites from the genome of this pufferfish species.

Thirty-two adult green spotted pufferfish were obtained from a local fish dealer in Singapore. Genomic DNA was extracted from muscle using a standard phenol–chloroform extraction protocol. A $(CA)_n$-enriched plasmid library was constructed using DNA from one fish as described previously (Yue *et al*. 2000). More than 7500 clones were obtained, most of them (approximately 99%) were white on plates containing X-gal. The insert length of clones was checked by colony polymerase chain reaction (PCR) using M13-20 and M13-reverse primers. Out of 192 clones tested, 58 contained inserts in the size range of 250–1000 bp. Colony PCR products of these clones were purified and sequenced as described (Yue *et al*. 2000).

Table 1 Characterization of six microsatellites on 32 individuals of the green spotted pufferfish (*Tetraodon nigroviridis*)

Locus	Repeat motif	Primer (5′–3′)	T_a (°C)	$MgCl_2$ (mM)	No. of alleles	Size range (bp)	H_O	H_E	GenBank Accession no.
PF12	$(CA)_{11}$	F HEX-CAGGCCTGGACAAACAAAAC R ATCTTCAAAGTGGCGCTATCATT	55	1.5	22	176–240	0.95	0.91	AF283467
PF29	$(GCA)_{13}$	F HEX-TGAGCCGATCAAGTAGTGAG R GAATGATAGTGCTGCTGGGG	55	1.5	11	120–159	0.84	0.84	AF283468
PF39	$(GT)_8$	F CTTGGATGTGACAGCGAAACAAAC R GCGCGTACGCACAGGCGGG	60	1.5	5	158–180	0.59**	0.75	AF283470
PF41	$(GA)_2(CA)_6GACTGAAG(CA)_3$	F ACAAACACGGTCAACAAGCACTAC R ACAGGTGTTCTTTGGCGTGACA	55	1.5	17	162–198	0.59**	0.90	AF283471
PF203	$(GT)_6(GA)_7(GT)_6(GA)_8(GT)_{10}$	F TGGTGACCATTAGGGTAAGG R GGGGGTGAAACGACCTC	45	3.0	12	220–258	0.22**	0.87	AF283472
PF204	$(GACA)_{10}$	F CTCGCCATGCAAAGAAAA R AAACGTTAAAGGTAGTGATGTGG	50	1.5	9	130–154	0.72	0.78	AF283473

T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; **, Loci showing significant ($P < 0.01$) deviations from Hardy–Weinberg equilibrium by using chi-square analysis. HEX: Primer labelled with the fluorescent dye HEX.

Out of the 58 clones sequenced, only 7 (~12%) contained microsatellite repeats, which is about 6 times lower than the percentage obtained from Asian arowana (Yue *et al*. 2000) by using the same enrichment procedure. This result might indicate low abundance of CA-repeats in the genome of *T. nigroviridis*. However, data from large scale sequencing performed on the *T. nigroviridis* genome do not support this hypothesis (Roest Crollius *et al*. 2000b). Alternative interpretation of the result might be that the CA-repeats in this compact genome are relatively short and are difficult to enrich by the method used. Among the seven microsatellites isolated, only five contained CA/GT repeats, the other two comprised GCA-repeats and GACA-repeats, respectively (Table 1).

Primer pairs were designed to the flanking sequences of repeats using software PrimerSelect (DNASTAR). Thirty-two *T. nigroviridis* individuals were genotyped for the seven microsatellites as described previously (Yue *et al*. 2000), except using different annealing temperatures (in the range of 45–60 °C) and $MgCl_2$ concentrations (1.5 or 3.0 mM; see Table 1). Six out of seven microsatellites showed specific products and polymorphism (Table 1), while locus *PF33* was not polymorphic. The average number of alleles at the polymorphic loci was 12.7 (range: 5–22), whereas the average observed heterozygosity ranged from 0.22–0.95 with an average of 0.63 (Table 1). Three loci (*PF12, PF29, PF204*) conformed to Hardy–Weinberg expectations when tested using chi-square analysis, while the other three did not. A significant heterozygosity deficit was displayed at locus *PF203*, suggesting the appearance of null alleles.

One duplex-PCR was established for the *PF12* and *PF29* loci according to Yue *et al*. (1999). The 25 μL reaction contained 10 mM Tris-HCl (pH 8.8), 150 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.2 μM each of *PF12* primers, 0.4 μM each of *PF29* primers, 30 ng genomic DNA and 1.0 U DyNAzyme II DNA-polymerase (Finnzymes). PCR cycling conditions were: an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 5 min. The detection of PCR products and the sizing of alleles were performed on the ABI 377 sequencer as described previously (Yue *et al*. 2000).

In order to become a good model not only for genomics, but also for genetics and developmental biology, *T. nigroviridis* must be bred routinely in captivity. The polymorphic microsatellite markers described here will assist the analysis of natural populations and breeding experiments.

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Polymorphic DNA microsatellites identified in the yellow dung fly (*Scathophaga stercoraria*)

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Sperm competition in yellow dung flies (*Scathophaga stercoraria*) has been extensively investigated since Parker's (1970a) seminal work (e.g. Parker & Simmons 1991; Ward 1993; Hosken & Ward 2000; reviewed in Hosken 1999). These flies serve as a model system for understanding the mechanisms and outcomes of sperm competition in internal fertilizers. Invariably however, these investigations have been laboratory based, and typically involved competition between only two males. How the results of such studies relates to free-living flies is unknown, but it is unlikely that the experimental conditions employed exist in nature, and therefore outcomes may not reflect true female sperm utilization patterns (Eady & Tubman 1996). This is exemplified by a study of sperm competition in pseudoscorpions, which showed that second-male mating advantage breaks down when females mate with more than two males (Zeh & Zeh 1994). In addition, Ward (2000) has shown that females are able to subtly alter paternity patterns under conditions that are likely to be common in the field. With this in mind, our aim was to develop appropriate genetic markers to allow paternity to be accurately assigned in clutches laid by free-living female yellow dung flies.

A subgenomic library enriched for CA repeat microsatellites was constructed following standard protocols outlined in Tenzer *et al*. (1999), with slight modifications. Genomic DNA isolated from a single *S. stercoraria* male using standard phenol–chloroform extraction and ethanol precipitation (Sambrook *et al*. 1989) was digested using *Tsp*509I (New England Biolabs). A 500–1000 bp size fraction was isolated from a LM-MP agarose (Boehringer Mannheim) gel by first excising the appropriate size range from the gel. The gel fragment was melted in a 65 °C water bath and volume was increased to 500 μL using double distilled water. An equal volume of equilibrated phenol (pH 8.0) was added, the solution vortexed briefly and then put at – 80 °C for 30 min. The sample was then thawed and extraction was completed following standard phenol–chloroform extraction methods (Sambrook *et al*. 1989). This isolate was used for ligation with TSPADSHORT/TSPADLONG linkers (Tenzer *et al*. 1999) and then amplified via the polymerase chain reaction (PCR), using TSPADSHORT as a primer. PCR was performed using the following conditions: Total reaction volume was 25 μL included 100 ng DNA, 1 U *Taq* DNA polymerase (Quantum-Appligene), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% TritonX100, 0.2 mg BSA (Quantum-Appligene), 100 μM of each dNTP (Promega), and 1 μM of TSPADSHORT. PCR was performed on a Techne Genius thermocycler (Techne Ltd) using the following thermotreatment: 2 min at 72 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. A total of 32 PCRs were carried out, pooled, cleaned and concentrated to minimize the likelihood of redundant products being detected during screening for positive clones. PCR products were hybridized to biotinylated $(CA)_{20}$ probes bonded to streptavidin-coated magnetic beads (Dynabeads M-280 Streptavadin, DYNAL, France) and amplified again. These final PCR products were cloned following the Original TA Cloning® Kit (Invitrogen) protocol. White colonies were dot-blotted onto nylon membranes (Hybond™-N^+, Amersham Pharmacia) and screened for CA repeats using the ECL 3′-oligolabelling and detection system (Amersham Pharmacia) and a 40mer CA oligonucleotide. All positive clones were sequenced following the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit protocol, version 2.0 (PE Biosystems) using M13 forward and reverse primers, and using the ABI 377 automated sequencing system (PE Biosystems). Primers were designed using Primer3 software (Rozen & Skaletsky 1998) and all oligonucleotides were synthesized by Microsynth GmbH (Switzerland). Initial tests for amplification and polymorphism were carried out at 55 °C and electrophoresed on 8%, nondenaturing, 14.5 cm × 17 cm acrylamide gels at 80 V overnight. Those primers that amplified polymorphic products using five test templates were used for all following analyses.

Only field-caught male *S. stercoraria* were used for PCR analysis, as almost every field-caught female is already mated (Parker 1970b), and extraction from fertilized females could therefore result in contamination by sperm DNA. Each sample male was extracted using the QIAamp® DNA mini kit (Qiagen). Twenty males were used to characterize suitable primers, and PCR was carried out using approximately 100 ng of template DNA and the following cycle treatment; initial step of 3 min at 94 °C, followed by 27 cycles of 30 s at 94 °C, 30 s at 58–61 °C (see Table 1), and 30 s at 72 °C, with a final extension step of 2 min at 72 °C. Total reaction volume was 25 μL and contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton × 100, 0.2 mg BSA (Quantum-Appligene), 100 μM of each dNTP (Promega), 0.5 μM of both forward and reverse primer, and 0.5 U *Taq* DNA polymerase (Quantum-Appligene). All products were electrophoresed on Spreadex™ EL-300 S-100 gels (Elchrom Scientific AG, Switzerland), using the SEA 2000™ advanced submerged gel electrophoresis apparatus (Elchrom Scientific AG, Switzerland). Gels were run at 100 V for 80–90 min, depending on allele sizes, then scored against the M3 Marker ladder (Elchrom Scientific AG, Switzerland). Expected and observed counts for homozygotes/heterozygotes were determined using GENEPOP version 3x (Raymond & Rousset 1995) and homozygote excess was tested for using Chi-square analysis (null hypothesis rejected at $P < 0.05$).

A minimum of five alleles were detected at each of the loci listed in Table 1. Tests for homozygote excess were only

Table 1 Primer sequence and related information for eight microsatellite loci developed for *Scathophaga stercoraria*. Both repeat motif and size of amplification product are based on that detected in the original sequenced clone (GenBank Accession nos: AF292121–8). *n*, number of individuals tested; T_a, annealing temperature; H_O, observed number of homozygotes; H_E, unbiased average heterozygosity estimate (Nei 1978)

Locus	Primer Sequences (5′–3′)	Repeat motif	T_a (°C)	*n*	No. alleles	Size (bp)	H_O	H_E
SsCA3	CCTCAACCCCCTCACTCAC CATCATCATTTAAGTCAACATTAGAAA	$(AC)_1(A)_2(AC)_{11}$ $(A)_3(C)_2(A)_3$	60	20	11	120	0.35	0.795
SsCA16	GACTTTGGTCCGTTGTAGTCC TTGGCGTCACCATACTCAAC	$(C)_3AT(AC)_{11}AT$ $(AC)_2(C)_3$	60	20	7	101	0.10	0.806
SsCA17	AATAAAAACTCAACCAACCATACAC CCTTACTCGATAAGTTGGTATTTGTG	$(TA)_2GA(CA)_4CG$ $(CA)_5$	60	18	6	108	0.40	0.695
SsCA20	TGTTTGCTGGTGCTACCG TGATCGTTGTTGTTTCATACG	$(CA)_{10}$	60	18	5	120	0.55	0.600
SsCA24	CACACACTCGCAGCTACACC AAACTTTAACTTCGATTTTTGCTG	$(C)_4AT(AC)_9$	60	20	8	120	0.30	0.821
SsCA26	TGCCACTTTTGGTGCTTTC CAGCAAAAACCGGCAAAC	$(CA)_{11}(T)_2(CA)_2CG(CA)_4CG$ $(CA)_4(T)_2(CA)_2(T)_2(GTT)_2$	61	20	8	110	0.25	0.845
SsCA28	GTTTGAAACCCTTAAGATAAAAACTC CCATCTTTCACGGGATTTTG	$(CT)_2(CA)_5AACG$ $(CA)_{10}$	58	20	13	127	0.35	0.890
Ss63T7	AAAGAATTTTACGAATTGTGTCTGG CAACAAATGCAACAAATGACC	$(CA)_6(A)_2(CA)_8$	58	18	8	129	0.20	0.869

significant at one locus, SsCa28, which may suggest one or more null alleles operating at this locus.

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Polymorphic microsatellite loci in vespertilionid bats isolated from the noctule bat *Nyctalus noctula*

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Prolonged sperm storage increases the possibility of sperm competition because several males could contribute sperm during the sperm-storing period prior to fertilization. The longest sperm storing capacity among mammals is documented in the noctule bat. Females can store sperm in the uterus after copulation in autumn for up to six months until fertilization in spring (Racey 1973). Therefore, noctule bats are likely candidates to show high levels of multiple paternity that could be analysed most efficiently with highly polymorphic nuclear markers, such as microsatellite loci.

Microsatellite loci of the noctule bat were isolated from a size-selected partial genomic library (Rassmann *et al*. 1991). Total genomic DNA was isolated from muscle tissue of a female bat and was digested with three restriction enzymes (*Alu*I, *Hae*III and *Rsa*I). Fragments ranging from 300–600 bp in length were ligated in *Sma*I digested M13mp18 and M13mp19 cloning vectors (Yanisch-Perron *et al*. 1985). Ligation products were transformed into competent XL-1 Blue cells (Stratagene) which were plated onto LB pates containing X-gal and IPTG. A total of 5400 clones were screened with different probes specific for microsatellites. Radioactive ^{32}P-labelled probes for di- and trinucleotide micosatellite loci were generated by slippage synthesis (Schlötterer & Tautz 1992) using the following pairs of oligonucleotides: $(AG)_7/(TC)_4$, $(GT)_7/(CA)_4$, $(TCC)_5T/(GGA)_3$, $(CCA)_5/(GGT)_3$, $(TGC)_5/(GCA)_3$ and $(TCG)_5T/(ACG)_3$. One hundred and forty-five 'positive' clones were detected with dinucleotide polymers, 54 with trinucleotide polymers, 11 with the ^{32}P-end-labelled oligonucleotide $(ATCC)_3$ and 14 with the ^{32}P-end-labelled oligonucleotide $(CTAT)_5$ representing 2.7, 1.0, 0.2 and 0.3%, respectively, of the total number of clones which were screened. Thirty-one of the 224 'positive' clones were sequenced and polymerase chain reaction (PCR) primers were designed for 14 loci.

A circular wing clip of 4 mm diameter was obtained from individual bats. Approximately 0.5 μg DNA was isolated after a 3-h incubation with 0.1 mg proteinase K in 500 μL digestion buffer (100 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 42 mM dithiotreitol, 2% sodium dodecyl sulphate) following the protocol of Müllenbach *et al*. (1989). PCR amplifications were carried out in a 10-μL volume containing approximately 10 ng DNA, 1.5 mM $MgCl_2$, 0.5 μM each primer, 0.025 mM each dNTP, 0.25 unit Goldstar Polymerase (Eurogentec) together with the reaction buffer provided by the supplier [final concentration: 75 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20]. PCRs were performed in a Perkin Elmer DNA Thermal Cycler TC1 and consisted of 30 cycles of 94 °C for 30 s, the annealing temperature (Table 1) for 20 s and 72 °C for 30 s. For each microsatellite locus one primer was labelled with a fluorescent dye. The PCR products were separated on 6% Sequagel®XR gels (National Diagnostics) in a LI-COR DNA sequencer (model 4000 L), and genotypes were determined using RFLPscan™ (Scanalytics).

Highly specific amplification products were obtained at 13 of the 14 loci. For each animal only one or two major amplification products were detected which were close to the length of the cloned allele. Allele sizes varied in multiples of the repeat size only and characteristic slippage bands could be detected at all loci. At two loci the proportion of homozygotes was much higher than expected under Hardy–Weinberg equilibrium. At locus P22a only one allele could be amplified in all males. In females no deviation from Hardy–Weinberg expectation was detected. This strongly suggests that locus P22a is located on the X chromosome. The deficit of heterozygotes at the other locus was not limited to males. Therefore, the presence of null alleles is a likely explanation. This locus was not evaluated further.

Cross-species amplification was tested in 11 European bat species of the family Vespertilionidae. Nine loci could be amplified in other species and eight in other genera than the source species. Allelic variation was usually high and only in five cases the loci seemed to be monomorphic in a particular species (Table 2). The applicability of primer pairs is high among species within the family Vespertilionidae but seems to be low in other families (Burland *et al*. 1998). Length of amplification products can vary substantially among species. For example the amplification product of locus P217 was about 400 bp longer in the three species of *Eptesicus* and *Plecotus* than in all other species.

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Table 1 Primer, annealing temperature and sequence of 12 microsatellite loci isolated from the noctule bat (*Nyctalus noctula*). Primers labelled at the 5′-end with the fluorescent dye IRD-41 or IRD-800 are marked with an asterisk

Locus	Primer	Sequence	Annealing temperature (°C)		Amplification of the cloned allele		GenBank accession no.
			N. noctula	other species	length (bp)	microsatellite sequence	
P2	Mü418	5′-ATATACTTAAGGATCAGAGC-3	48	48	98	$(AT)_2(GT)_2(AT)(GT)_2$ $(AT)(GT)_3(AT)_2(GT)_{12}$	AF141645
	Mü419*	5′-TATTGTTCTGTTCATTCAGT-3′					
P11	ER22*	5′-AAAACCAAAGTTATTTATTC-3′	52	—	132	$(GT)_{15}$	AF273675
	ER23	5′-CTTTCCTCAGAAATTATATC-3′					
P13	Mü360*	5′-CCTGATAAAACCTGT-3′	52	52	140	$(TG)_4C(GT)_{19}$	AF141646
	Mü361	5′-CTGAATCGGTGTTTC-3′					
P14	Mü397*	5′-TGGTGATTTGTTATG-3′	40	—	112	$(TG)_{17}$	AF273676
	Mü398	5′-CACTTATCATTTTCA-3′					
P19	ER28*	5′-TCTAATCTCTTTCTGCACCC-3′	53	—	114	$(AC)_{19}$	AF273677
	ER29	5′-GGGGCATGGAAATTGAACAG-3′					
P20	ER47*	5′-CTTATCTAATCAATATACTTAAAA-3′	45	40	176	$(TA)_{21}(TG)_{17}TAT(TA)_6$	AF141647
	Mü435	5′-AAAATGCATCAATATATGAG-3′					
P22a	ER37*	5′-CTTCTCCCTTCCCATAAATC-3′	48	40	113	$(AT)_5(AC)_4AT(AC)_{10}AT(AC)_{13}$	AF273678
	ER25	5′-TCTTATTTTGGGGGAAACTG-3′					
P217	ER49*	5′-TCCTAAGATTCTGTTCCTCC-3′	48	48	251	$(CTAT)_2CAT(CTAT)_{11}$ $(CATCTAT)_4(CTAT)_2$ $(CATCTAT)_2TAT(CTAT)_3$ $(CAT)_2CTAT$	AF141648
	ER6	5′-GGGCTGTATCATATGATTTT-3′					
P219	ER36*	5′-CAATTTAACTTTTCAACAAC-3′	48	48	157	$(CTAT)_7(CCAT)_6$	AF141649
	ER5	5′-TCTTCATTTCCTCTCCTCTC-3′					
P223	ER1*	5′-TCCATTTTTTCCCCTTCCCT-3′	48	48	110	$(CT)_7CCCTC(CTAT)_9$	AF141650
	ER2	5′-GGTCTCCTTTTCTTCACTTTG-3′					

Table 2 Polymorphism of microsatellite loci in different vespertilionid bat species. The source species was *Nyctalus noctula*. The loci NN8 and NN18 were previously published by Petri *et al*. (1997). No amplification is marked by a dash. Locus P22a is located on the X-chromosome. Therefore, the observed heterozygosity corresponds only to females at this locus. Cross-species amplification of the locus P14 was not tested, due to weak signals in *N. noctula*. Heterozygosities are given if more than 10 individuals were scored within a species. *Pipistrellus mediterraneus* corresponds to the 55 kHz phonic type of the pipistrelle bat (Helversen *et al*. 2000)

		Species											
Myotis	Parameter	*Nyctalus noctula*	*Nyctalus leisleri*	*Pipistrellus pipistrellus*	*Pip. mediter.*	*Pip. kuhli*	*Pip. nathusii*	*Eptesicus nilssoni*	*Vespertilio murinus*	*Plecotus austriacus*	*Plecotus auritus*	*Myotis myotis*	*Myotis bechsteini*
P2	number of individuals	43	10	37	16	5	4	6	5	3	5	—	—
	alleles detected	10	9	11	13	7	4	3	6	4	6	—	—
	observed heterozygosity	0.74	1.00	0.84	0.75								
	expected heterozygosity	0.83	0.83	0.89	0.86							—	—
NN8	number of individuals	43	10	55	3	1	5	5	5	9	16	57	5
	alleles detected	12	12	30	4	2	4	6	9	7	14	10	5
	observed heterozygosity	0.72	0.70	0.76							0.88	0.89	
	expected heterozygosity	0.79	0.87	0.93							0.88	0.85	
P11	number of individuals	41	—	—	—	—	—	—	—	—	—	—	—
	alleles detected	11	—	—	—	—	—	—	—	—	—	—	—
	observed heterozygosity	0.90											
	expected heterozygosity	0.84											
P13	number of individuals	42	5	25	20	3	2	5	5	10	16	—	—
	alleles detected	15	10	14	11	4	3	1	4	11	13	—	—
	observed heterozygosity	0.95		0.92	0.95					0.80	0.69		
	expected heterozygosity	0.91		0.83	0.85					0.87	0.89	—	—
P14	number of individuals	40	?	?	?	?	?	?	?	?	?	?	?
	alleles detected	9	?	?	?	?	?	?	?	?	?	?	?
	observed heterozygosity	0.92	?	?	?	?	?	?	?	?	?	?	?
	expected heterozygosity	0.86	?	?	?	?	?	?	?	?	?	?	?
NN18	number of individuals	30	10	53	21	5	6	8	6	4	14	5	—
	alleles detected	8	9	12	9	1	6	7	8	6	10	1	—
	observed heterozygosity	0.83	0.90	0.83	0.67						0.93		
	expected heterozygosity	0.78	0.82	0.85	0.79						0.81		
P19	number of individuals	37	—	—	—	—	—	—	—	—	—	—	—
	alleles detected	16	—	—	—	—	—	—	—	—	—	—	—
	observed heterozygosity	0.86											
	expected heterozygosity	0.90											

Table 2 *Continued*

Myotis	Parameter	Species											
		Nyctalus noctula	*Nyctalus leisleri*	*Pipistrellus pipistrellus*	*Pip. mediter.*	*Pip. kuhli*	*Pip. nathusii*	*Eptesicus nilssoni*	*Vespertilio murinus*	*Plecotus austriacus*	*Plecotus auritus*	*Myotis myotis*	*Myotis bechsteini*
P20	number of individuals	36	5	32	23	2	3	5	5	3	15	32	9
	alleles detected	14	8	3	3	2	6	1	5	4	4	18	7
	observed heterozygosity	0.83		0.53	0.04						0.67	0.94	
	expected heterozygosity	0.92		0.50	0.04						0.56	0.92	
P22a	number females + males	22 + 17	1 + 4	—	—	—	—	—	—	—	—	—	—
	alleles detected	14	3	—	—	—	—	—	—	—	—	—	—
	observed heterozygosity	0.67											
	expected heterozygosity	0.86											
P217	number of individuals	34	5	45	15	5	5	2	5	5	13	—	—
	alleles detected	18	8	12	10	8	10	4	6	8	11	—	—
	observed heterozygosity	0.88		0.89	0.93						0.85		
	expected heterozygosity	0.90		0.81	0.77						0.84		
P219	number of individuals	36	—	42	14	4	—	6	4	5	—	—	—
	alleles detected	7	—	14	8	3	—	4	6	1	—	—	—
	observed heterozygosity	0.72		0.76	0.79								
	expected heterozygosity	0.75		0.81	0.80								
P223	number of individuals	38	5	—	—	—	—	—	—	—	—	—	—
	alleles detected	13	5	—	—	—	—	—	—	—	—	—	—
	observed heterozygosity	0.76											
	expected heterozygosity	0.77											

Isolation and characterization of microsatellites in the seabird ectoparasite *Ixodes uriae*

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Ixodes uriae is a common parasite of many seabird species in the polar regions. Interest in this tick has risen in the past decade as more information is gathered on its potential impacts on host ecology and evolution. In particular, *I. uriae* is thought to affect the reproductive success and habitat choice of its seabird hosts (Boulinier *et al*. 2001), and to vector several avian arbo-viruses and disease agents (Chastel 1988), including the Lyme disease agent, *Borrelia burgdorferi* (Olsen *et al*. 1993). Thus, knowledge of dispersal ability of this ectoparasite within and among host populations is of vital importance if we are to understand its role in this host-parasite–disease interaction.

The independent migratory abilities of hard ticks are considered to be weak, and direct examination of dispersal of these parasites at large spatial scales is not possible (McCoy *et al*. 1999). Thus, to examine patterns of dispersion, indirect approaches, such as using genetic markers, are more plausible. To address the question of population structure and gene flow of *I. uriae* within and among its seabird hosts, we first attempted to amplify DNA using primers developed for *I. ricinus*, a common tick species of vertebrates in Europe (Delaye *et al*. 1998); no successful amplifications were achieved. In this note, we characterize nine microsatellite markers developed for *I. uriae*.

DNA was extracted from 42 unfed larval ticks originating from Atlantic puffin (*Fratercula arctica*) hosts on Hornøya, Norway (70°22′N, 31°10′ W). A genomic library was constructed following Estoup *et al*. (1993). Eleven μg of larval DNA was restricted with the enzyme *Sau*3A. Resulting fragments were separated on a 1.5% low-melting-point agarose gel and fragments between 400–800 bp were isolated, purified using a Q1Aquick Gel Extraction Kit (Qiagen) and ligated into a pBluescript vector II Sk + plasmid (Stratagene). Ligation products were then transformed into XL1-Blue MRF′ Supercompetent cells (Stratagene) and the resulting colonies were blotted on Hybond-N + membranes which were hybridized with a mixture of two probes $(CT)_{10}$ and $(GT)_{10}$. Two thousand clones from the library gave 65 positively hybridized clones from which 48 were sequenced. Primers were designed for 10 loci using Primer 0.5 (Lincoln & Daly 1991). Nine loci were polymorphic and gave clear polymerase chain reaction (PCR) results of expected size (Table 1).

Genomic DNA was prepared using a high-salt extraction method. PCR amplifications were performed in a 10-μL mixture containing 1 μL of genomic DNA (approximately 50 ng), 75 μM of each of dCTP, dTTP, dGTP, 7.5 μM of dATP, 0.4 μM of each primer, 1 μL of 10× *Taq* buffer (Tris-Cl, KCl $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7), 0.25 U *Taq* DNA polymerase (Qiagen) and 0.025 μCi [α^{33}P]-dATP (Amersham). Amplifications were performed in a PTC100 thermocycler (MJ Research) as follows: initial denaturation of 3 min at 94 °C followed by 30 cycles (30 s at 94 °C, 30 s at annealing temperature specified in Table 1 and

Table 1 Characteristics of nine polymorphic loci developed for *Ixodes uriae*. The number of observed alleles (N_A), observed (H_O) and expected (H_E) heterozygosities were calculated using ticks (*n* refers to number of ticks) sampled from two Atlantic puffin (*Fratercula arctica*) colonies. Hardy–Weinberg equilibrium was tested separately for each colony (Raymond & Rousset 1995); no significant deviations were found after correcting for multiple tests (Rice 1989)

Locus	Repeat array	*n*	Size (bp)	N_A	H_O	H_E	T_a* (°C)	Accession no.†	Primer sequence (5′–3′)
T1	$(GA)_3TA(GA)_2$-$(GA)_2$-$(GA)_4$-$(GA)_2CA(GA)_3$	63	158–164	7	0.63	0.70	57	AF293324	F: CTTCAATCACGTGGGATGC R: GACTTGTGCCTCTCCCAAAG
T3	$(CA)_4AA(CA)_3$-$(CA)_7$	64	112–114	2	0.05	0.08	57	AF293325	F: GCATTAGCGTCATAACATGAAC R: CTCTGTTTACCCTCTTCTTTGC
T5	$(GA)_{15}$	63	180–244	12	0.73	0.86	55	AF293326	F: AATTGGAAAGTAGCCATTCG R: ACTCTAATGCAACGGCGTATG
T22	$(GA)_7$-$(GA)_{13}$	64	157–187	11	0.83	0.83	57	AF293327	F: CAGACGCCGACAAATTATCC R: GACGTTTGTTTGGTGCTGTG
T35	$(CT)_{12}$	60	142–162	10	0.80	0.79	57	AF293328	F: CTCCTTTCACTCGCTTGTCC R: TCCTTCAAGCGTGTATCCAG
T38	$(TC)_{13}$	64	155–167	6	0.44	0.55	57	AF293329	F: GCATAACCAGATTCCTCCTTTC R: CAAGTGAAAGAAAACGGTGAC
T39	$(CA)_{31}$	63	188–255	26	0.89	0.94	55	AF293330	F: AACCGCAATATTAGGTCAGC R: GTTTTGGTTTCGCTTGTTTAG
T44	$(GT)_4AG(GT)_7AG(GT)_7$	63	153–185	6	0.32	0.33	57	AF293331	F: CATAACCCGACTGTCTCACTG R: GAACCACACCCAGACAACG
T47	$(GT)_5CT(GT)_7$	62	152–158	3	0.37	0.48	57	AF293332	F: GAAACGCAATGACGTACAGG R: TAATAACGCCGCACAAGGAG

*T_a, annealing temperature.
†Accession no. of the sequences available from GenBank.

1 min at 72 °C) and a final elongation step of 10 min at 72 °C. PCR products were denatured and separated on 6% polyacrylamide and 8 M urea sequencing gels using a M13 sequence as a size marker.

To characterize each locus, we genotyped ticks originating from two Atlantic Puffin colonies: Baccalieu Island, Newfoundland, Canada (48°08′ N, 52°48′ W) and Hornøya, Norway (Table 1). Expected heterozygosities were variable, ranging from 0.08–0.94 with an average of 0.61 (± 0.09). Neither tick population showed any significant deviation from Hardy–Weinberg equilibrium after correcting for multiple tests (Rice 1989).

Cross-species amplification of primers was tested on *I. ricinus*. PCR amplifications were attempted for all nine loci using 10 ticks originating from Bern, Switzerland; PCR protocols were identical except that the annealing temperature used for all primers was 52 °C. No successful amplifications were achieved for any locus.

In conclusion, based on their high polymorphism, the microsatellite markers developed for *I. uriae* should enable the examination of a diverse range of questions related to parasite dispersal among hosts over a range of spatial scales, from within colonies to between hemispheres. Likewise, patterns of parasite gene flow may provide insight into the large-scale movement of their seabird hosts. Such data will prove valuable for examining questions related to the evolution of local adaptation in this host-parasite system and for examining the epidemiology of tick-borne disease.

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Characterization of microsatellite loci in King George Whiting *Sillaginodes punctata* Cuvier and Valenciennes (Percoidei: Sillaginidae)

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Commonly known as 'whiting', the 25 species of the fish family Sillaginidae inhabit the western Pacific and Indian Oceans. The King George whiting, *Sillaginodes punctata*, is the most widespread whiting species in southern Australia where it forms significant fisheries, which have become subject to management controls due to catch declines (Kailola *et al*. 1993). If multiple stocks are present, then their identification can allow management to be more efficiently focused. In an allozyme study of stocks of Australian whiting, Dixon *et al*. (1987) identified too few usefully polymorphic loci in King George whiting. Microsatellite DNA markers can provide sufficient numbers of polymorphic markers in species that have low proportions of polymorphic allozyme loci. We describe the isolation and characterization of microsatellite loci from the King George whiting. We also evaluate the cross-species amplification of some of these loci on two other species of whiting of the genus *Sillago*, from southern Australian waters that also form significant fisheries.

Microsatellites were isolated, using $(AAAG)_6$ probes, with two methods, a polymerase chain reaction (PCR)-based procedure (Cooper *et al*. 1997) and magnetic bead enrichment (Gardner *et al*. 1999). A total of 32 clones were isolated by the first method and sequenced with the Sp6 vector primer using PE Applied Biosystems PRISM™ Dye Terminator Cycle Sequencing Kit with the products run on an ABI 373 instrument. Sequencing showed that inserts of 24 clones contained AAAG repeats. Of the 18 clones isolated with the second procedure, nine showed tandem repeats following sequencing. Of the 33 primer pairs designed, 19 produced amplifiable microsatellite loci. GenBank accession numbers for the sequenced clones are AF291469–80.

Each of the 19 loci tested for variability were amplified by PCR using 50–100 ng DNA, 10 pmol each primer, 0.2 mM each

Table 1 Primer sequences and variability measures for nine microsatellite loci genotyped in King George whiting. H_O and H_E are the observed and expected heterozygosity, respectively

Locus	Repeat sequence of clone allele	Primer sequences (5′–3′)	Fluoro-label type	No. of alleles	Size range (bp)	Mean H_O	Mean H_E	F_{ST}
Sp2	$(ATAG)_8(AAAG)_5$	F: ATGCGTGAAGATGGTGTCA R: CTGTTCTCAGCAGTGCTTCA	HEX	29	215–391	0.671	0.768	0.0027
Sp7	$(AAAG)_7$	F: AAGCTCATTTTCATCAGCGT R: CGGATCGGAATTTGAAGACA	HEX	9	119–147	0.466	0.530	0.0093
Sp19	$(AAAG)_4$	F: CGTGTAACCCAGAAACCTACT R: CATCGAAGCATTGCCTGTAA	FAM	3	197–205	0.361	0.331	0.0304
Sp22	$(AAAG)_7$	F: CTACTTCACTGCTGCACTCACA R: GGACCAACACAAGACACACAA	HEX	23	119–223	0.774	0.853	0.0016
Sp32	$(AAAG)_4(ACAG)(AAG)_3$	F: ACACAGATCGCGCACTTGTA R: CACTGTCCTCGCTGTGGTGA	TET	3	142–158	0.363	0.411	−0.0024
Sp35	$(AAAG)_4$	F: TCCTAGCTACGATGATGGATG R: TCTGGTCAGATTCGTCGATGG	FAM	6	124–144	0.438	0.404	0.0096
Sp36	$(AAAG)_6$	F: CCTCAGTAAGCGCCAGTAATAGAC R: CCTACAGCGATTGGTACAGCAC	TET	4	106–118	0.239	0.263	−0.0025
Sp38	$(CCT)_8$	F: CCGTGACCGGTTCCATTGAG R: TCCTCAACTGCGTCTGTGTTCA	FAM	4	274–283	0.019	0.538	*
Sp39	$(GTATC)_{11}$	F: TTGCTGACCATGTCAAGTTGA R: CACCAGGACAAGGCTGATATG	HEX	17	206–286	0.434	0.850	†

*†See text for explanation of locus departures from Hardy–Weinberg equilibrium.

of dNTP, 4 mM $MgCl_2$, 1× Promega *Taq* Gold dilution buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl) and 0.5 U Promega *Taq* Gold DNA polymerase in a 25-μL reaction volume. PCR cycling conditions for reactions involving King George whiting were: 94 °C for 3 min, 58 °C for 45 s, 72 °C for 1 min for one cycle; 94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min for 34 cycles; and 72 °C for 6 min, 26 °C for 10 s for one cycle. Genotyping of 10 individuals from across the species geographical range, showed that nine loci were variable (Table 1). A single primer from each variable pair was re-synthesized and labelled with ABI fluorescent dyes (Table 1) for genotyping on an ABI 377 instrument using the Genescan application (PE Applied Biosystems). Co-amplification was achieved for the following four groups of loci: Sp2–22, Sp7–19, Sp35–39 and Sp32–36–38. Loci were combined for electrophoresis as follows: Sp2–22, Sp7–19 and Sp32–35–36–38–39.

The nine microsatellite loci were genotyped for 288 individuals in 10 populations from across the species' geographical range. Inspection of the genotype arrays showed, for locus Sp38, a small number of individuals typed as homozygous for rare putative alleles in four populations that otherwise contained only the common allele. In view of these potentially anomalous typings and the high frequency of the common allele, either fixed or $P > 0.99$, the locus was omitted from further consideration. Tests for conformity to Hardy–Weinberg proportions in the remaining loci, after sequential Bonferroni adjustment (Hochberg 1988), produced significant results for locus Sp39 in all populations. Locus Sp39 was omitted from further analysis because of the possible presence of null alleles. Table 1 shows the values of F_{ST}, for the remaining individual loci estimated with GENEPOP (Raymond & Rousset 1995). Individual locus F_{ST} values are low, the typical picture for subpopulation differentiation seen in many marine fishes (Ward *et al*. 1994).

Cross-species amplifications, without extra optimization and at an annealing temperature of 50 °C, were successful for *Sillago bassensis* for Sp2, Sp11, Sp19, but were unsuccessful for Sp7, Sp22 and Sp32. For *S. schomburgkii*, Sp2, Sp7, Sp19, and Sp32 were successfully amplified. In all cases clean products were detected in the single individual of each species tested.

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Microsatellite primers from the Eurasian badger, *Meles meles*

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In man-dominated landscapes populations of once common species have become decreased both in range and density and been restricted to small habitat patches with reduced dispersal possibilities. Such fragmented populations become increasingly affected by stochastic population dynamics due to demographic, environmental and genetic risks, eventually leading to increased extinction probabilities (Bijlsma *et al*. 2000). The Eurasian badger (*Meles meles* L.) is a species that is threatened in many parts of Western Europe because of fragmentation and suffers greatly from agricultural activities (Moore *et al*. 1999). Moreover, increasing density of roads and traffic does not only considerably limit dispersal, but also highly increases mortality due to road-kills (Aaris Sørensen 1995). From a conservation perspective insights into the (meta)population structure of the badger is clearly needed. As allozyme variation was found to be low in badgers (Pertoldi *et al*. 2000), we have developed highly variable microsatellite markers that make noninvasive sampling and use of dead animals possible.

To isolate microsatellite markers, muscle tissue was obtained from 23 badgers, killed by traffic accidents, by sampling a piece of the ear or the tail. DNA extraction and small insert libraries were constructed using standard procedures (Ausubel *et al*. 1987). Overnight incubation in lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL proteinase K) at 55 °C was followed by two phenol extractions and one phenol:chloroform (24:1 v/v) extraction. Ethanol precipitated DNA was dried and dissolved in TE (10 mM Tris-HCL pH. 7.6, 1 mM EDTA). Total genomic DNA was digested to completion with *Mbo*I, size fractionated on a 1% agarose gel and the 200–1000 bp fraction was recovered by electroelution. These fragments were ligated into *Bam*HI digested pBluescript and used to transform competent XL1-Blue *Escherichia coli* cells to establish a small-insert library. This library was screened with synthetic $(CA)_7$ and $(GA)_7$ probes, end-labelled with [γ^{32}P]-ATP. Positive clones were sequenced using the T7-sequencing kit and [α^{35}S]-dATP. Out of an initial 2400 clones, 43 positive recombinants were identified, eventually yielding seven usable microsatellite loci.

Polymerase chain reactions (PCRs) were carried out in 10 μL volumes, containing 100 ng template DNA, 0.5 μM each primer, 0.2 mM dATP, dGTP and dTTP, 0.02 mM dCTP, 0.4 U *Taq* DNA polymerase (Pharmacia) and 0.14 μCi [α^{32}P]-dCTP (3000 Ci/mmol) in buffer (50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 9.0). After an initial 3 min at 94 °C, 30 cycles were performed with the following profile: 1 min at 94 °C, 2 min at T_a (optimal annealing temperature) and 1.5 min at 72 °C, followed by 10 min at 72 °C. Labelled PCR products were separated on a 5% denaturing polyacrylamide gel (Biozym, Sequagel XR) and exposed to medical X-ray film (Fuji) for 5–16 h at –70 °C, using intensifying screens. A sequence ladder of pBluescript was used as size reference.

The level of polymorphism was determined for a total of 105 badger samples collected from different localities in The Netherlands and Denmark. The characteristics of the seven microsatellite loci are shown in Table 1. All loci were found to be polymorphic and the mean number of alleles was 4.3 (range: 2–6) and mean expected heterozygosity (H_E) was 0.45 (range: 0.15–0.65). Although this is within the range observed for other mustelid species (O'Connell *et al*. 1996; Dallas & Piertney 1998; Fleming *et al*. 1999), mean expected heterozygosity in the badger was lower than in these other species (means ranging from 0.55 to 0.84). Except for two subsamples of locus Mel 2, mainly due to very low expected numbers for some genotypes, no significant differences in expected and observed levels of heterozygosity were observed for all loci, indicating the absence of null-alleles. The badger primer sets were also evaluated for use in two other mustelid species. In the pine marten (*Martes martes*, $n = 88$), two primer sets failed to produce an amplification product, two were found to be polymorphic and the other three monomorphic (Table 1). In the otter (*Lutra lutra*, $n = 5$), all primer sets yielded an amplification product (data not shown). However, the sample size was too small to reliably estimate number of alleles and expected heterozygosity.

Presently, the primer sets are used to assess the current genetic population structure of badger populations and of the pine marten.

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Table 1 Attributes of seven microsatellite loci derived from the badger (*Meles meles*) in 105 badgers and 88 pine martens (*Martes martes*) samples. T_a, annealing temperature; SR, size range observed in bp; *A*, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; NA, no amplification product. GenBank accession nos of these loci are AF300707–AF300714

				Meles meles				*Martes martes*			
Locus	Repeat structure	Primer sequences (5′–3′)	T_a (°C)	SR	*A*	H_E	H_O	SR	*A*	H_E	H_O
Mel 1	$(GT)_{20}$	CTGGGGAAAATGGCTAAACC AATGCAGGCTTTGCAATTCC	60	262–274	5	0.54	0.45	261–267	4	0.69	0.68
Mel 2	$(GT)_{12}$	TTGTGCGTATGCATGTGTGC TGCCCACGTTATAAACACTCC	55	126–128	2	0.15	0.08	NA	—	—	—
Mel 3	$(GT)_{13}$	CTAAAACCACCACCACAATGC GTGTATAGCCTGCGAACAAGG	60	128–134	4	0.69	0.67	NA	—	—	—
Mel 4	$(GT)_{16}$	TGAGTTTCCATCCTTGGTCC ATCTTTTTCCTGCTGAGACCC	60	141–147	4	0.21	0.21	144	1	0	0
Mel 5	$(GT)_{23}$	AATGTAAGGTACCCAGCATAGTCC GACACCATGTTAACCATATAAAGGG	60	105–119	6	0.65	0.53	188	1	0	0
Mel 6	$(GT)_{13}AC(GA)_4$	AAGTCCTCCTTGCAGTTTGG AGCAAGCTCTTGGTTCTTGG	60	149–155	4	0.33	0.29	137–139	2	0.29	0.31
Mel 7	$(GT)_{21}$	ATTCTTCCTTTTAGCTTTGGCC TCTCACAGTGTCAGCAGAAAGG	60	134–144	5	0.58	0.50	124	1	0	0

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Characterization of microsatellite loci in the eastern oyster, *Crassostrea virginica*

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Oysters of the genus *Crassostrea* are of great ecological and economic value worldwide. Eastern oysters, *C. virginica*, were once a keystone species of western Atlantic estuaries but now are depleted in many areas due to the combined effects of overharvesting, habitat alteration, and diseases caused by introduced parasites (Brown & Paynter 1991). Hopes of restoring oysters to these regions are tied to development of disease tolerant strains of the native *C. virginica*, and efforts to cultivate this and related species involve the use of marker-assisted selection. The preferred character for such studies currently is the microsatellite (Hare & Avise 1997; Magoulas *et al*. 1998; Huvet *et al*. 2000). In each published instance to date, dinucleotide repeats were investigated, exhibiting extremely high heterozygosities and therefore reduced power for multilocus heterozygosity surveys.

We surveyed a *C. virginica* library for the presence of tri- and tetranucleotide repeats. Genomic DNA was purified from *C. virginica* somatic tissue with an STE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 2% SDS, 0.5 mg/mL proteinase K) extraction (Hillis *et al*. 1996), which included treating the first aqueous phase following organic extraction with RNase A (final concentration 0.03 μg/μL) for 30 min. Detection of microsatellite sequences in a size-selected (400 and 900 bp) partial genomic library (pBluescript II SK+) was performed as described by Rassmann *et al*. (1991). Transformant colonies were screened using a cocktail of two digoxygenin-labelled oligonucleotide probes [$(ATG)_7$ and $(AAAC)_5$] and DNA inserts of 13 positive colonies were sequenced. Primers were designed with target annealing temperatures of 50–55 °C and expected amplicon lengths between 80 and 220 bp. Polymerase chain reactions (PCRs) were performed in reactions containing 100 ng genomic DNA, 0.5 μM unlabelled forward primer, 0.25 μM unlabelled reverse primer, 0.25 μM labelled reverse primer, 0.2 mM each dNTP, 0.5 U of *Taq* polymerase (Display Systems Biotech), 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 0.0005% gelatin, and sufficient diH_2O for a total volume of 15 μL. Amplification was conducted in PTC-100 thermal cyclers (MJ Research) using an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94° for 30 s, 50–55° for 30 s, and 72° for 15 s. Amplification products were resolved by ultrathin gel electrophoresis of fluorescent-labelled PCR products (using filter set

Table 1 Repeat structure, primer sequences, amplification characteristics, and polymorphism data for microsatellite loci examined in *Crassostrea virginica*. Observed numbers of alleles, heterozygosity values (observed and expected), and the *P*-values for exact tests of fit to Hardy–Weinberg equilibrium (HWE; for each *P*-value compared to Bonferroni-corrected alpha value, significant departure is shown by an asterisk '*') were determined for native populations in Virginia (n = 40) and Connecticut (n = 44; for Cvi6, 8, and 11 only), USA, the latter shown in parentheses

Locus Repeat	GenBank Accession no.	Primer Sequences (5′–3′) (F = forward, R = reverse)	Anneal (°C)	Expected size (bp)	Observed no. alleles	H_O	H_E	HWE
Cvi6	AF276247	F: AATATTACCACGTGACCTGTGATGAATCCTTGTAGC	50	198	13	0.54	0.87	<0.0001*
$(GAT)_{17}$		R: GTAAATATTGTATGTTCACTGTCCGGTCGTTGTGTTA			(15)	(0.55)	(0.90)	(<0.0001*)
Cvi7	AF276248	F: TCGAAACCGAACCCTTCACCAG	50	196	9	0.58	0.77	<0.0001*
$(CAAA)_6$		R: TAGTGTATATCAGTTCAGACAGGTCTTTTAATGG						
Cvi8	AF276249	F: CTGAGCTTAGACTACAGCCCTACACCAG	55	205	14	0.48	0.68	0.0001*
$[(CAAA)_2(CAA)]_2$		R: GATATCCTAAACCTACTCCTCTTTTGCATTTTTG			(6)	(0.18)	(0.47)	(<0.0001*)
Cvi9	AF276250	F: TCCAGAATTTATAAGATACTAACGATAATATACTTTATAATCCGT	55	124	14	0.83	0.90	0.1207
$(CAT)_{14}$		R: ACGAAACCGACCACAACGACGACT						
Cvi11	AF276252	F: CATCGGCCAGTGACTACCTTGTAAAAG	55	153	4	0.51	0.64	0.0465
$(CAAA)_5$		R: GCGATAACACTAAATACTTTGTTTCGGCCC			(4)	(0.48)	(0.53)	(0.2882)
Cvi12	AF276253	F: GAGTGAGAATTTCTCGGGTGGGGC	55	117	10	0.70	0.81	0.0399
$(CAAA)_6CAGAAAAA(CAAA)_4$		R: ACTTTTTGTCACATTGACCATCCCATTTCA						
Cvi13	AF276254	F: ACCGGAGATGGTGGTATTTCC	50	156	26	0.71	0.94	0.0027*
$(CAAA)_{10}$		R: GTGTTGCAAGACTTACAGAAGAAAC						

C and tamara size standard). Mendelian inheritance of alleles was determined by examining the amplified products in two or more full sib families per locus (both parents and 15–20 offspring in each family). To determine allele range and population-level variability, two wild groups of *C. virginica* were examined: one from Virginia Beach, Virginia (latitude 36°54′N, longitude 076°05′W; $n = 40$) and one derived from wild spat fall in Long Island Sound, Connecticut (latitude 41°06′N, longitude 73°25′W; $n = 44$). Primers also were tested with *C. gigas* ($n = 5$ each from two populations), *C. angulata* ($n = 5$), *Saccostrea glomerata* (formerly *S. commercialis*; $n = 5$) and *Tiostrea chilensis* ($n = 5$). Statistical analyses were performed using GENEPOP version 3.1 (Raymond & Rousset 1995).

Of the 10 primer sets, all amplified from *C. virginica* products of the size expected from insert sequences. Three yielded homologous products and seven loci (Cvi-6, Cvi-7, Cvi-8, Cvi-9, Cvi-11, Cvi-12, and Cvi-13) were polymorphic (Table 1). All seven polymorphic loci exhibited Mendelian segregation. The Virginia *C. virginica* population was surveyed for variation at all seven loci and the Connecticut population was surveyed for Cvi-6, Cvi-8, and Cvi-11. Only three of the seven loci conformed approximately to Hardy–Weinberg equilibrium (Cvi-9, Cvi-11, and Cvi-12). For all loci, observed heterozygosity was lower than expected, suggesting the common occurrence of segregating null alleles. No evidence for linkage was observed among these seven loci. Allelic distribution was significantly different between the two wild *C. virginica* populations (Fisher exact test $P < 0.0001$). When tested with *C. gigas*, *C. angulata*, and *S. glomerata*, four of the 10 primer sets (Cvi6, Cvi9, Cvi12, Cvi13) yielded various homologous products differing substantially in size from the allele sizes observed for *C. virginica*. No amplification was observed for *T. chilensis*.

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Microsatellite markers for *Rhytidoponera metallica* and other ponerine ants

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The ant genus *Rhytidoponera* (subfamily Ponerinae) contains 104 described species (Bolton 1995) which are remarkably diverse in their social organization and mating system (Crozier & Pamilo 1996). The greenhead ant *Rhytidoponera metallica* is among the most common ants in Australia, and it possesses an unusual social structure, as the reproductive role is almost invariably taken by multiple mated workers in lieu of queens (Haskins & Whelden 1965). This secondary loss of queens and partitioning of reproduction between morphologically undifferentiated workers offers a good opportunity to study how altruism is maintained in societies with low relatedness (Hamilton 1972). Such studies require detailed genetic data on the social organization and mating system. Recently, microsatellite markers have been described in three species of ponerine ants (Doums 1999; Giraud *et al*. 1999; Tay & Crozier 2000), but only two microsatellites from the most related species proved useful in *R. metallica*. Therefore, we characterized eight new microsatellite markers for *R. metallica*, and tested for cross-species amplification in 10 other species of ponerine ants.

A partial genomic library was constructed from 100 *R. metallica* workers, with gasters removed. DNA was extracted with a CTAB protocol (Hillis *et al*. 1990), digested to completion with *Sau*3A I and *Rsa*I, size-selected for fragments between 300 and 900 bp (Crozier *et al*. 1999), and ligated into a pUC19 vector. The library was screened with an $(AG)_{10}$ oligonucleotide probe end-labelled with ^{33}P, and 62 positive recombinant clones were isolated. Thirty positive clones were sequenced, and primers were designed for 14 of them.

These primers were assayed on a sample of workers collected from the You Yangs Regional Park in Victoria. DNA

Table 1 Characteristics of nine microsatellite loci for *Rhytidoponera metallica*. *n*, number of individuals analysed; *N*, number of nests analysed; H_O, observed heterozygosity; H_E, expected heterozygosity. Deviations from Hardy–Weinberg equilibrium are not significant (exact tests). GenBank accession nos: AF282988–AF282998, AF292086

Locus	Primer sequence (5′–3′)	Core repeat in cloned allele	*n*/*N*	No. alleles	Size range	H_O	H_E
Rmet3	F: TCTCGGAAAAGAAATAGAGACAG R: CATGTCTACCTGACCGAGAAC	$(GA)_{40}$	23/13	10	226–248	0.74	0.84
Rmet4	F: CATACTATCGCTTATCTCAGC R: GAACTAACCTCATCGTCCACT	$(CT)_{26}$	14/14	11	152–178	1.00	0.87
Rmet7	F: AGACTTCAATCACGAGAAGCG R: ATTGGCACTTGGTCGATAGG	$(AG)_{30}$	216 / 27	21	223–269	0.86	0.86
Rmet8	F: AAAACACGAGATACCGTCCTC R: CTGTTGACCCGCCTCCTG	$(CT)_{50}$	27/13	15	108–144	0.96	0.88
Rmet10	F: GTCATGGACGGAAATCGC R: TACCCCCATTCTATCTCGCA	$(CT)_{37}$	216 / 27	23	246–296	0.91	0.89
Rmet12	F: GGAGTTTCTACTCGCCTCTCG R: CTCATTCGTATCACGCAAGC	$(GA)_{20}$	216 / 27	15	275–315	0.85	0.87
Rmet15	F: CATTCGACCGCATTTTCC R: CGAGAGAGGGTGCGACAT	$(AG)_{28}$	216 / 27	9	154–202	0.44	0.42
Rmet16	F: TTTAGGGACAAGAGACATGGC R: ATTGATAGGTCGCGGTCTTG	$(CT)_{40}$	18/14	17	117–203	1.00	0.92
Rh12–13525	F: GACATACCGGGAGCGACC R: CGCCTTCTGACACCTTTGG	$(CT)_{11}$	216 / 27	7	178–192	0.72	0.70

from individual workers was extracted by incubating three crushed legs in 250 µL of 5% Chelex at 95 °C for 20 min (Crozier *et al*. 1999). Amplification was carried out in 10 µL final volume with 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1.7 µM each dNTP, 0.03–0.05 µM forward primer end-labelled with ^{33}P, 0.4 µM reverse primer, 5 µg of BSA, 0.4 U of *Taq* DNA polymerase (Promega) and 2 µL of template DNA. The polymerase chain reaction (PCR) profile consisted of a 3-min initial denaturation step at 94 °C, followed by 30 cycles of 30 s at 92 °C, 30 s at 50 °C and 30 s at 72 °C. PCR products were separated by electrophoresis through 6% denaturing polyacrylamide gels.

Eight primer pairs yielded suitable amplification products. All eight markers were highly polymorphic, with between nine and 23 alleles detected in the study population (Table 1). Alleles were somewhat difficult to score for Rmet8 and Rmet16, because of stutter bands. Additionally, the previously unpublished marker Rh12–13525, which was developed by W. Tek Tay for *Rhytidoponera* sp. *12*, (Tay & Crozier 2000) had seven alleles in *R. metallica* (Table 1).

The success of cross-species amplification in other genera was low (Table 2). Scorable amplification products were obtained in only 12 out of the 45 tests (27% of the five species assayed for nine markers). Polymorphism among three individuals was detected at a single marker in four species, i.e. in 9% of the 45 tests.

In contrast, the success of cross-species amplification within the genus *Rhytidoponera* was very high (Table 2). Priming sites were well conserved among the *Rhytidoponera* species tested, resulting in strong amplification products in 40 out of the 45 tests (89%). Overall, scorable polymorphism among three individuals was detected in 23 out of the 45 tests (51%). In each species of *Rhytidoponera*, between three and eight markers were polymorphic, and this figure should increase when more individuals are analysed. Hence, this panel of microsatellites will permit detailed studies of kin structure, breeding system, gene flow and population structure across species of *Rhytidoponera* with variable social structures. Additionally, these markers might help to distinguish between the species yet to be described that are currently lumped into the *metallica* species-complex.

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Table 2 Results of cross-species amplification in 10 other ant species from the subfamily Ponerinae

	Rmet3	Rmet4	Rmet7	Rmet8	Rmet10	Rmet12	Rmet15	Rmet16	Rh12–13525
Tribe ECTATOMMINI									
Rhytidoponera tasmaniensis	+5	+3	—	+5	+2	+3	+2	+2	+4
R. victoriae	+2	+1	+1	+1	+2	+2	+1	—	+2
R. purpurea	+1	+1	+2	—	+1	+3	+2	+1	+4
R. impressa	+1	+1	+5	—	+1	+2	s	+1	+4
R. confusa	+1	+1	+3	—	+2	+1	s	+2	+3
Gnamptogenys menadensis	+1	—	—	—	—	+1	+2	—	—
Tribe PONERINI									
Diacamma cyaneiventre	+3	—	—	—	—	—	+1	—	—
D. ceylonense	+2	—	—	—	—	—	+1	—	—
Harpegnathos saltator	+3	—	—	—	+1	—	+1	—	—
Streblognathus aethiopicus	—	—	—	—	—	+1	+1	—	—

+ *n*, scorable amplification product with *n* alleles detected in three individuals.
—, no scorable amplification product.
s, present of supernumerary amplification products.

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Rapid and efficient identification of microsatellite loci from the sea urchin, *Evechinus chloroticus*

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The New Zealand Fiords are characterized by a seawardly flowing surface low salinity layer (LSL), produced by prodigious rainfall. Maintenance of salt balance occurs by a weak oceanic inflow below this LSL. Because the flow of sea water is inwards, planktonic larvae of the fiords are thought to be retained within natal fiords, which could have important consequences on gene flow. This hypothesis was supported by allozyme analyses of *Evechinus chloroticus*, a sea urchin endemic to New Zealand (Mladenov *et al*. 1997). Despite high levels of gene flow found amongst all coastal populations of New Zealand, a population sampled within one fiord was found to be genetically differentiated. Our intention is to address the effects of oceanographic and hydrographic features of all 14 fiords on recruitment and population structuring of *E. chloroticus*. In order to do this we are using highly polymorphic microsatellite markers.

Traditional colony hybridization methods used for microsatellite cloning are time-consuming and relatively inefficient. Several enrichment techniques have previously been published (Gardner *et al*. 1999; Inoue *et al*. 1999). However, these either use radioactivity or include a number of lengthy steps. We report here on an alternative easy, fast, efficient and non-radioactive method of cloning microsatellite markers from the sea urchin *E. chloroticus*.

Size selected fragments (250–800 bp) of *Nde*II-digest genomic DNA from five individuals of *E. chloroticus* were ligated into pUC18 vector (Pharmacia). Inserts were amplified using universal primers (M13) and purified with High Pure PCR product purification Kit (Roche).

In order to hybridize DNA to probes, 100–500 ng of size selected amplified DNA (250–800 bp) was mixed, in separate tubes, with 2 pmol of GA_{12} and GT_{12} 5′-biotinylated repeat probes in 20 μL of extension solution containing: 0.2 mM of each dNTP, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.5 U *Taq* DNA polymerase (Roche). This mixture was subjected to one round of polymerase chain reaction (PCR) (5 min at 94 °C, 1 min at 55 °C, 10 min at 72 °C) using a PTC-100 thermal cycler (MJ Research). Purified products were added to Streptavidin MagneSphere Paramagnetic Particles (Promega) and incubated for 15 min at room temperature with 120 μL of 6× SSC/0.1% SDS, mixed continuously. After a series of washes in 150 μL of 6× SSC/0.1% SDS for 15 min: once at 60 °C, 65 °C, 70 °C, 75 °C and twice in 150 μL of 6× SSC at 80 °C, DNA was eluted with 100 μL of 0.1 M NaOH at 80 °C for 10 min. The solution was neutralized with 100 μL TE pH 7.5, purified and amplified as above. A further round of enrichment (hybridization, elution, PCR) was then undertaken.

Size selected fragments (250–800 bp) of *Nde*II-digest from enriched inserts were ligated into pUC18 vector. Ligation

Table 1 Characteristics of height *Evechinus chloroticus* microsatellite loci. H_O and H_E are observed and expected heterozygosities, respectively, calculated with GENETIX 4.1 (Belkhir *et al*. 1996). PCR programmes are: (1) 31 cycles of 15 s at 94 °C, 10 s at annealing temperature, 10 s at 72 °C; and (2) 4 min at 94 °C, 31 cycles of 1 min at 94 °C, 1 min at annealing temperature, 1 min at 72 °C and finished by 10 min at 72 °C

Locus	Primer sequences (5′–3′)	Repeat array	PCR programme	Annealing temp. (°C)	No. of alleles	Size range (bp)	H_O	H_E	Accession no.
C1	F: CTGCCCGGAAGTATTGTTATTG R: CATTTCGGCCACGGTCACT	$(AG)_{23}$	1	53	24	110–166	0.92	0.92	AF299134
C29	F: GAATAAACATTTACAAATCTGTC R: ATAAAAAGGGAAACGAAACAAGAA	$(AG)_{11}$	2	55	6	77–89	0.77	0.77	AF299135
G29	F: GATCGGTATGATAAACTT R: ATGCATGGGTAGGTGTG	$(CT)_3CC(CT)_3AT(CT)_{10}$	1	45	8	92–102	0.74	0.73	AF299136
A34	F: ACGGTTCGATTGAGAGAG R: TGACGGGGCAGGAAATGTG	$(AG)_{19}$	1	51	13	109–133	0.63	0.86	AF299137
B14	F: GATCATTGAGATGGCGATG R: GCACCCACACGTACGCGC	$(GT)_6$	1	51	4	58–64	0.31	0.36	AF299138
A12	F: CTGTGTTCTATTAAAATTGTCCTC R: TTGAAATTTGCTCTACCCCTATT	$(GA)_4TAT(GA)_7$	2	58	7	85–97	0.14	0.51	AF299132
D1	F: CGACAAGTCCACCGTTCAACTCCA R: ATCTACTGTTGTTGCCTGTGTCAC	$(CA)_2CT(CA)_2CT(CA)_4TG(AC)_2$	1	51	6	91–109	0.07	0.28	AF299139
A13	F: ATCCCCTTCAAATGTTGCCTGATT R: GGCGTAACGGTAATGACCCTGTC	$(AG)_2(GA)_5AA(AG)_4$	1	51	3	99–111	0.07	0.07	AF299133

reactions were transformed into *Escherichia coli* XL1-Blue competent cells. Recombinant clones were screened using two separate 10 μL PCR reactions, incorporating the repeat probe and either one of the M13 universal primers. Products were visualized on 2% agarose gel using ethidium bromide. Approximately 70% (19) and 45% (7) of the clones were positive for GA_{12} and GT_{12} probes, respectively. Screening of the same clones was attempted by using both M13 primers and the complementary probe in the same PCR reaction (as in Gardner *et al*. 1999). However, only 10% of clones for each probe were positive, indicating that this screening method is unreliable. Eighteen and six positive clones were amplified with M13 primers and sequenced using Big-Dye cycle sequencing kit (Applied Biosystems), separated on ABI 377 automated sequencer. Eighteen and five sequences contained microsatellites, respectively, and 11 and four were unique resulting in a final enrichment efficiency of approximately 45% for GA_{12} and 30% for GT_{12}.

Primer pairs were designed from sequences flanking repeats. PCR were performed in 10 μL reaction mixture: 20–200 ng DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.5 μM of [γ^{33}]-ATP-labelled forward primer, 0.5 μM of reverse primer and 0.25 U *Taq* DNA polymerase. Different PCR regimes were used (Table 1). Alleles were separated on a 5% denaturing polyacrylamide gel (Long Ranger, FMC) and visualized by autoradiography.

Eight polymorphic loci were identified and scored for 100 individuals from 14 sites along the fiords (Table 1). No linkage disequilibrium was detected between each pair of locus using GENETIX 4.1 (1000 permutations, $P < 0.05$) (Belkhir *et al*. 1996). To assess Wahlund effects, 26 individuals of the same site were analysed for deficit of heterozygotes. Loci A12 and D1 showed significant deviation from Hardy–Weinberg equilibrium (1000 permutations, $P < 0.05$) suggesting the possibility of null alleles.

We also tested the utility of these primers for two individuals from each of *Coscinasterias muricata* (Asteroidea), *Ophiactis savignyi* and *Amphipholis squamata* (Ophiuroidea). Only the less polymorphic locus (A13) seems to amplify clearly in such a large range of echinoderms.

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Polymorphic microsatellite DNA markers in the African elephant (*Loxondonta africana*) and their use in the Asian elephant (*Elephas maximus*)

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Poaching and rapid human population growth have put intense pressure on elephant populations, especially in the forests of west and central Africa. Conversion of rainforest to agriculture has resulted in the fragmentation and isolation of forest elephant populations in small reserves. Effective management of these populations will require information about census size, sex ratio, and the amount and distribution of genetic diversity. Although we can count savannah elephants from the ground or air, forest elephants are difficult to see in the dense vegetation and censusing them requires using indirect methods (Barnes & Jensen 1987).

For our genetic characterization of African forest elephant populations, we developed a panel of microsatellite loci. Genomic DNA was extracted from tissue samples of four unrelated African zoo elephants using the QIAamp Blood and Tissue Kit (Qiagen), then pooled in equal concentrations. We digested 10 μg with *Mbo*I and ligated fragments of 200–500 bp into M13mp18 (Rassmann *et al*. 1991). Transformation of competent DH5αF′ *Escherichia coli* (GibcoBRL) was performed by electroporation. Cells were plated on YT media and plaques were replicated on nylon filters (MSI). The probes $(CA)_{15}$ and $(GA)_{15}$ were labelled with [γ^{32}P]-dATP and hybridized with the plaque lifts. We selected 40 (1.6%) colonies that were strongly positive and isolated the DNA using the QiaPrep Spin Miniprep Kit (Qiagen). We sequenced these using the Sequenase 2.0 kit (Amersham Life Science), and determined that 32 contained microsatellites, 12 of which were uninterrupted and had sufficient flanking regions for primer design. Primer pairs were designed using PRIMER 0.5 (Whitehead Institute, Cambridge, USA).

We tested our primers on 10 African savannah elephants from the Frozen Zoo® of the Zoological Society of San Diego. Three primer sets revealed monomorphic loci and three were

Table 1 Characteristics of African elephant (*Loxondonta africana*) microsatellite loci and their use in the Asian elephant (*Elephas maximus*). Repeat motifs, primer sequences, allele numbers and sizes for elephants from the Frozen Zoo® and the forest elephants of Kakum National Park, expected (H_E) and observed (H_O) heterozygosity values for the Kakum elephants, and annealing temperatures for the loci developed in this study. Annealing temperatures (T_a) shown are for African elephants, these were lowered by 2 °C when amplifying DNA from Asian elephants. GenBank accession nos for the sequences of clones are AF311670–75

			Allele sizes		No. of alleles		Kakum N. P.		
Locus	Repeat Motif	Primer sequences	*L. africana*	*E. maximus*	*L. africana*	*E. maximus*	H_E	H_O	T_a (°C)
LA1	$(CA)_{10}(TA)_5$	F: TGGGTTGTTCCACCCTCTAC R: GTAACCGGGCAAGTGTGTG	139–149	—*	6	—	—*	—	53
LA2	$((CA)_6(CGTA))_2(CA)_6$	F: CTTGGTGGGAGTCATGACCT R: GGAGAAATGACTGCCCGATA	227–241	226–234	3	4	0†	0	58
LA3	$(CA)_{10}$	F: TACTCTGCTCCTCTGCCTATCC R: GCAGAATTTTGGTCTTGGAGG	165–171	166–172	3	3	0.521	0.527	55
LA4	$(CA)_{12}(CGTA)_4(CA)_7$	F: GCTACAGAGGACATTACCCAGC R: TTTCCTCAGGGATTGGGAG	117–137	111–117	11	4	0.760	0.747	54
LA5	$(CA)_{13}$	F: GGGCAGCCTCCTTGTTTT R: CTGCTTCTTTCATGCCAATG	130–154	142–144	7	2	0.575	0.377‡	52
LA6	$(CA)_{13}$	F: AAAATTGACCCAACGGCTC R: TCACGTAACCACTGCGCTAC	158–214	155–159	7	3	0.542	0.563	57

*Locus does not amplify.
†Locus monomorphic in this population.
‡Significant deviation from Hardy Weinburg expectation ($P = 0.002$, tested in GENEPOP 3.2a, Raymond & Rousset 1995).

unusable. To test our primers on Asian elephants (*Elephas maximus*), we used 12 samples from the Frozen Zoo®. Finally, our primers were screened on dung samples from 86 African forest elephants at Kakum National Park, Ghana. DNA from these samples was extracted using the protocol of Boom *et al*. (1990). To minimize the potential for allelic dropout or spurious alleles, genotypes were obtained from two different extractions of each sample in a 'multiple tubes' approach (Taberlet *et al*. 1996).

Amplifications were performed in 10 μL volumes containing 20–50 ng of template DNA, 1 μL reaction buffer (Promega), 0.2 μM radioactively labelled forward primer, 0.2 μM reverse primer, 0.2 μM dNTP mix, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Promega). Using a Hybaid thermocycler, the profile consisted of a denaturation step at 94 °C for 3 min, followed by 35–40 cycles of 94 °C denaturation for 30 s, 1 min of primer annealing at the temperatures shown in Table 1, and 1 min of primer extension at 72 °C. Alleles were separated in a 6% polyacrylamide gel, visualized by autoradiography, and scored by comparison with an M13 length standard.

All six loci were highly polymorphic in African elephants with between three and 11 alleles (Table 1). The smaller number of alleles found in our Asian elephant samples is not surprising, as it is generally assumed that microsatellite loci will be more polymorphic in the species from which they are cloned than in related species (Ellegren *et al*. 1995). As the Frozen Zoo® samples do not represent natural populations, only expected and observed heterozygosity values for the Kakum elephants are shown.

Previous work has shown that African forest elephants are genetically divergent from the savannah subspecies (Barriel *et al*. 1999), which may explain why locus LA1 could not be amplified in the Kakum samples. The significant deviation from the expected frequency of heterozygotes for locus LA5 may indicate the presence of one or more null alleles. However, we have no family groups with which to test for these.

Although African and Asian elephants diverged from a common ancestor approximately 5 mya (Maglio 1973), five of the six primer pairs amplify in Asian elephants. While some of the loci have less alleles in Asian than in African elephants, we believe that these loci will be useful for population studies in both species.

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The estuarine teleost, *Acanthopagrus butcheri* (Sparidae), shows low levels of polymorphism at five microsatellite loci

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The black bream, *Acanthopagrus butcheri*, is a member of the family Sparidae that is found throughout southern Australia (Kailola *et al*. 1993). Information on the population genetic structure of this species is of value for two reasons. First, black bream is one of a relatively small number of teleosts that typically spends its entire life-cycle within estuaries. Thus, studies of this species can be used to test hypotheses about the role that estuaries play in promoting genetic differentiation in those teleosts that breed within these systems (e.g. Chaplin *et al*. 1998). Second, such information has important implications for the management of this species, which supports significant commercial and recreational fisheries in three Australian states (Kailola *et al*. 1993) and is a target of a developing inland aquaculture industry in south-western Australia.

Microsatellite markers are particularly useful for elucidating the details of the population genetic structure of species that show low levels of polymorphism in other types of markers, such as allozymes and mitochondrial DNA (e.g. Shaw *et al*. 1999). The black bream is one such species (Chaplin *et al*. 1998; E. Yap *et al*. unpublished data). Here, we describe the isolation and characterization of microsatellite loci from black bream and then assess the levels of polymorphism at five loci.

Genomic DNA was extracted from the muscle tissue of black bream using CTAB buffer and a phenol–chloroform extraction protocol. The DNA was digested to completion with *Sau*3A and size fractionated in an agarose gel. Fragments of 200–600 bp were excised from the gel, purified and ligated into the *Bam*HI site of the vector pGEM 3Zf(+) (Promega). The ligation products were transformed into ElectroMAX-DH10B cells (Life Technologies), which were then plated onto agar containing ampicillin (100 mg/mL), IPTG and x-gal. The recombinant colonies ($n = 446$) were picked into 96-well microtitre plates, grown at 37 °C, vacuum blotted onto Hybond-N nylon membranes (Amersham), and screened with $(CA)_{12}$, $(AG)_{12}$, $(TCC)_5$, $(GACA)_4$, $(GATA)_4$ and $(GAA)_5$ oligo probes end-labelled with [α^{32}P]-dATP. Plasmid DNA was isolated from 12 positive clones and then subjected to dye-terminator cycle sequencing. The sequencing products were electrophoresed and the sequences of the plasmids and inserts were determined using an ABI 373 Sequencer (Perkin Elmer). All inserts contained microsatellite loci. Primers, for use in polymerase chain reaction (PCR), were designed for six of these loci on the basis that they contained 15 or more repeat units and that the sequencing of their flanking regions was sufficient to permit primers to be generated.

Five of the primer pairs amplified scorable alleles at the microsatellite loci (Table 1). The 'optimised' conditions for PCR amplification of these loci were: (i) 15 μL reaction mixture containing 50–100 ng DNA template, 1.5 mM $MgCl_2$, 0.20 mM of each dNTPs, 20–40 nM of each primer, with 25% of the forward primer end-labelled with [γ^{33}P]-ATP, 0.05 U *Taq* DNA polymerase, and 10 mM Tris-HCl with 50 mM KCl; and (ii) PCR profiles with an initial 5 min denaturation at 94 °C, followed by 26 cycles of 30 s denaturation at 94 °C, 30 s at annealing temperature (Table 1) and 90 s extension at 72 °C, and a final 7 min extension at 72 °C. Amplified alleles were resolved on a 6% denaturing polyacrylamide gel and their sizes estimated using pUC18 DNA sequencing standards.

The levels of polymorphism at the five microsatellite loci were assessed using at least 40 black bream from nine water bodies in Western Australia and 10 individuals from Gippsland Lake in south-eastern Australia. One locus (pAb4D5) was monomorphic in all samples, while another locus (pAb2D11) was polymorphic only within the samples from south-eastern Australia (Table 1). Only one (pAb2B7) of the remaining three loci, which were polymorphic in all 10 populations, was represented by a total of more than seven alleles and had an expected heterozygosity of greater than 0.56 (Table 1). Thus, the black bream appears to contain relatively low amounts of microsatellite polymorphism, especially in Western Australia, and particularly in comparison with, for example, two species of marine sparid (see Takagi *et al*. 1997; Batargias *et al*. 1999). Nevertheless, the four polymorphic loci have revealed greater amounts of variation in black bream than allozyme genes (see Chaplin *et al*. 1998). In addition, the genotype frequencies at each of the pAb1H1, pAb2B7 and pAb2A5 loci, in each of samples of 38 or more black bream from nine water bodies in Western Australia, did not show any statistically significant departures from those expected under Hardy–Weinberg equilibrium conditions. The four polymorphic microsatellite loci should, therefore, be useful for addressing population-level questions about the black bream.

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Table 1 Characteristics of five microsatellite loci in samples of black bream *(Acanthopagrus butcheri)* from nine water bodies in Western Australia and from the Gippsland Lakes in Victoria, south-eastern Australia. The Western Australian samples are the same as those used by Chaplin *et al*. (1998)

Locus	GenBank accession no.	Primer sequence (5′–3′)	Repeat unit*	T_a (°C)	Size range (bp)	No. of alleles	n	H_E	H_O
pAb1H1	AF284351	F: GGCTTTCATTTCCCCATTTGTG R: CACCTTTCTCCACGCCATAAA	$(TG)_{15}$	63	132–148	5	268	0.37	0.44
pAb2B7	AF284352	F: GGTGCGTGCATTGTTAATGTGT R: GATCTGCTTTCCTTTGACTCAGC	$(TG)_{24}$	65	98–128	14	274	0.70	0.72
pAb4D5	AF284353	F: ACCTCTTCATCTGCGTGACATCT R: GACAACACCCTCACTCAGCTGA	$(TG)_{60}$	54	199	1	50	0	0
pAb2A5	AF284354	F: AGTTACTTTCTCCAGAGTGGCGC R: GGCAACAGATAAGCACTGAGCATA	$(TG)_{19}$	63	105–119	7	273	0.56	0.62
pAb2D11	AF284355	F: CGGTCCAGTTTCACTCTGATGTT R: AACTGCTGTCATCGCCCTGTT	$(TG)_{15}$	65	106–110	4†	50	0.11	0.08

*determined from the sequenced insert; †polymorphic only within samples from the Gippsland Lakes. n, is the total number of individuals assayed per locus; T_a, is the optimal annealing temperature of each primer pair; H_E, is the expected heterozygosity, calculated as $1 - \Sigma(f_i^2)$, where f_i is the frequency of the ith allele; and H_O, is the observed heterozygosity.

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Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds

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The cooperatively breeding Seychelles warbler, *Acrocephalus sechellensis*, is a rare endemic of the Seychelles islands. By 1959, anthropogenic disturbance had pushed this species to the verge of extinction and only 26 individuals remained, confined to the island of Cousin. The population has since recovered and has been the focus of intense study since 1985 (e.g. Komdeur 1992; Komdeur *et al*. 1997).

We required a set of microsatellite markers to enable studies of mate choice, reproductive success and fitness. Genetic variability is relatively low within this species, possibly due to the recent population bottleneck. Consequently, many microsatellites had to be isolated and screened to provide sufficient polymorphic loci to enable parentage assignment and pedigree construction. We isolated 63 microsatellite loci from the Seychelles warbler and tested for their polymorphism in this and five other species of *Sylviidae*. We also examined the utility of a subset of these loci in 16 other passerine birds.

DNA was extracted following Bruford *et al*. (1998). A genomic library enriched for $(CA)_n$, $(GA)_n$ and $(TTTC)_n$ was prepared as described by Armour *et al*. (1994) using modifications suggested by Gibbs *et al*. (1997). DNA reactions were performed in a 10-μL volume containing 10–50 ng DNA, 1.0 μM of each primer, 0.2 mM of each dNTP, 0.05 units *Taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) and 1.0–2.0 mM $MgCl_2$ (Table 1) in 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween. Polymerase chain reaction (PCR) amplification was performed in a Hybaid Touchdown thermal cycler. Initially, a touchdown cycle was performed with a reaction profile of 95 °C for 3 min, then 94 °C for 30 s,

Table 1 Characterization of 50* polymorphic microsatellite loci from the Seychelles warbler (*Acrocephalus sechellensis*), *and* their polymorphism in five other members of the *Sylviidae* family

Locus	EMBL accession number	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	$MgCl_2$ conc. (mM)	Product size† (bp)	Number of alleles/ number of individuals			Number of alleles/number of individuals tested in				
							SW	H_O	H_E	CRW	AW	GRW	EMW	WW
*Ase*2	AJ287385	$[(GAAA)_2GCAA]_3$	F: TTGACAGAGTGTTATTCAATGTG R: GAGCAGATAATAGACCTTGCT	60	1.5	97	2/7	0.71	0.50	1/6	0	1/3	3/2	1/2
*Ase*3	AJ287386	$(CA)_{14}CCA$	F: ACAGGTATGGCGCTCAAGTC R: CTGAATCTTACACAGGAGACCGT	60	1.5	101	3/7	0.86	0.60	1/6	4/4	1/3	1/2	1/2
*Ase*4	AJ287387	$(CA)_{11}$	F: TCTCCATCATCACCACAAAGC R: TTCCCATTGCCCTAGTTATTCCA	60	1.0	103	2/25	0.40	0.37	0	3/4	1/3	0	0
*Ase*5	AJ287388	$AAA(CA)_{12}AAA$	F: TGAAACAAAATGGGATGGTCC R: CCTTTCTCGGAACTGATTGCTT	61	1.0	110	1/7	0.00	0.00	1/6	1/4	1/3	1/2	2/2
*Ase*6	AJ287389	$(CA)_3G(CA)_{17}$	F: TAAAAGCCAGCAGTGGAGCC R: CGAGCTTGCAGGGTTTCCT	60	1.5	119	4/25	0.76	0.70	1/6	2/4	1/3	0	0
*Ase*7	AJ287390	$(CT)_{13}$	F: AATCAACTTCAAATGCTCACAG R: ACTACATGACTCCAGGCTCAG	60	1.5	123	2/7	0.83	0.53	2/6	1/4	2/3	1/2	0
*Ase*8	AJ287391	$(GT)_4TTT(GT)_7$	F: TACCTCTCCTTGGCTGAGCA R: CCAGCCCTAGCTGTTTCACC	TD	1.5	125	1/7	0.00	0.00	3/6	2/4	1/3	2/2	1/2
*Ase*9	AJ287392	$(CA)_{15}$	F: GACTGAAGTCCTTTCTGGCTTC R: CACCAGGAATACAAGTCCATTG	60	1.5	125	3/25	0.40	0.44	3/6	5/4	5/3	2/2	1/2
*Ase*10	AJ287393	$(CCTTCCCT)_7$	F: CATTGGGGTACTATGGAAAGACC R: TCCTGAGTGGAAGGAACATAGG	TD	1.5	127	3/25	0.64	0.56	9/5	0	1/3	1/2	0
*Ase*11	AJ287394	$(AC)_{14}$	F: TCCCCAAATCTCTCAATTCC R: AGTTCTAAGCCTGCCTGTGC	60	1.5	128	2/5	0.40	0.53	7/6	4/4	5/3	3/2	0
*Ase*12	AJ287395	$(CA)_{11}$	F: TCAAGGAAACACAACTACAGCC R: TTTCCTCACAGCCTTGACTG	60	1.5	128	1/7	0.00	0.00	4/6	4/4	1/3	3/2	2/2
*Ase*13	AJ287396	$(GT)_{11}$	F: TGTGCTCCTCTGCTTTCC R: CAGATGGCCAGTGTTAGTCC	62	1.5	132	3/25	0.52	0.54	5/5	7/4	1/3	2/2	1/2
*Ase*16	AJ276374	$(TCTCC)_{13}$	F: TCAGTTCCTGAGTAAATGTCTC R: TGAATTACCCCTAAATACCTG	58	1.5	155	4/7	100.0	0.70	5/6	0	6/3	1/2	0
*Ase*18	AJ276375	$(GT)_{12}$	F: ATCCAGTCTTCGCAAAAGCC R: TGCCCCAGAGGGAAGAAG	60	1.5	176	3/25	0.56	0.50	1/6	5/4	3/3	1/2	3/2
*Ase*19	AJ276376	$(CA)_4GA(CA)_5$	F: TAGGGTCCCAGGGAGGAAG R: TCTGCCCATTAGGGAAAAGTC	60	2.0	177	4/8	0.88	0.64	3/6	3/4	1/3	4/2	3/2
*Ase*20	AJ276377	$(CTTC/CTTT)_{10}$	F: TCTAAAGCTGCCTGCCAGAA R: GCGGTTGCAGTGGACTTG	TD	1.5	178	1/7	0.00	0.00	1/6	7/4	1/3	1/2	0
*Ase*21	AJ276378	$(CTTTT)_2CTC(TTTC)_8$	F: TTAGAACCATTTGATAGTTGCCAC R: ATGGGTTTCTTGGGGAAGAG	58	2.0	180	1/7	0.00	0.00	9/6	5/4	1/3	1/2	2/2
*Ase*22	AJ276379	$(GT)_{13}$	F: TGAACCATTGTCACCAACAC R: GCTTTAGTTCAGATGCCCAG	58	1.5	181	2/6	0.50	0.53	1/6	0	0	1/2	0
*Ase*25	AJ276382	$(GAAA)_{31}$	F: GATGGCTATATGCTTCAAATGC R: TTGAAAGCCTTAAAGTGGGA	58	1.5	187	5/25	0.76	0.74	1/6	6/4	0	2/2	0

Table 1 *Continued*

Locus	EMBL accession number	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	$MgCl_2$ conc. (mM)	Product size† (bp)	Number of alleles/ number of individuals			Number of alleles/number of individuals tested in				
							SW	H_O	H_E	CRW	AW	GRW	EMW	WW
*Ase*26	AJ276383	$(CTC)_3(TC)_{12}$	F: GCTGGCCTTGCAAAAACTTC R: AACACCTCCCTGTCCCTGC	60	1.5	203	1/7	0.00	0.00	1/6	5/4	1/3	2/2	2/2
*Ase*27	AJ276384	$(TTTC)_{16}$	F: TTAACATTGCATGCTCCTGC R: AGTCAAGGTACAGGCTAGATAGCC	60	1.0	204	4/25	0.64	0.60	1/6	1/4	3/3	2/2	1/2
*Ase*29	AJ276386	$(AC)_7TTTG(AC)_6$	F: GATCAGTTTGGAGACGTTTTCT R: ACAGAGCCATAAGGAATGTGC	62	1.5	207	2/7	0.14	0.14	1/6	1/4	1/3	1/2	2/2
*Ase*32	AJ276635	$(GT)_{13}(TCAC)_2(GT)_9$	F: AATGAGCAATACCATGACAGC R: GATCTTTCAGTCAGGAACAAGC	58	1.5	218	1/7	0.00	0.00	1/6	5/4	0	0	0
*Ase*33	AJ289865	$(AT)_{10}$	F: CTTTGGAATGCCAGGCTGCT R: TGCTGGAACCACAGGACTT	TD	1.5	220	1/7	0.00	0.00	1/6	4/4	2/3	1/2	1/2
*Ase*34	AJ276636	$(CT)_{11}$	F: GTTAATTCTTTTGGCCCTCAGC R: GGAGACACCACACCAATGC	60	1.5	220	1/7	0.00	0.00	3/5	1/4	3/3	4/2	3/2
*Ase*35	AJ276637	$(GT)_{10}$	F: GTCCTTGGTCCTTAGCATCTGT R: GCTCCTGTTGTTCTGGGAATAG	58	1.5	224	3/25	0.44	0.62	1/6	1/4	0	2/2	0
*Ase*36	AJ276638	$(TGTGG)_7$	F: AAGTTCCATGGGGTGAATGC R: GAGCGTGTTCCTCCAATTCC	60	1.5	225	2/5	0.20	0.20	1/6	1/4	1/3	1/2	0
*Ase*37	AJ276639	$(AC)_9$	F: TAATTCATGGAGAAGCCCAG R: TCAAAACAACAGTTTTCACAGC	58	1.5	226	3/25	0.32	0.37	2/6	1/4	0	4/2	0
*Ase*38	AJ276640	$(CA)_{15}$	F: ATCCGAGAACCCAATCACTT R: GCAGCATTACAGTCTCAAAGAAC	58	2.0	226	2/4	0.50	0.43	0	3/4	1/3	3/2	1/2
*Ase*40	AJ276642	$(GT)_{10}$	F: CACTGCTCCAGGCACTCTG R: TCCAAGGCACACAAAGGTG	58	1.5	230	1/7	0.00	0.00	3/6	3/4	1/3	1/2	1/2
*Ase*42	AJ276644	$(GT)_4(AT)_6(GT)_8(AT)_2$	F: CATGGGTAGGTTGGGATGTC R: AGGTGAGGGTATGCAAACATG	62	1.5	243	2/25	0.32	0.27	1/6	1/4	4/3	1/2	2/2
*Ase*43	AJ276645	$(TA)_3(CA)_8(TA)_5$	F: ATTGTGTGGGATTTGCAT R: TTGCTGTGCAGTTTGCTTTT	TD	1.5	250	1/7	0.00	0.00	2/6	3/4	1/3	1/2	2/2
*Ase*44	AJ276646	$(GT)_{18}$	F: TTCCCGTAATTATGACCTCTCTTG R: ACCAGAACTTGTTGTCTGGGAG	TD	1.5	250	1/7	0.00	0.00	1/6	4/4	3/3	1/2	2/2
*Ase*46	AJ276775	$(TG)_{13}$	F: CTGGCTGTATCTTGGTGTGC R: CAGTGTTTTAGGTCTCCTGCTG	62	1.5	265	3/25	0.24	0.48	1/6	2/4	1/3	1/2	1/2
*Ase*47	AJ276776	$(CA)_{10} \ldots (CA)_4$	F: GATCACATTTGGCATTTACTGAT R: ACTCTTTAGGGCAAGGCACT	TD	1.5	267	1/7	0.00	0.00	4/6	0	1/3	1/2	2/2
*Ase*48	AJ276777	$(CCTTCT)_6$	F: TTTATTTCCTGGACTGGAACAATC R: GAACATTGGGCTACTGGGC	58	1.0	270	4/25	0.56	0.53	7/5	7/4	5/3	3/2	0
*Ase*49	AJ276778	$(AC)_{10}$	F: CCCCTGAAGTGTCCAACG R: ACTTTCCCAGCACATCTTGC	58	1.5	272	2/7	0.00	0.26	1/6	2/4	1/3	1/2	2/2
*Ase*50	AJ276779	$(CA)_{12}$	F: CTGTGGAATGCTGTCTGGC R: ATGGACTCCCGTCTAACTTGC	60	1.5	272	1/7	0.00	0.00	1/6	6/3	2/3	2/2	2/2

Table 1 *Continued*

Locus	EMBL accession number	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	$MgCl_2$ conc. (mM)	Product size† (bp)	Number of alleles/ number of individuals			Number of alleles/number of individuals tested in				
							SW	H_O	H_E	CRW	AW	GRW	EMW	WW
*Ase*51	AJ276780	$(CA)_{12}$	F: AATTCCCCTAGACAGGCAGC R: TCACTGGAGAGCCAAATTCC	60	1.5	277	1/7	0.00	0.00	1/6	7/4	2/3	2/2	1/2
*Ase*52	AJ276781	$(CA)_9(CA)_5$	F: TCTTAGCCTGCACTCATTTCA R: CAGTCACCGTAAGTTCATAGGC	60	1.5	278	1/7	0.00	0.00	1/6	2/4	1/3	1/2	1/2
*Ase*53	AJ276782	$(CTT)_{22}$ $(CTCCTT)_{10}$	F: ATGGAGAATTCTGGGTGCTG R: CCCAATAATGAGGTAACACCAA	60	1.5	285	2/7	0.43	0.54	1/6	8/4	0	0	0
*Ase*55	AJ276784	$(GT)_9$	F: GTGTGGACTCTGGTGGCTC R: TCCCAAAGCACTCAAACTAGG	62	1.5	292	1/7	0.00	0.00	1/6	6/4	2/3	2/2	2/2
*Ase*56	AJ276785	$(GT)_{18}$	F: TTCACTGAGAAGTGAGAATGTG R: GTCCTTGATTGATTACAGGCT	60	1.5	298	3/25	0.44	0.40	5/6	5/4	2/3	3/2	0
*Ase*57	AJ276786	$(AC)_{14}$	F: GCAAGTGCAGATGTTTCCCT R: CCAAAGCAGGACAATGCTG	TD	1.5	299	1/7	0.00	0.00	6/6	3/4	4/3	1/2	0
*Ase*58	AJ276787	$(CTTTTT)_{27}$	F: ATTCCAGGGATTGGGCAG R: CTCAAAGCGAAATTGAGCAGT	60	1.0	311	5/25	0.76	0.76	1/6	7/4	5/3	4/2	1/2
*Ase*60	AJ276789	$(GT)_9GG(GT)_8$	F: CATGAAAAGGAACTCTCCAGC R: TTCCATCTCTGTTCTACTGCG	62	1.5	353	1/7	0.00	0.00	0	5/4	4/3	1/2	3/2
*Ase*61	AJ276790	$(GAAAAA)_{13}$	F: AGGATTTTTAATGGGATATACACATCTG R: AGCCACATTTTAGCCCACAG	54	2.0	369	2/5	0.40	0.36	0	0	3/3	0	0
*Ase*62	AJ276791	$(CT)_2(GT)_8$	F: TCGCCAGGTCGTGTGTAGTC R: CAAAACCGTGTCGGGGAG	58	1.5	372	1/7	0.00	0.00	1/6	1/4	1/3	2/2	0
*Ase*63	AJ276792	$(GAGAAA)_8(GA)_7$	F: TTTGGGGTTTAGGAATAGCAGA R: GGCTTCAGCCTGAGAAAGTC	60	1.0	400	2/7	0.29	0.26	2/6	8/4	2/3	4/2	1/2
*Ase*64	AJ276793	$(AGGG)_9$ $(ATGG)_{12}$	F: CCACCTTTCATACTGGGGAG R: TTCAGCCAGTCAGTGTAGCC	TD	1.5	412	2/8	0.50	0.40	7/6	1/4	3/3	1/2	1/2

*An additional 13 loci were monomorphic in all species tested (EMBL accession numbers: AJ287384, AJ287397, AJ287398 AJ276380, AJ276381, AJ276385, AJ276387, AJ276634, AJ276641, AJ276643, AJ276647, AJ276783, AJ276788).

†Size in cloned allele.

SW, Seychelles warbler, *Acrocephalus sechellensis*; CRW, clamorous reed warbler, *Acrocephalus stentoreus australis* (M. Berg, personal communication); AW, aquatic warbler, *Acrocephalus paludicola* (P. Hedrich, personal communication); GRW, great reed warbler, *Acrocephalus arundinaceus* (B. Hansson, personal communication); EMW, European marsh warbler, *Acrocephalus palustris* (B. Hansson, personal communication); WW, willow warbler, *Phylloscopus trochilus* (B. Hansson, personal communication).

T_a, annealing temperature; TD, Touchdown cycle; H_O, observed heterozygosity; H_E, expected heterozygosity; 0, no product detected.

Table 2 Cross-species utility of 15 Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci in 21 other passerine birds

		Number of alleles/Number of individuals tested (n = 4 unless stated)														
Family*	Species	*Ase*8	*Ase*9	*Ase*13	*Ase*18	*Ase*19	*Ase*29	*Ase*34	*Ase*37	*Ase*40	*Ase*42	*Ase*43	*Ase*46	*Ase*48	Ase55	*Ase*56
Maluridae	Superb fairy-wren, *Malurus cyaneus*	0	1	1	0	0	1	1	0	1	0	1	1	0	0	0
Pomatostomidae	White-browed babbler, *Pomatostomus superciliosus*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Laniidae	Loggerhead shrike, *Lanius ludovicianus*	0	0	1	1	0	0	1	0	0	1	1	0	0	0	0
Corvidae	Azure-winged magpie, *Cyanopica cyana*	0	1	1	1	0	0	1	0	0	1	1	1	0	1	1
Cinclidae	White-throated dipper, *Cinclus cinclus*	0	1	1	1	0	1	1	0	1	1	1	1	0	1	0
Sturnidae	European starling, *Sturnus vulgaris*	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Certhiidae	Winter wren, *Troglodytes troglodytes*	7/6	2/6	2/6	5/6	3/6	5/6	2/6	3/6	2/5	—	3/6	3/6	—	5/6	6/6
Paridae	Blue tit, *Parus caeruleus*	0	1	1	1	0	1	1	0	0	1	1	0	0	1	0
Paridae	Long-tailed tit, *Aegithalos caudatus*	—	—	—	1	—	1	1	16/680	1	1	0	1	0	—	—
Hirundinidae	Sand martin, *Riparia riparia*	0	1	1	0	1	1	1	0	1	1	1	1	1	1	0
Pycnonotidae	White-spectacled bulbul, *Pycnonotus xanthopygos*	0	1	1	1	1	1	1	0	1	0	1	1	0	1	0
Zosteropidae	Seychelles grey white-eye, *Zosterops modestus*	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0
Sylviidae	Aquatic warbler, *Acrocephalus paludicola*	2/4	5/4	7/4	5/4	3/4	1/4	1/4	1/4	3/4	1/4	3/4	2/4	7/4	6/4	5/4
Sylviidae	Sedge warbler, *Acrocephalus schoenobaenus*	—	1/8	—	16/40	—	—	—	—	—	5/8	1/8	—	—	—	—
Sylviidae	European marsh warbler, *Acrocephalus palustris*	2/2	2/2	2/2	1/2	4/2	1/2	4/2	4/2	1/2	1/2	1/2	1/2	3/2	2/2	3/2
Sylviidae	Great reed warbler, *Acrocephalus arundinaceus*	1/4	5/4	1/4	3/4	1/4	1/4	3/4	0	1/4	4/4	1/4	1/4	5/4	2/4	2/4
Sylviidae	Clamourous reed warbler, *Acrocephalus stentoreus australis*	3/6	3/6	5/5	1/6	3/6	1/6	3/5	2/6	3/6	1/6	2/6	1/6	7/5	1/6	5/6
Sylviidae	**Seychelles warbler, *Acrocephalus sechellensis***	**1/7**	**3/25**	**3/25**	**4/25**	**4/8**	**2/7**	**1/7**	**3/25**	**1/7**	**2/25**	**1/7**	**3/25**	**4/25**	**1/7**	**3/25**
Sylviidae	Willow warbler, *Phylloscopus trochilus*	1/2	1/2	1/2	3/2	3/2	2/2	3/2	0	1/2	2/2	2/2	1/2	0	2/2	0
Nectariniidae	Seychelles sunbird, *Nectarinia dussumieri*	0	1	1	1	0	1	1	0	1	1	1	0	0	1	1
Passeridae	Seychelles fody, *Fodia sechallarum*	0	1	1	1	0	1	1	0	1	1	1	0	1	1	0
Fringillidae	European greenfinch, *Carduelis chloris*	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1
	Number of species tested for amplification	20	21	20	21	20	21	21	21	21	21	22	21	20	20	20
	% of species in which a product was amplified	50	95	100	90	55	86	100	38	86	90	95	80	52	90	50
	Number of species tested for variability	7	8	7	8	7	7	7	6	7	7	8	7	5	7	6
	% of species (tested for variability) with ≥3 alleles	29	50	43	75	86	14	57	67	29	29	25	14	100	29	83

*Following Sibley & Monroe (1990), except Seychelles warbler which follows Komdeur (1992).

—, sample not tested; 0, no reliable product; 1, product visualized on agarose gel (not tested for variability).

annealing temperature X for 45 s, 72 °C for 45 s for two cycles each at X = 60 °C, 57 °C, 54 °C, 51 °C then 25 cycles at X = 48 °C, followed by 72 °C for 5 min. To optimize the PCR amplification of the loci found to be polymorphic, further PCRs consisted of one cycle at 95 °C for 3 min then 35 cycles at 94 °C for 1 min, annealing temperature (Table 1) for 30 s, 72 °C for 45 s, followed by 72 °C for 5 min. For the cross-species amplifications, a touchdown cycle was performed as above.

PCR products were visualized on a 0.8% agarose gel stained with ethidium bromide. When testing for polymorphism, PCR products were run on 6% polyacrylamide gels and visualized by staining with silver (Promega) or by autoradiography (after PCR with one of the primers end-labelled with [γ^{33}P]-dATP; Sambrook *et al*. 1989).

We developed primers for 63 microsatellites, of which 50 were polymorphic in at least one of the tested species of *Sylviidae* (Table 1). Thirty loci were polymorphic, displaying up to five alleles, in a test panel of up to 25 unrelated Seychelles warblers. There was no significant difference at any locus between the observed and expected heterozygosity, though these comparisons were of limited power.

All 50 loci found to be polymorphic in the Sylviidae were tested for polymorphism in six unrelated individuals of the winter wren, *Troglodytes troglodytes* (M. Berg, personal communication). Fifteen of the loci that were also found to be polymorphic in the winter wren were selected and tested for utility in 16 other species, representing 15 passerine families (Table 2; following Sibley & Monroe 1990).

The high proportion of loci found to be polymorphic in the other *Sylviidae* will reduce or eliminate the need to develop new primers for future studies of these species. The cross-species amplification suggests that, after further testing, many of the primers presented here may also be useful for detecting polymorphic loci in other passerine families (Table 2).

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Variable microsatellite loci in red swamp crayfish, *Procambarus clarkii*, and their characterization in other crayfish taxa

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The red swamp crayfish, *Procambarus clarkii*, is a temperate freshwater crayfish native to the south-eastern United States. It is heavily exploited as a fishery product and is used widely in aquaculture. Its economic importance led to widespread introductions on four continents. The species has been used extensively in laboratory studies, but studies of its population biology in the wild have been rare (Huner 1988). Previous population work using allozymes found low levels of genetic variation in two *Procambarus* species, including *P. clarkii* (Busack 1988). We developed two microsatellite libraries for *P. clarkii* (f. Cambaridae) from which 23 variable microsatellite loci were optimized. The 18 clearest markers were tested in representative taxa of the other two crayfish families (Parastacidae and Astacidae), as well as two cambarid species in Orconectes and one congeneric species; characterization is reported here.

Genomic DNA was extracted from frozen (–80 °C) tail muscle of a red swamp crayfish (Putah Creek, Yolo County California) using the Tris sodium chloride EDTA sodium dodecyl sulphate (SDS) (TNES)-urea buffer extraction protocol (Asahida *et al*. 1996) with the following modifications. Approximately 200 mg tissue were added to 700 µL extraction buffer, containing 4 M urea and 0.5% SDS, and 0.035 mg Proteinase K. After overnight incubation (37 °C), samples were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated with 0.3 M sodium acetate pH 5.3 in a final ethanol concentration of 67%. The pellet was washed in 70% ethanol, air or vacuum dried, and resuspended in Tris low EDTA (TLE) buffer (10 mM tris + 0.1 mM EDTA, pH 8.0). Two subgenomic libraries were created by Genetic Identification Services (Chatsworth, CA) by partially digesting whole genomic DNA with a mixture of the following restriction enzymes: *Bsr*BR1,

Table 1 Summary of locus data for 23 microsatellite loci developed for *Procambarus clarkii*. GenBank Accession nos are AF290219-AF290941. n is the number of individuals screened; individuals were drawn from two or three (where $n \leq 10$) to four (where $n > 10$) populations. H_O and H_E are the observed and expected heterozygosities, respectively, calculated across all populations due to small sample sizes (Genes in Populations version 2, May *et al*. 1995); sample sizes precluded reasonable inference of the presence of null alleles

Locus ID	Primer sequences (5′–3′)	Repeat (cloned allele)	Product size range (bp)	n	No. of alleles	H_O	H_E	$MgCl_2$ (mM)	Primer (μM)
PclG-02	F: CTC CCC ATG CAC TCT GGC TCT GT R: TGG CGA ATT TTG CCT GTT TCT GTC	$(GATA)_3GAGAA(GATA)_5$	216–224	25	3	0.56	0.61	1.5	0.5
PclG-03	F: CTC TCC ACC AGT CAT TTC TT R: AAG CTT ACA ATA AAT ATA GAT AGA C	$(TCTA)_{20}$	216–420	26	12	0.73	0.89	2.0	0.5
PclG-04	F: TAT ATC AGT CAA TCT GTC CAG R: TCA GTA AGT AGA TTG ATA GAA GG	$(TCTA)_3 \ldots (TCTA)_2 \ldots (TCTA)_{29} \ldots (TCTA)_2$	170–290	26	15	0.77	0.89	2.0	0.5
PclG-07	F: CCT CCC ACC AGG GTT ATC TAT TCA R: GTG GGT GTG GCG CTC TTG TT	$(TCTA)_8$	100–160	19	11	0.84	0.85	1.5	0.5
PclG-08	F: ACG ATA AAT GGA TAG ATG GAT GAA R: CCG GGT CTG TCT GTC TGT CA	$(GATA)_{16}$	148–220	18	11	0.56	0.82	1.0	0.3
PclG-09	F: TAT GCA CCT TTA CCT GAA T R: TGT TGG TGT GGT CAT CA	$(TCTA)_{14}$	80–160	20	8	0.35	0.85	1.5	0.5
PclG-10	F: TGC TCA CGC AAA CTT GTA TTC AGT R: CAA TGG TCC TTG ATT TGG TGT TCT	$(TAGA)_2TA(TAGA)_{16}$	90–176	10	6	0.40	0.65	1.5	0.5
PclG-13	F: CTC TCC TGG CGC TGT TAT TTA GC R: TGA AGA GGC AGA GTG AGG ATT CTC	$(TCTA)_{12}$	130–150	17	3	0.53	0.54	1.5	0.5
PclG-15	F: GGC GTG ACG CCA ACG TGT CTT R: GGC TGG CCA CTT TGT TAG CCT GAG	$(TATC)_2TGTC(TATC)_{17}TATT(TATC)_3$	150–185	18	12	0.78	0.85	1.5	0.5
PclG-16	F: CTC GGA ATG TCC ACC TGA GA R: TCA TTA TGG ATT TTG TCA ATC TAT	$(TCTA)_{18}TCTC(TATC)_3$	80–160	19	11	0.95	0.86	1.5	0.5
PclG-17	F: GTC GGG AAC CTA TTT ACA GTG TAT R: AAG AGC GAA GAA AGA GAT AAA GAT	$(TCTA)_{14}$	156–190	19	8	0.84	0.78	1.5	0.5
PclG-24	F: CAA GGC ATT GAG GGG GTG AGA T R: CCG CGC CAC AGA ATT ACG AGT	$(GATA)_3AATA(GATA)_{24} \ldots (AC)_8T(CA)_{31}$	280–290	3	3	1.00	0.61	1.5	0.5
PclG-26	F: ATA TAG CCT CGC CCT TTT ACC C R: TCG TGT TCA CAT CAG CAG GAG A	$(CT)_5(CA)_{41}$	210–300	16	9	0.75	0.85	1.5	0.5
PclG-27	F: AAT CTT AAG ATC ATG AAA AAG GTA R: TTT AAG GAA CGT ATA AGA AAA GAC	$(TATC)_4CATC(TATC)_8$	80–150	8	11	0.63	0.84	1.5	0.5
PclG-28	F: CTC GGC GAG TTT ACT GAA AT R: AGA AGA AAG GGA TAT AAG GTA AAG	$(GATA)_{22}(GA)_5$	210–270	20	8	0.65	0.82	1.5	0.5
PclG-29	F: GAA AGT CAT GGG TGT AGG TGT AAC R: TTT TTG GGC TAT GTG ACG AG	$(TATC)_9$	95–165	19	7	0.58	0.82	1.5	0.5
PclG-32	F: CCC CCA CTC GTC TCT GTG TAT G R: TGT GCT TGC GGG AGT GAG C	$(CT)_7 \ldots (TC)_{37} \ldots (CA)_{15} \ldots (CA)_5$	150–250	19	14	0.74	0.91	1.5	0.5
PclG-33	F: TTC GAG GCG TTG CTG ATT GTA AGT R: CAA GGA AGC GTA TAG CCG GAG TCT	$(GT)_{21}$	120–180	19	11	0.63	0.85	1.5	0.5
PclG-34	F: CAG TCC ATG TGA TCA ATA CTG ACC R: CTC AGG TGG AAC ACT CAT AAA CAA	$(CA)_4CG(CA)_{22}TA(CA)_{15}$	80–160	4	6	0.75	0.75	1.5	0.5
PclG-35	F: TCC TCA CGT TTC TTT CCC ATC ATT R: TGC CTT TTC GAT CTC CAC CTT C	$(GT)_6AA(GT)_8AA(GT)_{11}AA(GT)_5$	152–190	18	6	0.56	0.68	1.5	0.5
PclG-37	F: TAA ATA AGT GGC GTG TAA GAC GAG R: TAA CTA AGC CAG GGT GGT CTC CAG	$(CA)_4CG(CA)_{15}CG(CA)_{13}$	80–180	20	12	0.85	0.90	1.5	0.5
PclG-45	F: ATA TAA ACC GGT GTC GGT GTA G R: CTT TGA CTT CAC CTT TTC TCT TAT	$(CA)_3 \ldots (GA)_6$	96–98	16	2	0.25	0.43	1.5	0.5
PclG-48	F: CTG TTG GTG ATT TCC GTC AAT TTT R: AGA TTC AAC GCT GTG TTC CTG ATC	$(CA)_{12}$	146–190	17	8	0.59	0.84	1.5	0.5

*Eco*RV, *Hae*III, *Pvu*II, *Sca*I, and *Stu*I. An oligonucleotide linker containing a *Hin*dIII site was ligated to fragments in the range of 300–700 bp. Magnetic beads were used to capture fragments containing $(CA)_n$ or $(TAGA)_n$. These were ligated into the *Hin*dIII site of pUC19; the products were used to transform competent *Escherichia coli* DH5α. Of the positive clones initially screened, 82% ($n = 11$) $(CA)_n$, and 58% ($n = 12$) $(TAGA)_n$ contained microsatellites. We plated additional clones and amplified approximately 300 recombinant clones by colony polymerase chain reaction (PCR) using the following protocol. We added a toothpick stab of each colony to 10 μL of 24 mM Tris-HCl (pH 8.4), 60 mM KCl, 0.075 mM each dNTP, 7.5 mM $MgCl_2$, and 0.6 mM pUC19 forward and reverse sequencing primers. We incubated the mixture at 100 °C for 10 min then placed the tubes on ice. Five μL *Taq* solution (12 mM Tris-HCl, pH 8.4, 30 mM KCl, 0.5 U *Taq* DNA polymerase, recombinant, GIBCO) were added to each tube. Fifteen μL reactions (final conditions: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.05 mM each dNTP, 5 mM $MgCl_2$, 0.4 mM each primer, 0.5 U *Taq* DNA polymerase) were placed in a preheated thermal cycler (MJ Research PTC 100) set to cycle as follows: 94 °C for 4.5 min, 25 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, then 72 °C for 2 min. Approximately 1 μL product was run on a 3% TAE agarose gel made with 0.03× GelStar nucleic acid stain (BioWhittaker Molecular Products) to identify inserts of 300–800 bp. Colonies containing these inserts were grown overnight in Luria broth from which plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). More than 150 clones were sequenced using the Big Dye™ Terminator cycle sequencing protocol and visualized on an ABI 377 DNA sequencer (Applied Biosystems) by Davis Sequencing (Davis, CA). Fifty-four primer pairs were designed from approximately 100 unique sequences using 'PrimerSelect' (DNAStar, Inc.). Ten to 20 ng DNA from up to four crayfish populations sampled within the Sacramento Valley, California, were combined with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM each dNTP and 0.5 U *Taq* DNA Polymerase in a 10 μL reaction volume; $MgCl_2$ and primer concentrations are indicated in Table 1. Cycling conditions were 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, then 72 °C for 5 min. Amplification products were mixed 1:1 with 98% formamide loading dye, denatured for 3 min at 95 °C, placed on ice, then run on 5% denaturing acrylamide gels and stained by agarose overlay containing 0.5 μL SYBR GreenI nucleic acid stain (BioWhittaker Molecular Application). Staining otherwise followed Rodzen *et al*. (1998). Products were visualized on a Molecular Dymamics FluorImager 595. Locus details are reported in Table 1. Eighteen primer pairs were also tested on *P. zonangulus*, *Orconectes virilis*, *O. rusticus*, *Pacifasticus leniusculus*, and *Cherax quadricarinatus*. Amplification success is reported in Table 2. These results indicate the utility of these microsatellite loci for genetic studies involving *P. clarkii*, and their potential utility in related species.

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Table 2 Cross-species amplification with 18 of the primers listed in Table 1. *n* indicates number of individuals tested unless otherwise indicated in parentheses in each cell. Numbers in cells indicate the number of observed (presumed) alleles; '–' indicates amplification but unclear; '0' indicates no amplification or smear only

Species	PclG-02	PclG-03	PclG-04	PclG-07	PclG-08	PclG-09	PclG-13	PclG-15	PclG-16	PclG-17	PclG-27	PclG-28	PclG-29	PclG-32	PclG-37	PclG-45	PclG-47	PclG-48
Procambarus zonangulus ($n = 4$)	7	1	5	3	—	1	0	1	0	0	2(2)	1	1	3	—	2	—	2(3)
Orconectes virilis ($n = 2$)	3	0	1	1	2	1	0	1	0	0	0	0	0	1	1	1	0	—
Orconectes rusticus ($n = 2$)	2	0	3	1	2	1	0	1	0	0	0	0	0	1	1	1	1	—
Pasifasticus leniusculus ($n = 4$)	1(2)	0	1	1(3)	1(1)	1(3)	1(2)	1	1(3)	0	0	0	0	1	1(1)	1	2(3)	1(3)
Cherax quadricarinatus ($n = 4$)	—	0	0	1	1	1	0	1	0	1(2)	0	0	0	1	–(2)	0	0	1

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