

## PRIMER NOTES

### Polymorphic trinucleotide microsatellite loci for a neotropical parrot, the green-rumped parrotlet, *Forpus passerinus*

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This paper characterizes eight pairs of PCR primers that amplify microsatellite loci in green-rumped parrotlets, *Forpus passerinus*, Psittacidae. We examined polymorphism of these loci in this species and in five other parrot species.

The green-rumped parrotlet is a neotropical member of one of the world's most endangered bird families, the parrots, Psittacidae (Bennett & Owens 1997; Collar & Juniper 1992). Parrots are particularly worthy of attention as they are among the best flagship species in South America for stimulating conservation efforts. For this reason, and to investigate the population biology of *F. passerinus*, we developed microsatellite loci. High heterozygosity, neutrality, and ease of assay make microsatellite loci ideal tools for studies of behavioural ecology and population genetics (Queller *et al.* 1993).

As primers developed in one species may amplify loci in related species, we investigated the utility of our loci for some other neotropical parrots (Primmer *et al.* 1996; Hughes *et al.* 1998). Loci could provide species and population identification, confirming that birds purported to come from captive breeding or sustainably harvested populations did originate from such environments (Beissinger & Bucher 1992; Derrickson & Snyder 1992). Such distinctions are critical to enforcement of the Exotic Wild Bird Conservation Act of 1992.

Three partial genomic libraries were made in Lambda Zap Express (Stratagene, La Jolla, CA) (Hughes & Moralez Deloach 1997). We screened approximately 300 000 clones, sequenced several hundred positives, and developed primers for clones containing  $\geq 8$  uninterrupted repeats of the sequence AAT. Most positive clones contained loci with seven or fewer AAT repeats. Libraries were also screened for other trinucleotide, and dinucleotide, repeats, with less success.

DNA amplification reactions (5 or 10  $\mu$ l) contained about 10 ng of DNA, 50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 0.1% NP40, 250  $\mu$ M each dNTP, and 500 nM each primer. Using the 'tube control' function of a Hybaid thermal cycler (where a thermistor monitors temperature in a dummy

tube), reactions were cycled: 90 s at 92 °C, then 0 s at 92 °C, 5 s at 55 °C, 5 s at 72 °C, 30 times, and finally 90 s at 72 °C. When genotyping, 0.05  $\mu$ l of 3.3  $\mu$ M, <sup>35</sup>S-labelled, dATP was included per reaction.

We found microsatellite loci unusually difficult to develop in this species. It took three iterations of cloning and sequencing to find eight polymorphic loci (GenBank Accession nos AF035366–AF035373, Table 1). Five loci that contained six or seven AAT repeats in original clones were monomorphic; no other primer pairs were bought for loci containing fewer than eight repeats. Bird genomes are apparently under selection to be small (Hughes & Hughes 1995), and this may partly explain why microsatellite loci containing longer runs of repeats (those most likely to be polymorphic) are rare. However, the extreme paucity of these loci in *F. passerinus* is remarkable.

We found that these loci are unexpectedly monomorphic in other species (Table 2). We attempted PCR amplification of these loci in five other species, using annealing temperatures 5 °C lower than in Table 1. Work in passerines suggests that about 50% of primers that amplify an appropriately sized product will be polymorphic (Primmer *et al.* 1996). Assuming that mutation rates are similar in psittacines as passerines, this result is consistent with small effective population size in the species tested.

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**Table 1** Polymorphic microsatellite loci in *Forpus passerinus*. Length, and number of AAT repeats were determined from the original clone. The number of alleles was counted in 10 unrelated birds sampled from a population at Hato Masaguaral in the llanos of Venezuela.

Name	Primer sequences (5'-3')	T anneal	Length	No. of repeats	No. of alleles
FpAAT5	GAGACTTGCCTCCATAATA CAGAAATCCTGGAAATTACTC	50	112	17	4
FpAAT7	CTGCAACTTTACACCCAGCTACTC TGCCAGAATTTTAACGCTTGTAC	50	223	9	7
FPAAT54	GTAATCCACAGGTTGAAATA TCCCCTCGTTGTTCTTATTA	60	72	7	7
FpAAT91	TGTGGAAAGTCCCTGGATTAT TGTCCCATAAAAGAGCAAATA	50	86	10	9
FpAAT93	ATGGGGAAGAGAATAAAAAGAACATA GCAGGTGATGAGCGGTTGTAT	60	165	8	3*
FpAAT98	CCACATTCAGAGGAAGGGAAGT CCTGCTCGAAGGTGACCAACT	60	108	11	7*
FpAAT189	ATGAGGCCACAAAGCATAG ATCTGGCTGTACATCTCTA	60	153	14	4
FpAAT198	TGGCAATGAGCAGTTGTATT ACTCCCATGGATTGAGATAAT	60	137	7+4	6*

\*Alleles more readily scored when TaqStart (Clontech, Palo Alto, CA) was used.

**Table 2** Heterospecific amplification with the polymorphic loci.

Species	Primer pair							
	5	7	54	91	93	98	189	198
<i>Brotogeris jugularis</i>	M	M	M	M	P	P	M	M
<i>Aratinga pertinax</i>	M	M	M	M	M	M	M	M
<i>Aratinga canicularis</i>	M	M	M	M	M	M	M	M
<i>Amazona auropalliata</i>	M	M	M	M	M	Ø	M	M
<i>Amazona albifrons</i>	M	M	M	M	M	Ø	M	M

M, monomorphic; P, polymorphic; Ø, no amplification.

## Microsatellite primers for the Eurasian otter

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The Eurasian otter, *Lutra lutra*, displays great differences in density, habitat and behaviour throughout its range (Kruuk 1995), and is a focus of conservation effort following rapid population declines in the mid-1950s (Strachan & Jefferies 1996). Similarly, most otter species are of biological interest and are important targets for conservation (Foster-Turley

*et al.* 1990). However, even basic information such as numbers present is difficult to obtain because otters are rarely observed, and are very difficult to trap, mark and recapture. As an alternative, we are developing methods for individual identification using DNA profiles derived from otter scats. Here, we describe primers for 13 highly polymorphic microsatellites in *L. lutra*. These markers are also polymorphic in between one and six other otter species, but not in other carnivore species, and are not detectable in two common prey species.

Four genomic libraries of one male *L. lutra* were constructed using standard (Sambrook *et al.* 1989) or enrichment (Armour *et al.* 1994) methods: (i) 400–600 bp *Sau3A* fragments/ $\lambda$  ZAP Express (11 200 clones); (ii) 400–600 bp *Sau3A* fragments enriched for GATA microsatellites/pUC18 (7500 clones); (iii) 10–12 kb *Bam*HI fragments/ $\lambda$  ZAP Express (17 400 clones); and (iv) *Bam*HI fragments not selected for size/ $\lambda$  EMBL 3 (21 500 clones). Libraries were screened with

(CA)<sub>n</sub> obtained commercially (Pharmacia), and (CAAA)<sub>n</sub>, (GAAA)<sub>n</sub>, (GATA)<sub>n</sub>, (TAA)<sub>n</sub>, (TAG)<sub>n</sub> and (TAAA)<sub>n</sub> polymerized by PCR from complementary partially overlapping oligonucleotides, e.g. (GATA)<sub>10</sub> plus (TATC)<sub>8</sub>. Probes were labelled with [ $\alpha^{32}$ P]-dATP using random priming, but without random primers present. Positive clones were detected for CA, TAG and GATA, but not for CAAA, GAAA, TAA and TAAA. The TAG probe detected fragments having a common *AluI* length of 380 bp; therefore these were abandoned.

The inserts of positive plaques were prepared by PCR using T3 and T7 primers from libraries (i) and (ii), and by the plate lysate method from libraries (iii) and (iv). In the latter two cases, microsatellite-containing subfragments of each clone were identified by digestion with *AluI*, *RsaI* or *Sau3A*, followed by Southern blotting and filter hybridization with the appropriate probe. Subfragments were cloned into pUC18 (Pharmacia), and then inserts were amplified using vector primers. All PCR fragments were purified using Qiaquick columns (Qiagen), then sequenced on an Applied Biosystems 377 automated DNA sequencer.

PCR primers were designed using OLIGO (National Biosciences, Inc.), then tested for single-fragment detection in one individual using nonradioactive PCR: 1× NH<sub>4</sub> buffer, 1–4 mM MgCl<sub>2</sub>, 0.01 U/ $\mu$ L *Taq* DNA polymerase (both BioLine), 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dATP, dCTP, dGTP, dTTP, and 0.5  $\mu$ M of each primer. The PCR program used was:

90 °C/2 min, and 20 cycles of 90 °C for 30 s, 65 or 60 °C minus 0.5 °C per cycle for 30 s, and 15 cycles of 90 °C for 30 s, 55 or 50 °C for 30 s, and finally 72 °C/1 min. Primer sets detecting single fragments were tested for levels of polymorphism in a panel of *L. lutra* individuals using radioactive PCR. Forward primers were end-labelled with [ $\gamma^{32}$ P]-ATP, and the PCR conditions were 0.25  $\mu$ M both primers, 2.5 mM MgCl<sub>2</sub>, other reagents as above. PCR products were resolved on denaturing PAGE gels, then detected by autoradiography at –75 °C.

The levels of polymorphism detected for 13 microsatellites in 32 *L. lutra* from Scotland, southwest England, Wales, southwest Ireland and northeast Germany are within the range found in other mammalian species (Table 1). The alleles of the GATA loci were well defined whereas those of the CA loci were more difficult to score as alleles due to stuttering and background smears. The 13 microsatellites were tested for polymorphism in six other species representing all four otter genera, and in five other carnivore species using the same PCR program as described above with the lower annealing temperature (Table 2). All loci were polymorphic in between one and six other otter species. Again, the GATA loci were much easier than the CA loci to interpret as alleles due to the lack of stutter products. Levels of polymorphism appeared to be much lower in representatives of four other mustelid genera, and nothing was detected in one representative carnivore. No PCR products were detected in

**Table 1** Attributes of 13 microsatellites in 32 *Lutra lutra* from Britain, Ireland, and Germany; EMBL Accession nos are Y16292 to Y16304

Locus	Primers: 5' to 3'	Repeats	H <sub>O</sub>	Alleles	Sizes (bp)
Lut435	F TGAAGCCAGCTTGGTACTTC R ACAGACAGTATCCAAGGACCTG	(CA) <sub>29</sub>	0.61	10	170–200
Lut453	F AGTGCCTTTGTAAGTGGTAATGG R AGACTGAAAGCTCTGTGAGGTC	(CA) <sub>26</sub>	0.50	8	175–203
Lut457	F CAGGTTTATGGCTTTATGGCTTTTC R CAGGGTTTGATTTCTGGTGAGG	(CA) <sub>26</sub>	0.52	9	224–252
Lut604	F TATGATCCTGGTAGATTAACCTTTGTG R TTTCAACAATTCATGCTGGAAC	(CA) <sub>26</sub>	0.48	5	197–211
Lut615	F TGCAAAATTAGGCATTTTCATTCC R ATTCTCTTTTGCCCTTTTGCTTC	(CA) <sub>27</sub>	0.65	7	244–262
Lut701	F GGAAACTGTTAAAGGAGCTCACC R CAGTGTTCATAAGGATGCTCCTAC	(GATA) <sub>11</sub> GAA(GATA) <sub>2</sub> GAA(GATA) <sub>4</sub>	0.57	5	192–208
Lut715	F TTCACAATAGCCAAGATATGGAC R TGGCATAATATCCTTTCTCATGG	(GATA) <sub>6</sub> GAT(GATA) <sub>7</sub> GAT(GATA) <sub>5</sub>	0.52	6	197–217
Lut717	F TGTTGCCTTCAGAGTCCTGTG R GTCAGGCATTGTAACATATTCTCAG	(GATA) <sub>12</sub>	0.61	6	175–203
Lut733	F GATCTCATTTTAAATGTTCTTACCAC R TGGTTCTCTTGCAGGATCTG	(GATA) <sub>4</sub> GAT(GATA) <sub>12</sub>	0.56	5	164–192
Lut782	F GAGATATCACTAAGCAATACACGATG R ACAAAGACTGAGCAAAACAAGC	(GATA) <sub>6</sub> GAT(GATA) <sub>10</sub>	0.47	6	161–197
Lut818	F AAGGATGTGAAACAGCATTG R CCATTTTATACATAAATCGGAT	(GATA) <sub>11</sub>	0.59	6	150–178
Lut832	F TGATACTTTCTACCCAGGTGTC R TCCTTAGCATTTATCTTATTTACCAC	(GATA) <sub>11</sub>	0.44	6	178–198
Lut833	F CAAATATCCTTTGGACAGTCAG R GAAGTTATCTAATTTGGCAGTGG	(GATA) <sub>15</sub>	0.59	8	155–183

H<sub>O</sub>, observed heterozygosity.

Table 2 Cross-species utility of 13 microsatellite primer sets from *Lutra lutra*

Species	Locus *													
	N	Lut435	Lut453	Lut457	Lut604	Lut615	Lut701	Lut715	Lut717	Lut733	Lut782	Lut818	Lut832	Lut833
<b>Otters</b>														
<i>L. canadensis</i> , river otter	12	7	4	5	3	m	5	5	m	6	4	6	m	m
<i>L. maculicollis</i> , spotted-necked otter	6	4	2	3	5	4	4	3	m	m	3	m	3	m
<i>Aonyx capensis</i> , Cape clawless otter	5	2	3	4	2	4	4	3	m	m	2	3	2	2
<i>A. cinerea</i> , Asian short-clawed otter	2	3	2	3	m	3	3	2	2	3	2	2	3	2
<i>Enhydra lutris</i> , sea otter	4	m	3	4	2	2	2	m	m	m	m	m	2	m
<i>Pteronura braziliensis</i> , Brazilian otter	4	m	3	m	m	m	3	m	m	3	2	m	2	m
<b>Other mustelids</b>														
<i>Martes martes</i> , pine marten	5	m	m	m	m	2	-	-	m	m	m	m	m	m
<i>Mustela vison</i> , American mink	4	m	m	m	4	m	-	-	m	3	2	2	-	m
<i>Meles meles</i> , European badger	4	-	m	m	m	m	-	-	m	m	-	m	-	m
<i>Gulo gulo</i> , wolverine	4	-	m	m	2	2	-	-	m	m	m	m	m	m
<b>Carnivore</b>														
<i>Crocuta crocuta</i> , spotted hyena	4	-	-	-	-	-	-	-	-	-	-	-	-	-
<b>Contaminants of spraint DNA</b>														
<i>Anguilla anguilla</i> , European eel	4	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Salmo salar</i> , Atlantic salmon	4	-	-	-	-	-	-	-	-	-	-	-	-	-

N, number of individuals tested; \*, values are the number of alleles detected; m, monomorphic; -, no product.

European eel or Atlantic salmon, two common prey species whose DNA is probably copurified with otter DNA from spraints. These markers should provide useful genetic data for studies in otter ecology and conservation.

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## Characterization of (GT)<sub>n</sub> microsatellites from native white shrimp (*Penaeus setiferus*)

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*Penaeus setiferus*, native white shrimp, are part of a large commercial and recreational fishery along the western Atlantic and Gulf of Mexico (Pérez-Farfante 1969), yet little is known about the population genetics. Previous work with allozymes suggested limited genetic variability and population differentiation (Lester 1979), but recent studies in other penaeids with mitochondrial DNA, RAPD's, or microsatellites suggest otherwise (Benzie *et al.* 1993; Wolfus *et al.* 1997).

We have developed microsatellite genetic markers from *P. setiferus*. Thirty-nine loci containing (GT)<sub>n</sub> repeats were iden-

tified and the microsatellites classified by length according to specific guidelines (Weber 1990). Six primer sets were tested on several penaeid species. *P. aztecus*, *P. duorarum*, and *P. setiferus* are native to the western Atlantic and Gulf of Mexico. *P. vannamei* and *P. stylirostris* are found in the eastern Pacific from the Gulf of California to Peru and are important mariculture species in the Americas (Pérez-Farfante 1988).

The genomic library was constructed and screened essentially according to Brooker *et al.* (1994), except genomic DNA from tail-muscle tissue digested with *DpnII* was ligated to *BamHI*-digested bacterial alkaline phosphatase-treated pUC18 (Pharmacia) and used to transform DH5 $\alpha$  maximum competent cells (BRL). The resulting colonies were transferred to nylon filters and screened with a (GT)<sub>15</sub> oligonucleotide end-labelled with [ $\gamma^{32}$ P]-ATP. Plasmid DNA from positive clones was prepared using Wizard Preps (Promega) and then sequenced using Sequenase 2.0 (Amersham). Primers identified from flanking regions were synthesized at the Medical University of South Carolina.

For DNA isolations for amplifications, muscle tissue (100–200 mg) from frozen samples was lysed in 1% Sarkosyl/6 M urea at 60° overnight. DNA was isolated according to Vogelstein & Gillespie (1979). Standard PCR reactions included 10 ng of template DNA, 0.3  $\mu$ M forward and reverse primer, 0.2 mM each dNTP, 3 mM MgSO<sub>4</sub>, 17 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM  $\beta$ -mercaptoethanol, 67 mM Tris-HCl pH 8.8 and 0.25 units of *Taq* polymerase (Promega) in 10  $\mu$ L. One primer was end-labelled with [ $\gamma^{32}$ P]-ATP at 0.1  $\mu$ Ci/pmol primer. Cycling parameters were 3 min at 94 °C followed by 35 cycles of 40 s at 94 °C, 40 s at the annealing temperature, and 40 s at 72 °C. Amplifications were performed in Ericomp Delta I thermal cyclers. Gel electrophoresis was performed as described (Brooker *et al.* 1994).

We screened  $\approx$  4000 clones, sequenced 55 positive clones, and found 27 clones which contained one or more (GT)<sub>n</sub> repeats (for a total of 39 repeats). Classification of the repeats is shown in Fig. 1. These repeats are, in general, longer than those found in mammals (Weber 1990; Brooker *et al.* 1994), and comparable to those found in *P. vannamei* and *P. monodon* (A. Alcivar-Warren, A. Brooker, personal communication). Eight loci were identified where the total number of repeats was over 60; however, the longest uninterrupted repeat was 59 units.

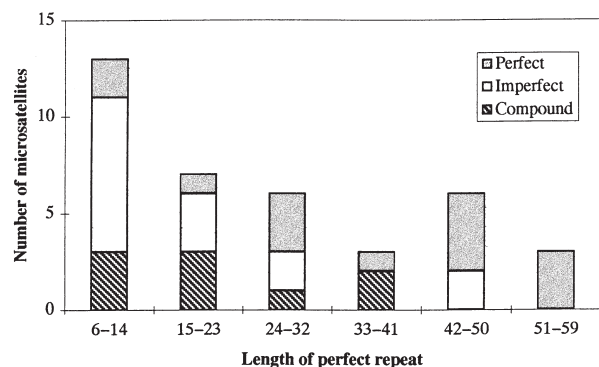


Fig. 1 Frequency of different types of microsatellite repeats classified by the number of repeats in the longest uninterrupted array.

**Table 1** Primer and repeat sequences, amplification conditions and results of amplification of six microsatellite loci in five penaeid species

Locus	Primer sequences (5' to 3')	Repeat sequence	Type of repeat	Size range (bp)	Anneal °C	<i>P. setiferus</i> *				
						Observed heterozygosity	<i>P. aztecus</i>	<i>P. duorarum</i>	<i>P. vannamei</i> †	<i>P. stylirostris</i>
Pse002	CTGAAATACAACCACCTTTGC CGGGATTCTGCTTGAGGG	(AC) <sub>5</sub>	Perfect	88#	55	0.00 (20)	0.55 (11)	0.60 (10)	0.27 (11)	0.53 (19)
Pse004	GATCACGTGACTCTGCAAAG CGTTCAGATTGTCAACTTCGGG	(GT) <sub>47</sub> AT(GT) <sub>7</sub>	Imperfect	160–210	50	0.81 (32)	0.60 (10)	–	–	0.67 (46)
Pse017	GATCTCGCTCAATCGCTTCAAGC TTGTGAAAATCGTAAGCGCTGTC	(GT) <sub>44</sub>	Perfect	120–140	55°	0.43 (7)	–	–	–	–
Pse028	GATCCTTCTAGCTAAATGGG GATCGAAGGTAACTTTAATTATC	(CA) <sub>24</sub>	Perfect	136–264	50°	0.67 (90)	0.57 (7)	0.60 (10)	0.56 (9)	0.20 (5)
Pse035	CACGTGAGGACAAAGAGCAFTG CTTTCATACTCACGCTAACAAFTTG	(AC) <sub>57</sub>	Perfect	180–250	60°	0.50 (6)	–	–	–	–
Pse036	GACTTTGTAFTTTTCATAAAGCTG CGGTATAFTTTCGACAGTAAGGCTAC	(AC) <sub>30</sub>	Perfect	105–161	55°	0.81 (90)	–	0.25 (8)	0.56 (9)	0.45 (49)

\*Samples (wild shrimp): *Penaeus setiferus*, Charleston Harbor, Charleston, SC, January 1997; *P. aztecus*, offshore Charleston, SC, July 1996; *P. duorarum*, offshore Florida, October 1996.†Samples (cultured shrimp): *P. vannamei*, Waddell Mariculture Center, Beaufort, SC, November 1996; and *P. stylirostris*, Island Fresh Seafood, Megget, SC, September 1997.# Size range given is for *P. setiferus*, ranges varied in other species.

Ten sets of primers were designed; six amplified *P. setiferus* DNA, five were polymorphic, and two have been used for amplification of large sets of samples (Table 1). Four of the six loci cross-reacted with other species, and two produced strong products in all species. *P. aztecus* and *P. duorarum* are classified as *Farfantepenaeus*, while the other three are classified as *Litopenaeus* (Pérez-Farfante 1988). However, the cross-reactivity did not reflect any particular species grouping.

Pse028 and Pse036 were tested more extensively on *P. setiferus* (Table 1). No linkage disequilibrium was observed at these loci (GENEPOP 3.1, Raymond & Rousset 1995). For Pse028, observed heterozygosity, 0.67, was significantly different than expected heterozygosity, 0.97,  $P < 0.001$ . This could be due to null alleles, selection, population substructure, or assortative mating. Using Brookfield's (1996) calculations when null homozygotes are observed (1 of 90 samples), the frequency of null alleles was estimated to be 16%. At this frequency, all apparently homozygous individuals are predicted to be heterozygous for a visible and a null allele, and  $H_z = 0.99$ . Therefore null alleles cannot be rejected as an explanation for this observation of heterozygote deficiency. At locus Pse036, observed heterozygosity, 0.81, was not significantly different from the expected heterozygosity, 0.87.

The microsatellites isolated from *P. setiferus* were typical except for the extreme length. The cross-reactivity confirms that primers developed for one penaeid may be useful in others. These markers will be useful for population studies and for broodstock selection programs.

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## Isolation and characterization of microsatellite markers in the periwinkle *Littorina striata* King & Broderip, 1832 (Mollusca, Gastropoda, Prosobranchia)

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The highly polymorphic, planktotrophic developing periwinkle *Littorina striata* is an inhabitant of the intertidal rocky shores of Macaronesia (Azores, Madeira, Canary Islands and Cape Verde Islands), where its spatiotemporal morphological and genetic structuring has been analysed with allozyme and RAPD data (e.g. De Wolf 1997). Both types of marker showed considerable variation and revealed tendencies towards population genetic substructuring (De Wolf 1997). Microsatellite markers are expected to provide more sensitive markers to substantiate or reject these supposed patterns.

Hitherto, no microsatellite amplifying primers were available for *L. striata* and only few were available for molluscs in general (Jarne *et al.* 1994; Naciri *et al.* 1995; Shaw 1997). We developed five specific pairs of primers for microsatellite loci in *L. striata* and tested their cross-species amplification in four other littorinid species (*L. arcana*, *L. saxatilis*, *L. obtusata* and *Nodilittorina punctata*).

DNA was extracted (Winnepeninckx *et al.* 1993) from the digestive gland of five *L. striata* individuals, collected on the island of Faial (Azores, Portugal). A partial genomic library was constructed by digesting 2.5 µg of DNA with both *Mbo*I and *Sau*3AI. Fragments of 300–500 bp were ligated into the *Bam*HI site of pUC18 (Pharmacia). Screening ≈ 5000 DH5α *Escherichia coli* recombinant clones with [<sup>32</sup>P]-dATP 5' end-labelled (CA)<sub>15</sub>, (GC)<sub>15</sub>, (AT)<sub>15</sub> and (GATA)<sub>7</sub> probes yielded 62 positive clones. Plasmids were isolated (Qiagen) and sequenced in both directions using the Thermosequenase fluorescent labelled primer cycle sequencing kit (Amersham) and two universal M13 primers. Sequence reactions were run on the ALF Express automated sequencer (Pharmacia). Of all 62 positive clones, 35 contained one or more repeat sequences. These yielded 38 repeat arrays, from which we developed 10 *L. striata*-specific primer sets using the program EUGENE 1.0 (Danibens Systems).

**Table 1** Microsatellite loci from the periwinkle *Littorina striata*.  $T_A$  is optimal annealing temperature,  $H_O$  is observed and  $H_E$  is expected heterozygosity

Locus	Primers (5'-3')	$T_A$	EBI accession number	Repeat sequence	No. of alleles	No. of specimens	$H_O$	$H_E$
Lstri1.4	AGCGTTCTCTTTCCGGC ACCCAGCCCTGCTCGTTC	54	Y14839	(TG) <sub>24</sub> CG(TG) <sub>2</sub> CG(TG) <sub>13</sub> CG(TG) <sub>4</sub> CG(TG) <sub>2</sub>	19	24	0.250	0.946
Lstri1.131	ATGCATAACCATAAGGATGTTTG GAATTGTTTCGTTACCAAGCC	59	Y14840	(AC) <sub>19</sub> (AG) <sub>25</sub>	15	16	0.188	0.954
Lstri1.151	GGAGACGGTTCCAGAGCG TCAGTTGCTCACGCTGTC	54	Y14842	(CA) <sub>3</sub> (GACA) <sub>4</sub> (CA) <sub>21</sub>	27	35	0.829	0.962
Lstri1.152	AGGTCTTCGAAAGTCTCGGC TGAATATTCCAGAGGAGCG	59	Y14843	(CA) <sub>22</sub>	17	32	0.406	0.930
Lstri2.3	AGCGGACACGCGAAGTCAGGG CGCTCGCATCAACAATGCG	58	Y14844	(CA) <sub>25</sub>	18	16	1.000	0.954

DNA for genotyping was extracted from the foot muscle of single individuals (Winnepeninckx *et al.* 1993). Instead of crunching tissues under liquid N<sub>2</sub>, they were minced using a scalpel. PCR reactions (10 µL) consisted of template DNA, 2.25 pmol forward primer 5' end-labelled with either [<sup>32</sup>P]-dATP or CY5 (Pharmacia), 2.25 pmol reversed primer, 0.4 units of *Taq* polymerase (Boehringer Mannheim), 1× the supplier's PCR buffer (1.5 mM MgCl<sub>2</sub> final concentration), and 1.3 nmol of each dNTP (Pharmacia). An Omn-E thermal cycler (Hybaid) was used with the following cycle parameters: 1 × 94 °C, 3 min; 25 × 94 °C, 30 s; 54–59 °C, 1 min; 72 °C, 1 min; 1 × 72 °C, 5 min. PCR products were separated by electrophoresis in 7% denaturing polyacrylamide gels using either a standard vertical electrophoresis unit (Gibco BRL) or the ALF Express automated sequencer.

PCR amplification of four loci failed to yield products of the expected size, even after applying a wide range of conditions. The amplification products of one marker were too short to allow appropriate scoring. Data on the five loci which were successfully amplified are listed in Table 1. These five markers were used to analyse samples from two localities on São Miguel (Azores) and two on the Cape Verde Islands. Both the [<sup>32</sup>P]-dATP and the CY5 detection system yielded the same results. The five loci revealed a high amount of allelic polymorphism (Table 1) (average number of alleles/locus = 19.2), suggesting a high degree of genetic variability, which seems higher than that found for microsatellite markers in other molluscan species (Jarne *et al.* 1994; Naciri *et al.* 1995; Shaw 1997). For three loci (Lstri1.4, Lstri1.131 and Lstri1.152), there was a large discrepancy between the observed and expected heterozygosity (mean value 0.281 and 0.943, respectively). As scoring of the gels was straightforward, the discrepancies may be explained by the limited sample size or substructuring of the samples. Yet, as there is an inconsistency in discrepancy between different loci, the latter explanation seems rather unlikely. As for several samples, no amplification products of loci Lstri 1.4, Lstri 1.131 and Lstri 1.152 were observed; the occurrence of null alleles may be another valid explanation for the heterozygote

deficiency in these loci. However, it seems unlikely that null alleles would occur in that many loci. Further investigation of this topic is necessary.

The attempted cross-species amplification of DNA from the four other littorinid species failed for the five primer sets of Table 1. This failure seems to support the suggestion that *L. striata* is a rather unique species that is only distantly related to other littorinids and probably reflects the ancient separation of these taxa (Reid 1996; B. Winnepeninckx, unpublished results). *L. striata* diverged from the other *Littorina* species ≈ 40 Ma (Reid 1996).

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## Universal PCR primers for S7 ribosomal protein gene introns in fish

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Lessa (1992) introduced intron-targeted PCR, in which a non-coding intron was amplified using primers designed from highly conserved exon sequences. Introns appear to harbour a much greater degree of genetic polymorphism within and between species than exons. On the other hand, length and nucleotide sequence of exons, and exon-intron arrangement can be highly conserved between considerably distant animal taxa. These characteristics may allow us to design sets of primers based on exon sequences to amplify flanking intron regions. Such sets of primers might function in very distant species. This study introduces two pairs of primer sets which were designed for amplifying the 1st and 2nd introns of the S7 ribosomal protein gene in fish. These primers were applied to distant fish species in order to determine their universality, and polymorphism in the amplified fragments was investigated.

The DNA sequence data of the S7 ribosomal protein gene of puffer fish (*Fugu rubripes*), frog (*Xenopus laevis*) and human were derived from Cecconi *et al.* (1996), Mariottini *et al.* (1993) and Annilo *et al.* (1995), respectively. Exons 1, 2 and 3 of these species were aligned to determine conserved sequence regions. Because exon 1 of humans showed very poor homology with exon 1 of other species, data from puffer fish and frog were used for aligning exon 1. By contrast, highly conserved regions among these distant species were observed in exons 2 and 3. Two sets of primers were designed from the conserved sequence regions. The primer sequences to amplify the 1st intron (RP1) were 5'-TGGCC-TCTTCCTTGGCCGTC-3' (S7RPEX1F) and 5'-AACTCGTCTGGCTTTTCGCC-3' (S7RPEX2R), and those for the 2nd intron (RP2) were 5'-AGCGCCAAAATAGTGAAGCC-3' (S7RPEX2F) and 5'-GCCTTCAGGTCAGAGTTCAT-3' (S7RPEX3R). The PCR reaction mixture contained 0.2 U of *Taq* DNA polymerase (Perkin Elmer Cetus), 0.2 mM of each dNTP, 1  $\mu$ L of the manufacturer's supplied 10 $\times$  buffer, 2 mM MgCl<sub>2</sub>, 10 pmol of each primer and 10–50 ng of template DNA, in a final volume of 10  $\mu$ L. Amplification was carried out with an initial denaturation at 95 °C for 1 min, followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min). PCR products and those digested by endonuclease were electrophoresed on a 2.5% agarose gel (Biogel) in TBE buffer (50 mM Tris, 1 mM EDTA, and 48.5 mM boric acid).

Using the standard phenol–chloroform method, crude DNA was extracted from frozen or ethanol-preserved muscles of chum salmon (*Onchorhynchus keta*), tuna (*Thunnus* spp.)

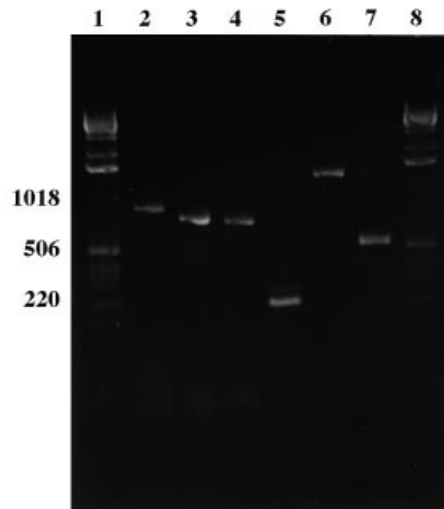


Fig. 1 Result of PCR amplification targeting the 1st (lanes 2–4) and 2nd introns (lanes 5–7) of the S7 ribosomal protein gene of three distant fish species. Lanes: 1 and 8, molecular weight marker (Gibco BRL 1 kb ladder); 2 and 5, chum salmon (*Onchorhynchus keta*); 3 and 6, yellowfin tuna (*Thunnus albacares*); 4 and 7, puffer fish (*Fugu rubripes*).

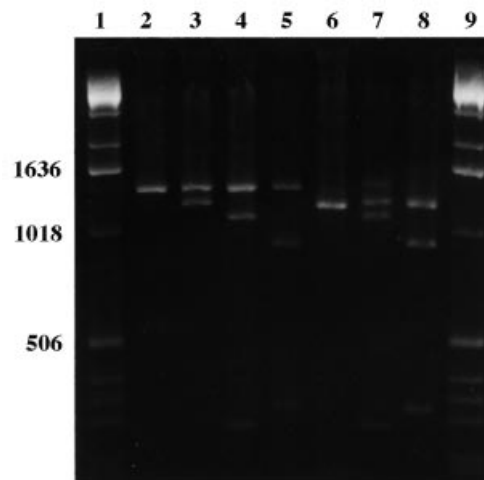


Fig. 2 *Hha*I restriction profiles observed in the 2nd intron of the S7 ribosomal protein gene of yellowfin tuna. Lanes 1 and 9, molecular weight marker (Gibco BRL 1 kb ladder). Deduced genotypes are: lane 2 AA; lane 3, AB; lane 4, AC; lane 5, AD; lane 6, BB; lane 7, BC; and lane 8, BD.

and puffer fish (*Fugu rubripes*), each of which belonged to a different order. Results from PCR amplifications of RP1 and RP2 are shown in Fig. 1, where amplification of a single fragment was eminent in all species. Amplified fragments of salmon, tuna and puffer fish were all different in length with respect to each other, while no length difference was observed among eight tuna species (data not shown).

A battery of 4-bp cutter endonucleases was applied to PCR products of yellowfin tuna (*Thunnus albacares*) in order to investigate intraspecific restriction site polymorphism.

Restriction site polymorphisms were observed in both RP1 and RP2 fragments, where the total length of restriction fragments in all endonuclease digestions never exceeded twice the size of the uncut PCR product. Relatively simple restriction patterns obtained by *Hha*I digestion of the RP2 fragment are shown in Fig. 2, which allow us to interpret the presence of four alleles. Observed and expected heterozygosities were 0.213 and 0.239 for a Pacific Ocean sample ( $n = 38$ ), 0.390 and 0.435 for an Indian Ocean sample ( $n = 39$ ), and 0.434 and 0.416 for an Atlantic Ocean sample ( $n = 42$ ), respectively. These genetic variations were comparable with the results of allozyme analysis obtained by Ward *et al.* (1994), and all populations analysed in this study were found to be in accordance with the Hardy–Weinberg equilibrium. These results support the use of polymorphic intron within the S7 ribosomal protein gene as Mendelian marker, at least in tunas.

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## Genetic distinction of scorpionflies (*Panorpa vulgaris*) by microsatellites

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The scorpionfly *Panorpa vulgaris* represents an interesting model for studying sexual selection and life history. The scorpionflies mate promiscuously in both sexes (Sauer *et al.* 1998). There is evidence that the promiscuous mating behaviour of the females is forced selectively by their inability to determine the quality of their potential mates prior to mating (Sauer *et al.* 1998). Females appear to discriminate among males of varying phenotypic quality (nutrition status) only

by mating longer with high-quality males. Similarly, lifetime mating duration is the decisive proximate determinant of male fitness. To achieve meaningful interpretations on sexual selection and lifetime history, genetic relationships must be determined unambiguously. Multilocus DNA fingerprinting provides high interindividual resolution power but is not suitable for population genetic studies in insects where limited amounts of DNA are obtained. Here we describe three highly informative microsatellites with individual exclusion probabilities of > 0.80 for *P. vulgaris*.

*P. vulgaris* specimens were collected in the field near Freiburg (Germany) or used after breeding. For DNA preparation, larvae or adult *P. vulgaris* were grinded with a pestle in 450  $\mu$ L of 6% DTAB. A volume of 450  $\mu$ L of DTAB and 20  $\mu$ g of RNase A was added and incubated for 15 min at 68 °C. After chloroform extraction (900  $\mu$ L) the supernatant ( $\approx$  500  $\mu$ L) was poured into a 2 mL Eppendorf tube containing 100  $\mu$ L of 5% CTAB, 90  $\mu$ L of H<sub>2</sub>O. By inverting the tube a DNA/CTAB precipitate formed. After centrifugation (2 min, 10 000 g) the pellet was dissolved in 300  $\mu$ L of 1.2 M NaCl and the DNA precipitated with 750  $\mu$ L of 99.5% ETOH. After washing (70% ETOH) the DNA was redissolved in 10 mM Tris/1 mM EDTA. All other procedures followed the methods outlined in Sambrook *et al.* (1989).

For library construction, restriction enzyme-digested DNA of the selected size was ligated into the vector pBluescript KS+™ and *Escherichia coli* SURE cells were transformed. Individual bacterial colonies were collected and gridded automatically by a Biomek™ workstation (Beckman). A total of 10 464 clones were generated, i.e. > 900 from unfractonated *Sau*3AI digests, > 6700 from 200 to 1000 bp long *Sau*3AI fragments and > 2800 from *Rsa*I fragments of 100–1000 bp. A total of 98% of clones were recombinant as judged from PCR amplifications of inserts. Nylon membranes were screened with 19 simple repetitive oligonucleotides. Thirty-two clones generating strong hybridization signals revealed simple repeats upon sequencing, and PCR primers were designed (Table 1).

The PCR reactions (10  $\mu$ L), containing approximately 50 ng of template DNA, PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 1–3 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, 1  $\mu$ M of each primer, and 0.5 U of *Taq* polymerase (Beckman), were performed in a Crocodile III™ thermocycler (Appligene). The initial denaturation time was 5 min at 95 °C, thereafter 30 s. Annealing time was 60 s with the following temperatures: 2Pv at 60 °C, 5Pv at 54 °C, 7Pv at 56 °C. In the first two cycles the temperature was raised 6 °C and 3 °C above the final annealing temperature. Elongation lasted for 60 s at 72 °C and 5 min in the final step. Fluorochrome- (6-FAM, TET, HEX) labelled fragments were analysed automatically using an ABI 377 sequencer (GENESCAN program).

The highly polymorphic microsatellites 5Pv and 7Pv represented perfect (AT)<sub>n</sub>/(TA)<sub>n</sub> blocks; 2Pv is a cryptic (CT)<sub>n</sub> repeat. As the original 2Pv 5' primer (double underlined in sequence no. 2, Table 2) did not always amplify both parental alleles, a null allele was suspected. Therefore new primers (Table 1) were synthesized and alleles of different lengths were sequenced (Table 2). Obviously the amplification failure was due to a 1 bp insertion in sequence no. 1 at the 3' end of the primer attachment site. In addition, an 8 bp (GCG-



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## Isolation and characterization of highly polymorphic microsatellites in the water vole, *Arvicola terrestris*

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The water vole, *Arvicola terrestris*, has become a species of particular conservation importance in the UK due to an 80% reduction in numbers in recent years (Strachan & Jefferies 1993). This decline has been attributed to the loss of its natural habitat, and to predation by feral American mink, *Mustela vison*. Consequently, gaps have appeared in a formerly contiguous distribution, and groups of adjacent water vole colonies may now function as metapopulations (Lawton & Woodroffe 1991). The persistence of this species may in future depend on the balance between local extinction and effective dispersal (Hastings & Harrison 1994). In this Primer Note, we describe highly polymorphic tri- and tetranucleotide microsatellite loci in *A. terrestris*. These markers will be invaluable in estimating levels of individual dispersal and gene flow among populations. A better understanding of how dispersal underpins metapopulation persistence in this species will be an essential contribution to a workable conservation strategy.

Two approaches were used to isolate the microsatellites. First, a 300–800 bp partial genomic library was constructed by ligating *Sau3AI*-digested water vole DNA into  $\lambda$ -ZAP (Stratagene) and screened with the trinucleotide polymer (TAA/ATT)<sub>n</sub>. Second, enrichment cloning (Armour *et al.* 1994) was used to obtain further microsatellites more efficiently. A *Sau3AI*-digested 300–800 bp fraction of water vole DNA was enriched for (GATA)<sub>n</sub>, (GAAA)<sub>n</sub>, (CAA)<sub>n</sub> and (TAAA)<sub>n</sub> according to Piertney *et al.* (1998). The enriched fragments were ligated into pUC18, transformed into *E. coli* Invaf' (Invitrogen), then screened with radiolabelled polymer probes. Inserts of positive clones were sequenced using an ABI 377 automated sequencer. Forty-eight clones were sequenced, and sixteen primer pairs flanking the microsatel-

lite arrays were designed using the program OLIGO (National Biosciences Inc., version 4.0). Twelve primer pairs detected clear highly polymorphic PCR products. Enrichment cloning proved to be the more effective technique, producing 11 out of the 12 microsatellite loci.

Non-destructive sampling of live animals was carried out with the minimum of distress to the individual. Levels of polymorphism of the 12 loci were estimated using DNA isolated (Müllenbach *et al.* 1989) from small ear discs taken during ear-tagging of 96 water voles from the river Ythan in northeast Scotland. PCR amplifications were carried out in a total volume of 10  $\mu$ L, which contained 20 ng of template DNA, 75 mM Tris-HCl (pH 9.0), 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2 mM of each nucleotide, 5 pmol of each primer (forward primer end-labelled with [ $\gamma$ <sup>32</sup>P]-ATP) and 0.5 units of *Taq* DNA polymerase (BioLine). Salt concentrations were optimized for each primer set resulting in final concentrations of MgCl<sub>2</sub> being 2–3 mM (see Table 1). The PCR programme was: 2 min at 91 °C, followed by 30 cycles of 30 s at 91 °C and 30 s at the annealing temperature, then a final 2 min at 72 °C. PCR fragments were resolved by electrophoresis in 6% denaturing acrylamide gels. Allele sizes were determined relative to an M13mp8 DNA sequencing ladder.

All 12 of the microsatellite loci were highly polymorphic (Table 1), a property which makes them highly informative tools for studies of population genetic structure and relatedness in *A. terrestris*. In addition, null alleles for these markers do not appear to be common. The observed heterozygosity values are very close to the expected heterozygosity values within subpopulations, which are generally in Hardy-Weinberg equilibrium for all 12 microsatellites (data not shown).

The utility of these microsatellite primers was examined in four other microtine species: *A. richardsoni*, *Microtus agrestis*, *M. epiroticus*, and *M. oeconomus* (Table 2). The PCR conditions used were the same as those given above. None of the primers gave a product of the expected size in *A. richardsoni*, whereas most of the primers detected a product of or close to the expected size in *M. agrestis* and *M. epiroticus*, but showed low levels of polymorphism. Although sample numbers were low, it does not seem that these microsatellites will be useful for population studies of these microtine species. However, six out of the 12 primers detected polymorphism (2–5 alleles) in *M. oeconomus*, suggesting that these primers will be useful for studying gene flow and population structure in this species.

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**Table 1** Characteristics of 12 microsatellites of the water vole

Locus name	Repeat motif	EMBL Accession no.	Primer sequences	Annealing temp (°C)	MgCl <sub>2</sub> (mM)	Fragment size (bp)	Allele no.	Observed heterozygosity
AV1	(CCTT) <sub>17</sub>	Y16548	F: ctctgagctgagtatgtgtcc R: ggtctacaagagctagtccag	55	1.5	205	11	74%
AV3	(TAA) <sub>20</sub>	Y16555	F: ggatcaaccaggctccagcac R: aaagagctagtggttcctaaag	51	2.5	205	13	68%
AV4	(GATA) <sub>14</sub> AGGA(GATA) <sub>19</sub>	Y16556	F: gaattacacatgggagctctgag R: cacagccacaaggtagaaaag	55	2.0	226	11	79%
AV7	(GATA) <sub>12</sub>	Y16557	F: agatgataaacacgtagatgc R: tatccatctgtccatctgtc	53	2.5	177	8	65%
AV8	(GATA) <sub>18</sub>	Y16558	F: gcaccaaacataactccac R: aggatgccgagatactccag	58	2.5	330	6	77%
AV9	(GATA) <sub>15</sub>	Y16559	F: tggctcagattcaagactac R: ggaaagctaggtcacagat	52	3.0	240	14	78%
AV10	(GATA) <sub>16</sub>	Y16549	F: gctgagccatctctccagac R: cagcatttgaaggcagagg	59	3.0	350	7	60%
AV11	(GATA) <sub>13</sub> (GATG) <sub>3</sub> (GATA) <sub>10</sub>	Y16550	F: tggccttatcaggaacatac R: ctctgtcctccacctctct	56	2.5	225	11	75%
AV12	(GATA) <sub>12</sub>	Y16551	F: ggccaggagataacaagattgag R: tctccaagatgagttccaacag	59	2.5	210	8	63%
AV13	(GATA) <sub>14</sub>	Y16552	F: ctggctctatctatctgtctatc R: acaattacagcatccagaag	52	2.5	200	11	78%
AV14	(GATA) <sub>16</sub>	Y16553	F: tatgtgatatggcactagcatgt R: agcctgtctcagcagaagg	56	2.5	250	10	77%
AV15	(GATA) <sub>14</sub>	Y16554	F: tatatggaaggtcgtagattcag R: attaaagcatttggagaaagc	54	2.5	205	10	72%

**Table 2** Cross-species amplification of 12 microsatellites of the water vole

	Species			
	<i>Arvicola richardsoni</i> (n = 1)	<i>Microtus agrestis</i> (n = 4) (field vole)	<i>M. epiroticus</i> (n = 5) (common vole)	<i>M. oeconomus</i> (n = 6) (root vole)
AV1	–	1	–	2
AV3	–	1	–	–
AV4	–	–	2	–
AV7	–	2	–	4
AV8	–	–	–	–
AV9	1	1	1	1
AV10	–	3	–	1
AV11	–	–	–	–
AV12	–	3	–	2
AV13	–	1	2	4
AV14	–	1	–	4
AV15	–	2	–	5

Where amplification was successful the numbers of alleles detected are given; – indicates a smear or no amplification.

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## Characterization of microsatellite loci in *Pinus sylvestris* L.

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Recently, interest has been directed towards the study of the genetics of natural populations. For this purpose nuclear SSRs (simple sequence repeats) have been characterized in a number of tree species (Smith & Devey 1994; Kostia *et al.* 1995; White & Powell 1997a). A greater increase in SSR isolation efficiency has been achieved since the introduction of various enrichment procedures (Edwards *et al.* 1996; Fisher *et al.* 1996). SSR enrichment protocols have been successfully applied in gymnosperms (Echt *et al.* 1996; Pfeiffer *et al.* 1997), but the large genome size and complexity (Kinlaw & Neale 1997) have rendered the identification of single-locus, reproducible markers for conifers a challenging task (Echt *et al.* 1996; Morgante *et al.* 1996) and hindered their application in population studies.

In this study we successfully used an enrichment procedure (White & Powell 1997a) to isolate SSRs in *Pinus sylvestris*. We generated two genomic libraries enriched for SSR repeats and produced a set of markers that identify single variable loci. Total genomic DNA was extracted from leaf and megagametophyte tissue following Wagner *et al.* (1987). Two libraries enriched for (AC)<sub>n</sub> and (AG)<sub>n</sub> repeats were constructed following the procedure described in White & Powell (1997a). Enrichment of dinucleotide repeats was achieved through hybridization with (AC)<sub>13</sub> or (AG)<sub>13</sub> 3'-biotinylated oligonucleotides bound to streptavidin-coated magnetic beads. The enriched fraction containing small frag-

ments (200-600 bp) was then cloned in λ-Zap phage vector. Positive recombinant clones were identified through hybridization with either an end-labelled (AC)<sub>13</sub> oligonucleotide probe or a poly(AG) probe by random priming. Hybridization was carried out in 5× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS at 68 °C or 65 °C, respectively, for AC and AG probes. Two washes were performed in 2× SSC, 0.1% SDS at 65 °C followed by one wash in 1× SSC, 0.1% SDS at 68 °C or 65 °C, respectively. Recombinant clones were sequenced with an ABI 377 sequencer.

Approximately 15% of the plaques were scored as positive in the primary screen for both libraries. Assuming the efficiency of recovery for (AC)<sub>n</sub> repeats in a nonenriched library to be the same as estimated for *Pinus strobus* (0.14%; Echt *et al.* 1996), and the proportion of duplicates is not significant this procedure achieved an overall 100-fold enrichment. Sixty-five out of the 90 positive (AC)<sub>n</sub> clones sequenced contained a microsatellite repeat. The four repeat types described by Weber & May (1989) were detected, the compound perfect (44.6%) and simple perfect types (35.3%) being the most frequent. In 90% of the compound clones (AC)<sub>n</sub> repeats were associated to an AT motif; this is consistent with (AT)<sub>n</sub> being the most frequent dinucleotide found in plant genomes (Morgante & Lagercranz *et al.* 1993; Morgante & Olivieri 1993). In general, compound (AC)<sub>n</sub> repeats appeared to be more frequent in Scots pine (55%) in comparison to *P. strobus* (24%; Echt *et al.* 1996) and Norway spruce (15%; Pfeiffer *et al.* 1997); this could be due to a higher stringency of the enrichment method used here. In the 54 (AG)<sub>n</sub> microsatellites characterized, the simple perfect type alone accounted for ≈ 54% of the total repeats. The average length of an uninterrupted simple dinucleotide stretch was 27.5 and 25.5 units for the AC and AG library, respectively.

Thirty-seven primer pairs were designed using the software package Primer (Version 5.0). PCR reactions were carried out as described in White & Powell (1997a). Fifty per cent of the clones produced a multilocus PCR amplification pattern, 25% a poor or null amplification profile, 5% or a

**Table 1** Primer sequences and diversity values (heterozygosity) of *Pinus sylvestris* SSRs characterized in the present study

Locus	Repeat	Primer sequence (5'-3')	Expected size (bp)	$T_m$	Megagametophytes analysed	No. of alleles	$H$	Accession no.
SPAC 11.4	(AT) <sub>5</sub> (GT) <sub>19</sub>	TCACAAAACACGTGATTCACA GAAAATAGCCCTGTGTGAGACA	150	60	14	8	0.901	AJ223766
SPAC 11.6	(CA) <sub>29</sub> (TA) <sub>7</sub>	CITCACAGGACTGATGTTCA TTACAGCGGTTGGTAAATG	165	55	11	7	0.927	AJ223767
SPAC 11.8	(TG) <sub>16</sub>	AGGGAGATCAATAGATCATGG CAGCCAAGACATCAAAAATG	142	55	13	2	0.538	AJ223770
SPAC 12.5	(GT) <sub>20</sub> (GA) <sub>10</sub>	CITCTTCACTAGTTTCTTTGG TTGGTTATAGGCATAGATTGC	155	54	10	10	0.924	AJ223772
SPAG 3.7	(TC) <sub>45</sub>	GTAAAGAAAATAATGACGTCTC AATACATTTACCTAGAATACGTCA	171	52	12	8	0.924	AJ223769
SPAG 7.14	(TG) <sub>17</sub> (AG) <sub>21</sub>	TTCGTAGGACTAAAAATGTGTG CAAAGTGGATTTTGACCG	209	55	14	6	0.868	AJ223771
SPAC 11.5	(AT) <sub>8</sub> (GT) <sub>19</sub> - (TA) <sub>11</sub>	TGGAGTGGAGTTTGAGAAGC TTGGGTTACGATACAGACGATG	194	60	14	6	0.868	AJ223768

single, monomorphic band while only seven (20%) identified a single variable locus. The seven primer pairs that identify functional markers were tested on a panel of 11–14 DNA samples extracted from megagametophyte tissue; between two and 10 alleles per locus were detected. Genetic diversity was estimated using unbiased Nei's heterozygosity index (Nei 1987), expressed as  $H = (n/n - 1) 1 - \sum p_i^2$  (Table 1). Diversity values ranged between 0.538 and 0.945. The segregation of alleles at three polymorphic SSR loci (SPAC11.6, SPAC11.5 and SPAG3.7) was examined in a full-sib family ( $n = 60$ ). A  $\chi^2$  test (3 d.f.) was performed: none of the three SSRs deviated significantly from the expected Mendelian segregation ratio.

The use of SSRs as codominant markers in plant population studies is related to their ability to unambiguously define genotypes. Only 20% of the primer pairs analysed in this study resulted in locus-specific amplification of a polymorphic locus; the majority of the remaining primer pairs gave either no amplification or produced multiband patterns. A similar proportion of functional markers has been reported in studies of other conifer species (Echt *et al.* 1996; Morgante *et al.* 1996; Pfeiffer *et al.* 1997), and can be attributed to the high complexity of conifer genomes. Pfeiffer *et al.* (1997) showed that the majority of bands observed in multiple PCR profiles corresponded to fragments coamplified with the SSR locus but not containing the microsatellite region. Kinlaw & Neale (1997) showed that gene duplication is a common process in the evolution of gymnosperm genomes. Furthermore, hybridization of clones with labelled total genomic DNA showed that 25% of SSRs are located within highly repeated regions and are therefore likely to produce multiband patterns (Smith & Devey 1994).

The identification of markers suitable for population studies is therefore a crucial process in conifers. The characterization of functional SSRs in conifers is a difficult and costly task, but the hypervariability typical of these markers is such that only a limited number are required for population studies. The possibility of transferring SSR information across species is currently being investigated (C. S. Echt, personal communication; White & Powell 1997b). Otherwise, the development of sequence-tagged sites (STS) and bi-allelic polymorphisms could provide an attractive alternative to SSRs.

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## Characterization of tetranucleotide microsatellite markers in the Scottish crossbill (*Loxia scotica*)

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The taxonomic status of the Scottish crossbill (*Loxia scotica*) is a contentious issue. It is currently designated as Britain's only avian endemic, and one of only three UK bird species of global conservation concern (Gibbon *et al.* 1996). However, it differs only slightly (in call, size, morphology and plumage) from other European crossbills (*L. curvirostra* and *L. pytyopsittacus*). Crossbill taxa are ecologically distinct, each adapted to feed on the seeds of specific conifers (Benkman 1993). *L. scotica* is adapted to feed on Scots pine but the reduction in native pineforest, combined with large-scale planting of exotic conifers and immigration of *L. curvirostra*

and *L. pytyopsittacus*, has resulted in widespread sympatry in Scotland (Summers *et al.* 1996) with anecdotal evidence of interbreeding. With the prospect of species loss, there is an urgency to quantify the levels of divergence between crossbill taxa and the extent of individual hybridization and genetic introgression.

Here we describe the isolation of several polymorphic tetranucleotide microsatellite markers that will be invaluable in examining population structure within the crossbill types and the genetic relationships among them. We also highlight the suitability of these primers in other passerine species.

The method used to obtain the microsatellites is described in Pierny *et al.* (1998). In brief, total genomic DNA was extracted from an adult Scottish crossbill using standard proteinase K digestion and phenol–chloroform procedures (Sambrook *et al.* 1989). DNA was digested using *Sau3AI* restriction endonuclease, and a 300–800 bp fraction isolated. Fragments were ligated to a SAU linker molecule made by annealing equimolar amounts of SAU-L-A (5'-GCGGTACC-CGGGAAGCTTGG-3') and SAU-L-B (5'-GATCC-CAAGCTTCCCGGTACCGC-3') oligonucleotides. The fraction was denatured and hybridized to a 1 cm<sup>2</sup> piece of Hybond N+ membrane saturated with (GAAA)<sub>n</sub> polymer in 2.5× SSC, 0.1% SDS at 60 °C. After three low-stringency washes of 3× SSC; 0.1% SDS to remove nonrepetitive DNA from the membrane, the enriched microsatellite fraction was removed by heating to 95° for 5 min in sterile water. The enriched fraction was precipitated from the wash solution using isopropanol, and double-stranded conformation reformed in a PCR reaction (29 cycles with 91°C denaturation, 55 °C annealing and 72 °C extension), using the SAU-L-

A linker as a primer. SAU linkers were removed by restriction with *Sau3AI*, and fragments were ligated into pUC18/*Bam*HI plasmid vectors (Pharmacia Ltd). Vector molecules were heat transformed into INVαF' One-Shot™ *Escherichia coli*, then grown overnight at 37 °C on Luria–Bertani (LB) medium containing 50 µg/ml ampicillin and surface-streaked with 40 µL of 40 mg/ml X-gal. White colonies were streaked onto a second LB plate then regrown. Plate lifts were made onto Hybond N+ membrane and hybridized to radiolabelled GAAA polymer using standard procedures (Sambrook *et al.* 1989) in 1× SSC, 0.1% SDS, 1× Denhardt's solution at 65 °C to detect microsatellite repeats. Positive clones were sequenced (dye-terminator cycle-sequencing using an ABI 377 automated sequencer according to the manufacturer's protocols) and *L. scotica*-specific PCR primers designed using the criteria described in Pierny & Dallas (1997).

DNA was extracted from the blood of 50 unrelated *L. scotica* individuals (identification being based on bill depth measurement), and from muscle tissue of a variety of other passerine species (listed in Table 2). Extraction procedures have been described by Bruford *et al.* (1992). PCR amplifications were performed in 10 µL reactions in an MJ Research PTC-100 thermal cycler. Individual mixes contained 10 ng of DNA, 0.2 mM of each nucleotide, 5 pmoles of each primer (forward primer end-labelled with [<sup>32</sup>P]-dATP), 2.5 mM MgCl<sub>2</sub>, 0.5 units of *Taq* polymerase (Bioline Inc) and 1× NH<sub>4</sub> buffer. PCR profiles consisted of 30 cycles of 30 s denaturation at 92 °C, and 30 s annealing at the temperature specified for each primer pair in Table 1. No extension step was included in the reaction, except for a final 5-min step at 72° immediately following the 30th cycle.

**Table 1** Description of seven microsatellite loci for *Loxia scotica*. Sequences are given in the 5'–3' orientation together with the expected PCR fragment length (based on the clone sequence), optimal annealing temperature, array type, number of resolved alleles and heterozygosity. The clone sequences from which the primers were designed have EMBL accession numbers Y16820–Y16825

Locus	Sequence (5' – 3')	Fragment size (bp)	Annealing temperature	Repeat type	No. of alleles	H <sub>O</sub>	H <sub>E</sub>
LOX1	F: ATGATGGTAAGTCTAATGAAAGC R: CCACACACATTCACCTATTG	341	54	(CTTT) <sub>30</sub>	31	0.90	0.97
LOX2	F: CAGGCAGAGTGGACATTTATG R: CAGTTTCATGTGGATTTTTAG	195	57	(CTTT) <sub>17</sub>	25	0.96	0.96
LOX3	F: TTCTGTGGTGAAGTTTCTGGAG R: CCAACCCATTCCATGACAAC	235	61	(CTTT) <sub>21</sub>	24	0.95	0.96
LOX4	F: TATGTGCTGAAGTGAACCATCC R: TTCCCTCACAATTTTCCGAC	172	60	(CTTT) <sub>14</sub>	11	0.92	0.91
LOX6	F: ACAAATAACATAGGTGAGAAGC R: GCTCTATAACTTTGTGATTTTGC	184	54	(CTTT) <sub>19</sub>	17	0.91	0.94
LOX7	F: AACCTAAGCACATTTATTTCAGC R: ACAAATAACATAGGTCAGAAGC	151	55	(CTTT) <sub>19</sub>	15	0.88	0.93
LOX8	F: TTGTGAAGTTTGGGACATAAG R: AGTTGAGGCCATTA AAAAGATTC	282	58	(CTTT) <sub>25</sub> (CCTT) <sub>13</sub>	19	0.94	0.95
Mean					20	0.92	0.95

**Table 2** Cross-species amplification of seven pairs of *Loxia scotica* microsatellite primers with 14 other passerine bird species. Where a single PCR product was obtained, the number of alleles resolved is provided.

Common name	Scientific name	LOX1	LOX2	LOX3	LOX4	LOX6	LOX 7	LOX 8
Sand martin (2)	<i>Riparia riparia</i>	2	1	3	2	–	1	–
Grey wagtail (3)	<i>Motacilla cinerea</i>	2	2	–	–	2	–	1
Wren (2)	<i>Troglodytes troglodytes</i>	2	4	–	2	–	–	–
Blackbird (5)	<i>Turdus merula</i>	4	–	–	2	3	2	–
Long-tailed tit (3)	<i>Aegithalos caudatus</i>	4	3	2	–	2	2	–
Treecreeper (2)	<i>Certhia familiaris</i>	3	2	–	1	1	1	–
House sparrow (5)	<i>Passer domesticus</i>	3	–	–	2	2	–	4
Chaffinch (4)	<i>Fringilla coelebs</i>	–	–	1	3	–	1	–
Bullfinch (3)	<i>Pyrrhula pyrrhula</i>	2	–	4	–	–	–	–
Greenfinch (3)	<i>Carduelis chloris</i>	6	4	2	4	–	–	2
Siskin (1)	<i>Carduelis spinus</i>	1	2	–	1	–	1	1
Linnet (1)	<i>Carduelis cannabina</i>	2	–	–	2	–	–	–
Common crossbill (50)	<i>Loxia curvirostra</i>	25	20	24	7	18	15	21
Parrot crossbill (50)	<i>Loxia pytyopsittacus</i>	23	19	20	5	15	12	19
Corn bunting (2)	<i>Miliaria calandra</i>	1	–	–	2	–	–	1
Yellowhammer (3)	<i>Emberiza citrinella</i>	2	1	2	–	2	–	3

–, a multiband pattern, a smear, or no PCR product was detected.

Approximately 1000 clones were screened for the presence of microsatellite arrays, of which 40 ( $\approx 4\%$ ) gave a signal following hybridization. Twenty of these clones were sequenced and 10 sets of primers designed. The discarded clones contained arrays that were either short (< 10 motifs long) or compound. Seven primer pairs gave well-resolved microsatellite patterns that were easily scorable with minimal stutter-banding (Table 1). Each locus was highly polymorphic with a mean number of alleles per locus of 20 and a mean observed heterozygosity of 0.92. Alleles were shown to segregate according to Mendelian expectations, with no evidence of null alleles or linkage disequilibrium.

Table 2 shows the suitability of the primer sets in other passerine species. In several cases single PCR products of appropriate size were resolved, suggesting that these markers may be useful for examining population structure and gene flow in a range of passerine species.

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