Molecular Ecology (2000) **9**, 1919–1952

PRIMER NOTES

Highly polymorphic microsatellites in the lacertid *Gallotia galloti* from the western Canary Islands

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Keywords: *Gallotia galloti*, *Gallotia stehlini*, microsatellite markers

Received 2 March 2000; revision accepted 31 May 2000

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Microsatellite loci are increasingly used in population genetic and evolutionary studies, see Schlötterer & Pemberton (1994) and Jarne & Lagoda (1996) for reviews. Although primers may amplify a homologous microsatellite locus in related taxa (Schlötterer *et al*. 1991; Primmer *et al*. 1996) they tend to be specific to species/species groups and have to be isolated *de novo*.

We report here the isolation and characterization of five highly polymorphic microsatellite loci for phylogenetic and population genetic use in *Gallotia galloti*, which have also been tested on the related species, *G. stehlini*. A *G. galloti* genomic library was established in XL-1 Blue MRF' (Stratagene) transformed with *Alu*I/*Hae*III fragments (300–600 bp), ligated into the *Sma*I site of pUC18 (Pharmacia). A total of 2500 recombinant clones were transferred on plates (2× TY medium) and replica plated onto nylon membranes. Colonies were screened with end-labelled oligonucleotides $(CA)_n$, $(CT)_n$, $(GATA)_n$ and $(GACA)_n$. From 100 selected positive clones, primers were designed for 10 loci using OLIGO 5.0 (National Biosciences, INC. Plymouth, MN, USA). At least five of the primer sets produce clear, highly polymorphic amplification products of the expected size range (Table 1).

Tissue samples come from noninvasive biopsies (tail tips naturally autotomized) taken from individuals of *G. galloti* from several geographically distinct localities in Tenerife and from *G. stehlini* in Gran Canaria. An average number of 30 individuals per population has been analysed. DNA extractions were performed using a standard protocol (Sambrook *et al*. 1989). Radioactive polymerase chain reaction (PCR) amplifications were carried out in 10 μL of a mixture containing 15–30 ng of DNA, 200 nM of each primer (100 nM of one of them labelled with [γ^{32}P]-dATP), 30 μM of each dNTP, 1.5 mM $MgCl_2$, standard $MgCl_2$-free BRL 1× reaction buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4) and 0.4 U of *Taq* DNA polymerase (BRL). Initial denaturing step of 3 min at 94 °C was followed by 35 cycles [94 °C for 30 s, 55 °C (53 °C for locus A348 for 30 s and 72 °C for 15 sec] and 2 min at 72 °C. PCR products were run through 6% denaturing sequencing polyacrylamide gels and visualized by autoradiography. Allele lengths were determined by comparison to the original clone and those previously scored. Observed (H_O), and expected (H_E)

Table 1 Characteristics of *Gallotia galloti* microsatellites including, GenBank accession nos, core sequences, product length in bp (length) of cloned alleles, and primer sequences. Polymorphism data are from three populations on Tenerife (codes from Thorpe & Brown 1991 and Thorpe *et al*. 1996), with the range in size of the repeat region in bp (Range), number of alleles (*A*), observed (H_O) and expected (H_E) heterozygosity and *P*-values for departure from Hardy–Weinberg equilibrium (*$P < 0.05$) derived from an exact test using a Markov chain (length: 100 000)

Locus	Accession no.	Core sequence	Length	Primers	Pop code	Range	*A*	H_O	H_E	*P*
A348	AF070978	$(AC)_{19}$	228	AATGCTGCTCTCTTGTGTCC	63	20–42	11	0.622	0.722	0.024*
				TTTGTGTTCCTGTCTCTTTC	48	20–40	9	0.590	0.690	0.359
					51	20–42	12	0.763	0.740	0.725
					All	20–42	12	0.658	0.717	
A49	AF070979	$(CA)_{10}$	198	AGAGGAAGTGGGTAATA	63	16–46	14	0.944	0.893	0.459
				GATAGAGGATGGGTGAT	48	16–38	11	0.816	0.873	0.015*
					51	16–40	11	0.763	0.885	0.61
					All	16–46	15	0.841	0.883	
B81	AF070980	$(TC)_{19}$	163	GGCAGGTAGAGGAAATC	63	14–44	11	0.838	0.877	0.176
				ATAGGGAAATGAACAGG	48	12–42	12	0.872	0.845	0.752
					51	14–44	14	0.763	0.836	0.145
					All	12–44	16	0.824	0.853	
B821	AF070981	$(AC)_{12}$	261	CCAGAGAGAGGTTTGAC	63	22–44	11	0.838	0.876	0.440
				GGTTTGAAGATAGAGAA	48	22–42	11	0.770	0.826	0.554
					51	22–42	11	0.658	0.750	0.062
					All	22–44	12	0.755	0.818	
B967	AF070982	$(GT)_3AT(GT)_{10}$	149	CACTGCTGTTCCAAAAGACCAC	63	16–46	15	0.919	0.907	0.738
				CCCTCCCTCCCACTCACC	48	16–44	12	0.795	0.882	0.218
					51	22–48	13	0.816	0.887	0.250
					All	16–48	17	0.843	0.892	

Table 2 *Gallotia stehlini* alleles sequences for the two loci which exhibited primers conservation and polymorphism within both species. The sequences are compared with the sequence of the original cloned allele from *G. galloti*

Locus	Species	Sequences						
B821	*G. galloti*	CTCCTACTGG	CAGCAGTGTG	AATTGCAGCA	TGATGACAGC	CTTACATTTT	TAGGTATATA	
	G. stehlini	......T...	....	....	....	....	....	
	G. galloti	GAGGA$[AC]_n$	GGATGGATAT	TGTTACTAGG	GGAAAACAAG	AATTGCAAGA	GGGTCCTTCA	$8 > n > 24$ repeats
	G. stehlini	.....$[AC]_{n'}$	....	....	....	G........	....	n' = 8, 9 or 12
	G. galloti	GATTAGAAGT	TTGTCAGAGG	ATCTGGTGTC	TGCACATGGA	GCAGCCCAAT	TTAATCCACA	
	G. stehlini	....	.....G....	....	....	...A......	....	
	G. galloti	TTCCAGATTA	AGGAACATCT	ATCACCAA				
	G. stehlini	....	....	....				
A348	*G. g* clone	AGCCATTTCA	ATAGAATCGG	TCTAAAGCAG	TATTCACCAA	CTTTTGGGGG	CAAGTGGGCT	
	G. s allele2	.A........	.......T..	....	....	.......T..	....	
	G. s allele4	.A........	.......T..	....	....	.......T..	....	
	G. s allele5	.A........	.......T..	....	....	.......T..	....	
	G. g clone	CATTTGGAAT	TTTGAGAAAA	TG---TATGT	GCCAGTCACA	AATTGGCTAC	TGGGGAGATG	
	G. s allele2	T.........	......C...	..CTG.....	.T........	....	....	
	G. s allele4	T.........	......C...	..--------	-T........	.....A....	....	
	G. s allele5	T.........	......C...	..CTG.....	.T........	.....A....	....	
	G. g clone	TGACTAACAC	ATAATGGCTG	CCACATATTT	AAA [CA]19	-- length	= 188 bp	
	G. s allele2	....	....	....	... [CA]8	--	= 167 bp	
	G. s allele4	....	....	....	... [CA]9	AA	= 164 bp	
	G. s allele5	....	....	....	... [CA]9	--	= 171 bp	

heterozygosity and departure from Hardy–Weinberg equilibrium were calculated (Raymond & Rousset 1995).

There are from 9 to 17 alleles per locus in the sampled Tenerife localities (Table 1) and high gene diversity (0.717–0.822). There was no significant evidence of null alleles from heterozygote deficit. Four out of the 30 possible combinations of loci and populations showed a significant departure from independence, but as these combinations revealed no consistent linkage, the loci were considered to be in linkage equilibrium. Two loci (A348, B821) could be amplified in *G. stehlini* (Table 2) and they both exhibit some size polymorphism. For B821 the sequence is highly conserved as only four transitions are observed between the original *G. gallotia* cloned allele and *G. stehlini* observed alleles. The polymorphism is very low in *G. stehlini* with only three alleles, one of which has a frequency of 0.90 in our sample. A348 size polymorphism is not only due to different numbers of repeats; deletion and insertion of short sequences are also found (Table 2). For the other loci, we found major sequence differences between the two species. There was no amplification for some individuals, or multiple amplification products for some others.

Acknowledgements

This work was supported by the EU-(grant ERBCHRXCT-940585) to RST who thanks the corecipient, Dr Baez, for permissions and Per Palsbøll for reading the manuscript. MR acknowledges the advice of Dr Jubier.

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Characterization of microsatellite loci in western redcedar (*Thuja plicata*)

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Keywords: conifer, Cupressaceae, genetics, microsatellites, redcedar, *Thuja plicata*

Received 31 March 2000; revision accepted 31 May 2000

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Conifers are among the most genetically diverse plants (Hamrick & Godt 1996) and are predominantly outcrossed (Barrett & Eckert 1990). In contrast, western redcedar (*Thuja plicata* Donn, Cupressaceae) has shown low diversity based on leaf oil terpenes (von Rudloff & Lapp 1979), isozyme loci (El-Kassaby *et al*. 1994) and restriction fragment length polymorphism (RFLP) (Glaubitz *et al*. 2000). Population outcrossing rates for western redcedar based on one isozyme locus indicates a mixed mating strategy in this species (El-Kassaby *et al*. 1994; LM O'Connell *et al*., unpublished). *Thuja plicata* is a widespread conifer found along the west coast of North America from southern Alaska to northern California, and in the interior from east-central British Columbia into northern Idaho (Minore 1990). The coastal and interior populations are geographically isolated from each other and may be genetically differentiated. Estimating outcrossing rates in plants usually requires several polymorphic loci, however, in species with low genetic diversity the lack of isozyme polymorphism prevents us from obtaining accurate estimates of outcrossing rates with this marker. Because microsatellites are highly polymorphic, codominant and neutrally selected markers they are well suited for mating system studies. We designed microsatellites in *T. plicata* to study its population genetic structure and mating system.

Microsatellite markers were isolated from redcedar genomic DNA using modifications of biotin-enrichment strategies of Kijas & Fowler (1994). Genomic DNA was digested with *Hae*III and individual fragments were ligated to double stranded oligonucleotide adapters (M28, M29) on their 5′ and 3′ ends, respectively. Adapted fragments were then denatured, hybridized with 5′ biotin labelled $(TG)_{12}$ and enriched by selection with magnetic streptavidin affinity supports (Dynal M-280). Biotin selected genomic fragments were then amplified using primer M30 and the resulting mixture was cut with *Eco*RI and ligated into standard cloning vectors (pGEM3Z+, Promega) for propagation in bacteria. Individual microsatellites containing clones were isolated by colony hybridization with P^{32}-labelled $(AC)_{12}$ and picked into glycerol cultures for long-term storage and isolation.

We sequenced 96 clones directly from glycerol stocks using SequiTherm EXCEL™ II Long-Read DNA Sequencing Kits-LC (Epicentre Technologies) on a LiCor 4200. We chose 35 clones to design microsatellite primer sets. In each primer pair, one of the primers was tailed (Table 1; Oetting *et al*. 1995).

Table 1 Characterization of *Thuja plicata* microsatellites in two parts of its range

Locus	Primer sequences (5′–3′)	T_a (°C)	Repeats of cloned allele	Size range (bp)	Alleles (n)	Coastal			Interior			GenBank accession no.
						n	H_O	H_E	n	H_O	H_E	
TP1	TTGCAATCAATTTATTACGCGGG* AGTCCAACGAAAGCATTGTCTCC	60	$(CA)_{21}$	153–179	11	22	0.591	0.674	20	0.500	0.774	AF245205
TP2	TAACATCTAGCTTTGTACATG TAGAAATGTTAGAAATTAAAA*	52	$(TG)_{21}(AG)_{>50}$	196–334	36	22	0.955	0.907	22	1.000	0.941	AF245206
TP3	ATCAGTCTCAAGATCCACTAA* ATAAAAATAACTTGGAAATGC	55	$(TG)_{18}$	157–203	12	21	0.953	0.824	21	0.714	0.757	AF245207
TP4	CCCATCTTGCCACTTATTGTA GGAAGCCATCAGATCCTGGAG*	55	$(TG)_{22}$	263–281	10	20	0.600†	0.832	22	0.773	0.774	AF245208
TP5	AATATTGATGTTTATAGCTTC CATTGTAGATGTGTATTGATA*	55	$(GT)_{14}$	266–276	6	22	0.591	0.735	22	0.545	0.704	AF245209
TP6	AGCAATGAATGAATAAGCAAGAAC GGACTAGGGCTATACACACGTATG*	58	$(GC)_6(GT)_{13}(ATATGT)_8\ldots(GT)_{19}$	225–279	21	22	0.727	0.856	19	0.841	0.853	AF245210
TP7	ACACAAGGGTGATCTAATGGA TAGGACCACTTAGAACCCATT*	55	$(CA)_{15}$	228–244	8	21	0.714	0.833	19	0.734	0.650	AF245211
TP8	TTAGGTTAATAGCTAGTTG TAAGTGGCAATAAAAAATA*	52	$(CA)_9CG(CA)_{>20}$	185–239	16	20	0.750	0.877	22	0.455	0.765	AF245212
TP9	TCTCCTTGTCTTGGATTTGG CGGAAAGTAGTCTCATTATCAC*	58	$(AC)_{>20}$	244–306	20	22	0.955	0.912	22	0.909	0.888	AF245213
TP10	TAGTTGTGTCCATTCAGGCAT* GCTCTTATCTTCTTTTAGGGC	60	$(GT)_4GC(GT)_{12}$	168–172	3	17	0.353†	0.659	20	0.350	0.434	AF245214
TP11	CCTGATCCGCTTTGATGGGT GATAAGAGGCATCACTCGAG*	65	$(CT)_{12}(CA)_{16}$	203–211	5	19	0.579	0.630	22	0.727	0.718	AF245215
TP12a	CCGGATCATTAAGGGCTCTA GTCGCATCTATTCAGGCATA*	65	$(CA)_{29}$	179–221	15	19	0.632†	0.909	21	0.857	0.816	AF245216
TP12b				165–177	6	18	0.556	0.716	19	0.211	0.323	

*Primer to which a forward or reverse tail sequence was added; T_a, annealing temperature; n, total number of individuals; H_O, observed heterozygosity; H_E, expected heterozygosity. †Significant deficiencies of heterozygotes relative to Hardy–Weinberg expectations (calculated for the coastal population only).

Total DNA from *T. plicata* was isolated using a CTAB method (Doyle & Doyle 1987). To test for polymorphism we screened individuals from one coastal population (south-western BC, $n = 22$) and two interior populations (south-eastearn BC, $n = 11$; and northern Idaho, $n = 11$). The DNA samples for the interior populations were obtained from J Glaubitz (personal communication).

Polymerase chain reaction (PCR) amplifications were performed using 10 μL total reaction volumes with 1× *Taq* buffer (10 mM Tris, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3; Roche), 1 pmol dNTP, 0.5 pmol each of forward and reverse primers, 0.5 pmol M13 IRD-labelled primer, 1 Unit *Taq* DNA Polymerase (Roche), and between 10 and 30 ng of genomic DNA template. Samples were amplified on a PTC-100 thermocycler (MJ Research) denaturing at 95 °C for 5 min, followed by 33 cycles of 95 °C for 45 s, annealing temperature (Table 1) for 45 s, 72 °C for 45 s and ending with one cycle of 72 °C for 5 min. Following amplification, 3 μL of loading dye (100% formamide, 1 mg/mL pararosaniline basic red 9) was added to each reaction. For final screening the microsatellites were detected on a LI-COR 4200 sequencer with a 7% polyacrylamide (Long Ranger™) gels.

Of the 35 microsatellites, 12 amplified interpretable polymorphic loci. One of these (TP12a,b; Table 1) showed two loci for the primer pair. Observed and expected heterozygosities were higher for a greater number of loci in the coastal population than in the interior population. There was a significant deficiency in heterozygotes at three loci (TP4, TP10 and TP12a) in the coastal population indicating possible null alleles. The number of alleles ranged from 3 to 36 for a total of 189 alleles for the 13 loci. Together these loci have enough variability to study the population genetic structure *T. plicata*. Loci with high number of alleles will be particularly useful for the study of the mating system of this species.

Acknowledgements

We thank C. Newton for his assistance in developing the microsatellites and J. Glaubitz for DNA samples. This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) grant to K. Ritland, and NSERC and Killam Scholarships to L. M. O'Connell.

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Development of microsatellite markers in the guanaco, *Lama guanicoe*: utility for South American camelids

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Received 2 March 2000; revision accepted 8 June 2000

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In this paper we describe the development and characteristics of microsatellite primers designed for the guanaco, *Lama guanicoe*, and their utility for the South American camelids. To our knowledge, these are the first microsatellites developed for a wild South American camelid, although Penedo *et al*. (1998, 1999) have developed them for the closely related domestic Llama, *Lama glama*.

We isolated microsatellite loci using a modified non-radioactive capture–hybridization method (Refseth *et al*. 1997). Aliquots (30 μg) of guanaco DNA were digested with *Sau*3AI, and electrophoresed on a 2% agarose (TAE) gel. The 300–600 bp DNA fragments were excised from the gel and purified using Geneclean (BIO 101). Oligonucleotides EP-1 (CCCCCACCTCCTGCCCATCATAAAAAATC, HPLC-purified) and EP-2 (GATCGATTTTTTATGATGGGCAGGAGGTGGGGG, HPLC-purified and 5′-phosphorylated, Life Technologies) were modified versions of the prinker sequences described by Muth *et al*. (1996), which when annealed have an *Sau*3AI-compatible end. A double-stranded linker was made by combining 5 μL of EP-1 (500 nm/mL), 5 μL EP-2 (500 nM/mL), 2.5 μL 10 × annealing buffer (100 mM Tris pH 7.5, 1 M NaCl, 10 mM EDTA) and 12.5 μL of water, incubating at 65 °C for 10 min, and then room temperature for 1 h. The linker concentration was adjusted to 1 μg/μL with 12.5 μL of 1 × annealing buffer. For linker addition, 1 μL of linker, 200 ng of the 300–600 bp *Sau*3AI-digested DNA, 2 μL 10 × ligase buffer (New England Biolabs) and

Table 1 Characterization of guanaco, *Lama guanicoe*, microsatellites across each species of South American camelid. Characteristics are based upon 10 individuals from each species

Locus	Repeat motif	Primers (5′ → 3′)	Size (bp)	GenBank accession no.	Species	Size range	No. of alleles	H_O	H_E
Lgu49	$(TG)_{16}$	*TCTAGGTCCATCCCTGTTGC	222	AF237488	Guanaco	218–242	9	0.90	0.86
		GTGCTGGAATAGTGCCCAGT			Alpaca	224–248	9	0.90	0.82
					Llama	219–249	9	0.80	0.82
					Vicuña	224–248	6	1.00	0.75
Lgu50	$(TG)_{12}$	*CTGCTGTGCTTGTCACCCTA	195	AF237489	Guanaco	189–195	3	0.50	0.52
		AGCACCACATGCCTCTAAGT			Alpaca	185–195	5	0.80	0.68
					Llama	187–201	5	0.40	0.49
					Vicuña	183–187	3	0.20	0.18
Lgu51	$(GT)_{13}$	*CCTTCCTCTTGCAAATCTGG	195	AF237490	Guanaco	200–212	4	0.30	0.34
		GCACCTGATGTCATTTATGAGG			Alpaca	201–213	5	0.40	0.70
					Llama	201–217	5	0.30	0.42
					Vicuña	197–211	4	0.70	0.62
Lgu52	$(GT)_{10}$	*GTGGTTGGAACCTGCAACTC	186	AF237491	Guanaco	180–194	5	0.90	0.69
		TTGGGACCTGCTTCCATAAC			Alpaca	182–206	5	0.90	0.72
					Llama	182–188	4	0.90	0.63
					Vicuña	182–208	5	0.70	0.76
Lgu56	$(GT)_{16}$	*TTGCTGTACCGGAGATGTTG	178	AF237492	Guanaco	178–186	5	0.50	0.74
		TTGAGGCAAGAATGCAGATG			Alpaca	160–180	5	0.80	0.71
					Llama	168–182	4	0.60	0.69
					Vicuña	160–192	4	0.80	0.62
Lgu68	$(GT)_{10}$	*CATCTACATGCCCCTGTGTG	215	AF237493	Guanaco	199–231	7	0.80	0.75
		TGCAGGGAGGACTAACAGGT			Alpaca	209–221	6	0.80	0.81
					Llama	207–229	8	0.90	0.80
					Vicuña	211–229	6	0.80	0.78
Lgu75	$(GT)_{20}$	*GATCAGCTTTGGTGGTTGGT	184	AF237494	Guanaco	178–196	4	0.80	0.65
		CACCTCTTCCCCATGCATAA			Alpaca	196–202	5	0.60	0.48
					Llama	178–198	6	0.70	0.74
					Vicuña	178–202	6	0.80	0.64
Lgu76	$(GT)_{11}$	*TTCCTTCCATTGAAGCAGGT	225	AF237495	Guanaco	233–251	6	0.90	0.75
		TGAGATGCACTGCTTTGGATA			Alpaca	241–259	6	0.80	0.80
					Llama	241–261	8	0.90	0.84
					Vicuña	233–259	7	0.70	0.73
Lgu79	$(CA)_{10}$	*TAAGGTAGGAGCGAGCCAAA	200	AF237496	Guanaco	202–230	6	0.90	0.77
		ACCTGCTCGCTAATCTCTGC			Alpaca	198–224	7	0.90	0.80
					Llama	198–214	5	0.30	0.54
					Vicuña	204–222	5	0.50	0.75
Lgu80	$(TG)_{12}$	*GCGTGTGTTTATGCATGAGG	213	AF237497	Guanaco	213–221	5	0.50	0.68
		ATGGCCCATTAGCAGAGATG			Alpaca	215–229	4	0.50	0.68
					Llama	209–219	4	0.80	0.64
					Vicuña	217–245	9	0.60	0.83
Lgu83	$(GT)_{28}$	*TCACAAACTTGGAGGAAGCA	160	AF237498	Guanaco	142–166	6	0.30	0.73
		ATAGCCCTGGGGTTCTATGG			Alpaca	138–140	2	0.00	0.18
					Llama	126–140	5	0.40	0.48
					Vicuña	136–142	4	0.20	0.41
Lgu91	$(TG)_{11}$	*GTCTCCGGTTTGCAGATGAG	233	AF237499	Guanaco	231–241	5	0.70	0.70
		AGAAGCAGAATGGTAGTGAAGAA			Alpaca	225–237	4	0.60	0.54
					Llama	231–237	3	0.10	0.40
					Vicuña	235–245	5	0.90	0.76
Lgu93	$(TG)_{14}$	*CCTGTGGAGCTAGGCCTTC	200	AF237500	Guanaco	188–196	4	0.70	0.68
		TTTGTAATCCCAGCCTGCTC			Alpaca	188–214	8	0.80	0.81
					Llama	188–204	6	0.80	0.78
					Vicuña	184–196	4	0.20	0.41
Lgu96	$(AC)_{15}$	*GGACCTGGGACAAGCATTAT	163	AF237501	Guanaco	159–171	5	0.10	0.76
		CGATCGTGTGAGGAGAACAA			Alpaca	149–161	5	0.90	0.70
					Llama	147–167	5	0.40	0.66
					Vicuña	155–171	5	0.60	0.48

H_O, observed heterozygosity; H_E, expected heterozygosity; estimates of H_E calculated according to Li (1997). *Indicates labelled primer.

1 μL of ligase (New England Biolabs, 400 units/mL) were combined in a 20 μL reaction, and incubated for 1 h at 37 °C and for 10 min at 65 °C. Excess linker was removed with two washes in a Microcon 100 (Amicon), and the volume brought to 15 μL with water. A 1 μL aliquot of the linkered DNA was polymerase chain reaction (PCR)-amplified using primer EP-3 (CCCCCAC-CTCCTGCCCATCAT, HPLC-purified, Life Technologies). PCR was performed in a Perkin Elmer 9700 thermal cycler with 1.2 U Ampli *Taq* DNA polymerase (Perkin Elmer), 1 × Perkin Elmer PCR buffer (10 mM Tris pH 8.3, 50 mM KCl), adjusted to 2 mM $MgCl_2$ final, 2 μM EP-3, 250 μM each dNTP in a total of 30 μL, at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 65 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 2 min.

The biotinylated capture probe was annealed to the linkered DNA by combining 10 μL of linkered DNA, 1 μL (0.1 μg/μL) 5′-biotinylated capture probe (5′ biotin $(CA)_{15}$, HPLC-purified, Life Technologies) and 89 μL of 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate pH 7.0), heating to 95 °C for 10 min, cooling on ice for 30 s, then incubating for 5 min at room temperature. Washed magnetic beads (Dyna beads M-280 streptavidan, Dynal Corps) as previously described by Refseth *et al*. (1997), were added to the DNA and incubated for 15 min at room temperature, washed three times with 200 μL of 2 × SSC at room temperature, and three times with 200 μL of 1 × SSC at 65 °C for 3 min. The DNA was eluted from the beads into 50 μL water, incubated for 5 min at 95 °C, washed three times in a Microcon 100, and concentrated to 15 μL. PCR amplification of DNA was performed with the EP-3 primer. The PCR product was run on a 3% TAE gel and the desired size fragment was recovered by the Geneclean procedure.

The final PCR product was ligated directly into the pT7 Blue T-Vector (Novagen Inc.) following the manufacturer's directions. Following transformation, white colonies were selected and plasmid DNA was isolated using a REAL prep 96 plasmid kit (Qiagen). DNA was sequenced using PE Applied Biosystems Big Dye Terminator Kit on an ABI 377 DNA sequencer.

Fourteen microsatellites were amplified in 10 individuals of the four South American camelid species (Table 1). All loci were polymorphic and the number of alleles per locus was variable (Table 1). The guanaco demonstrated the highest average heterozygosity (0.69), followed by the alpaca, *Lama pacos* (0.68), llama (0.64) and vicuña, *Vicugna vicugna* (0.62). The domestic llama and alpaca exhibited an average of 5.5 and 5.4 alleles per locus, respectively, while the wild guanaco and vicuña exhibited 5.3 and 5.2 alleles per locus, respectively. On the basis of this initial screening, these 14 guanaco microsatellites appear to be a promising tool to study camelid population genetics and phylogeography, which could ultimately influence camelid conservation and management.

Acknowledgements

We thank the Chilean National Forestry and Park Service (CONAF), along with Mike Bank, Dr Russ Hunter DVM and Dr Nicholas Soto DVM, for assistance collecting guanaco blood samples in southern Chile. Dr Cristian Bonacic DVM, Benito Gonzalez and Dr Beatriz Zapata DVM provided vicuña blood samples from northern Chile. Dr Brad Smith DVM provided llama and alpaca samples from the Oregon State University captive herd. Eduardo Eizirik, Carla Ferris, Amanda Garfinkel, Jae-Heup Kim, Jan Martenson, Marilyn Raymond-Menotti, and Amy Snyder provided technical assistance. Permits to import blood samples were issued by the USDA and US Fish & Wildlife Service to R.J.S.

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Isolation and characterization of microsatellite loci in the ascidian *Ciona intestinalis* (L.)

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Received 12 May 2000; revision accepted 8 June 2000

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In polluted areas, ascidians can take up and accumulate metals and toxic substances, acting as active filters for purification and cleaning of coastal waters and becoming important pollution bio-indicators (Papadopolou & Kanias 1977; Naranjo *et al*. 1996). The ascidian *Ciona intestinalis* forms relatively dense populations, with a filtration rate of 0.3 cm^3 individuals/second, characterized by cyclic reduction and re-colonization of preferential areas (Riisgard *et al*. 1998). The study of the genetic structure of *C. intestinalis* in distinct populations could help in understanding the *tempo* and *modo* of colonization of new environments and of re-colonization of particular sites. Such results could shed light on the effective dispersal of the planktonic larval stage in this important benthic organism and give important insights for the management of the coastline. The high rates of mutation commonly observed at microsatellite loci make this class of marker suitable for addressing the above objectives.

High-molecular-weight DNA from *Ciona* sperms was prepared with Proteinase K buffer and purified using chloroform:isoamyl alcohol (24:1) following a standard protocol (Sambrook *et al*. 1989). DNA from five *C. intestinalis* individuals was pooled to increase the number of gene copies to be scored

Table 1 Primer sequences (F, forward; R, reverse), repeated motif, annealing temperature and allelic designation and size, expected and observed heterozygosity, for the five polymorphic microsatellite loci identified in *Ciona intestinalis*

Locus	Primer (5′ → 3′)	Repeat sequence	Annealing temp. (°C)	Allele size (number of repeats)*	H_E	H_O	Accession no.
Cin-4	F-cacgggttgtcctggtattgcagt R-actcttcgaaactgtaaggcgggg	$(AATA)_4$ AACA AATA AACA	64	144, 148, **152**, 156	0.318	0.065†	AF274065
Cin-22	F-tgaatctggaattaatgaattgag R-cccatgttttgattcatttcttca	$(AAC)_7$	58	99, 105, 108, **111**, 120, 132, 135, 141, 147, 150, 153, 156, 159	0.745	0.676	AF274066
Cin-49	F-gcggaaagcgagaaattacttctg R-tgacaaagaaattagacaaccatt	$(GTT)_5$ GTA GTT AAT $(GTT)_4$	58	214, 232, **235**	0.406	0.458	AF274067
Cin-67	F-cttggtctttaagtactgcttttg R-agaacgaaaacatgtcttataccc	$(GTA)_4$ $(GTT)_6$	56	**208**, 211, 214, 217, 220	0.603	0.622	AF274068
Cin-95	F-tgccatatttgttaatgtaacata R-aaaataagttatattgactcaaaa	$(TG)_8$	52	151, **153**, 155	0.212	0.016†	AF274069

*Alleles of the expected molecular size are indicated in bold; †P = 0.0000 (Fisher's exact test for Hardy–Weinberg equilibrium).

during hybridization. A size-selected genomic library (200–400 bp fragments) was prepared in pBluescript SK+ vector (Stratagene) from the pooled DNA (Sambrook *et al*. 1989). For microsatellite identification, the protocol described by Procaccini & Waycott (1998) was utilized. Pooled *C. intestinalis* DNA was digested to completion with *Sau*3A restriction enzyme and aliquots were dot-blotted on Hybond N+ membrane (Amersham). Dot blots were hybridized with 17 synthetic oligos, end-labelled with γ-^{32}P, and the four oligos giving the strongest signals (TG/AC, AGC/TCG, ACC/TGG, AGG/TCC) were chosen for screening almost 25 000 colonies. Only the colonies giving the strongest signals were manually sequenced using the dideoxy chain termination method (Sanger *et al*. 1977). Of the total number of sequenced clones, only five were chosen for designing primers on the flanking regions of repeated sequences. PCR reactions were optimized in a total volume of 10 μL to a final concentration of 50 mM KCl, 10 mM Tris–HCl pH 9.0, 200 μM of each dNTP, 2 mM $MgCl_2$, 1.7 pmol of each primer, 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus) and 10–20 ng of template DNA. Thirty-five amplification cycles were run in a Perkin Elmer model 9700 thermal cycler with the following cycling conditions: 15 s denaturation (94 °C), 30 s annealing (see Table 1) and 30 s extension (72 °C). Results were obtained by running γ-^{32}P-labelled PCR products (forward primers were labelled) on 6% polyacrylamide gels and visually scoring bands on autoradiographs.

Microsatellite polymorphism was assessed in 60 individuals collected at three sites along the Southern Tyrrhenian coasts (Italy). The five selected microsatellite regions include two simple repeats (one dinucleotide and one trinucleotide) and three complex repeats (Table 1). Correct assignment of bands to alleles was performed in two different ways: (i) in all individuals, molecular weight was determined in comparison with a known nucleotide sequence; and (ii) in few selected individuals, PCR bands were excised from the agarose gel and either directly sequenced or cloned. Ten individual colonies were sequenced for each of the cloned PCR products. Alleles of different molecular weight resulted from the sequences of clones obtained from PCR products of heterozygous individuals.

Twenty-eight alleles were present in total; the multi-locus profiles from all individuals were compared, and were always found to be unique. Consistent genetic diversity was detected for the three sample sites, with average values of expected heterozygosity per site of 0.385 (SE 0.134), 0.461 (SE 0.097) and 0.529 (SE 0.069), respectively. Values of expected and observed heterozygosity calculated for each locus indicate significant differences only in two loci (Table 1).

Overall, the five selected microsatellite regions show promising results for detecting genetic polymorphism in the ascidian *Ciona intestinalis*. We are currently applying microsatellite marker polymorphism to analyse population genetic parameters from natural populations and to monitor genetic variability in inbred lines constructed to provide a less polymorphic, genotyped, genome resource.

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Isolation and characterization of microsatellite loci from the tapeworm *Schistocephalus solidus*

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Keywords: cestode, hermaphrodite, microsatellite, paternity, *Schistocephalus solidus*, sex allocation

Received 8 May 2000; revision accepted 9 June 2000

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Schistocephalus solidus, a pseudophyllidean cestode parasite, is an outstanding model organism for studying the evolution of hermaphroditism, as it is a simultaneous hermaphrodite that appears to reproduce by self- and cross-fertilization. Learning more about, for example, sex allocation, selfing and out-crossing strategies or decisions in the hermaphrodite's dilemma requires determination of selfing and out-crossing rates and the extent of sperm competition (Jarne & Charlesworth 1993). A necessary prerequisite for such studies are high-resolution, co-dominant molecular markers that allow assignment of paternity and the assessment of levels of heterozygosity. In this report, we describe the isolation of five microsatellite DNA loci in *Schistocephalus solidus* and the development of appropriate PCR primers. These are, to our knowledge, the first microsatellite primers for any cestode species.

Aliquots (75 μg) of genomic DNA from three individuals from Bochum (Germany) were isolated using the Genomic Tip Kit from Qiagen (Basel, Switzerland). The DNA was sent to the Genetic Identification Services (GIS, http://www.genetic-id-services.com; Chatsworth, California, USA), to develop two enriched microsatellite genomic libraries, one containing inserts with $(CT)_n$ repeats and the other containing $(CA)_n$ repeats. The inserts (cut with *Hin*dIII) were cloned into dephosphorylated pUC18 cut with *Hin*dIII. The libraries (stored as plasmid DNA) were used to transform *E. coli* DH5α competent cells. Individual bacterial clones were picked, suspended in 20 μL sterile water and the insert DNA was amplified by polymerase chain reaction (PCR) using primers complementary to the regions flanking the polycloning site (5′-ACGACGTTGTAAAACGACGGCCAG-3′ and 5′-TTCACACAGGAAACAGCTATGACC-3′, respectively). PCR products were isolated for sequencing using 'Qiaquick' PCR purification columns (Qiagen), and eluted into 30 μL of distilled water. Insert DNA samples were subject to dideoxy sequencing using the ABI BigDye ready reaction kit (PE Biosystems) and the M13 universal forward (5′-TGTAAAACGACGGCCAGT-3′) and reverse (5′-ACAGGAAACAGCTATGACC-3′) primers. Sequencing reactions were resolved on urea–5% polyacrlamide gels and the gels were analysed on an ABI 377 DNA Sequencer (PE Biosystems). Primers for amplification of the microsatellite loci were designed with the help of primer 3 (Rozen & Skaletzky 1996–1997). Genomic DNA of two individuals each from various geographical locations including Loirston (near Aberdeen, UK), Belfast (UK), Perth (UK), Inverness (UK) and 15 individuals from Bochum (Germany) was isolated as follows: approximately 1 mg of tissue was suspended in 100 μL solution containing 10 mM Tris, 2 mM EDTA, 0.01% Triton X-100 and 20 μg Proteinase K (Sigma). The solution was incubated for 2 h and then heated at 95 °C for 10 min. PCR reactions were carried out in a 10 μL volume containing: 1 μL of extract, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.5 U of Amplitaq Gold DNA polymerase (PE Biosystems), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of locus-specific fluorescent-labelled forward primer (fluorescent dyes were 6-FAM and HEX) and non-labelled reverse primer (see Table 1). PCR cycling parameters were the following: 10 min at 95 °C, 30 cycles of 95 °C for 30 s, 15 s at 57 °C and 75 s at 72 °C, followed by a final step of 72 °C for 7 min. Fluorescent PCR fragments were separated on a 5% polyacrylamide gel and visualized on an ABI 373 DNA Sequencer.

All five loci were polymorphic and their characteristics are summarized in Table 1. For the Bochum population

Table 1 Characterization of five microsatellite loci in the tapeworm *Schistocephalus solidus*

Locus	Primer (5′ to 3′)	PCR product size of the sequenced allele (bp)	Observed allele sizes (bp)	Observed allele numbers	Repeat structure	H_O*	GenBank accession no.
SsCTA22	F:TGATCCCAACCCTACTGCTG						
	R:GCACAAGTCACCGTCCCTG	152	146–160	8	$(CT)_{14}$	0.73	AF247830
SsCAB6	F:GTTGGTGATGGTCGAGAAAG						
	R:GAATGTGTGATTTTCAGGGAAC	120	96–130	10	$(CA)_{11}$	0.33	AF247829
SsCTB24	F:ACGCAGTCCGAGTTATACCG						
	R:CGGTGGTCTGATTGTGAATG	188	162–196	9	$(CT)_{21}$	0.33	AF247831
SsCA25	F:CGCAATAAGGTTGGATCGTC						
	R:TAAATCACGCGCAAGTCATC	170	132–194	8	$(CA)_{19}$	—	AF247832
SsCA58	F:GGAGTCAGACATACCGGGTG						
	R:GAAAAGGTCGTGCTTCAACC	169	143–179	8	$(CA)_{26}$	0.2	AF247833

*Observed heterozygosity (H_O) is only given for the Bochum population (n = 15).

($n = 15$), observed heterozygosity was low for three loci (SsCTAB6, SsCTB24 and SsCA58). A significant excess of homozygotes, and hence a positive inbreeding coefficient ($f = 0.30$), was found in the 15 individuals of the Bochum population using the AMOVA procedure implemented in Arlequin 3.1 (Schneider *et al*. 1997). This indicates that individuals of this hermaphroditic species exhibit selfing. However, due to the small sample size, it was not possible to test for the presence of null alleles (Van Treuren 1998). No significant linkage disequilibrium could be detected between any locus pair using the software Arlequin 3.1 ($P > 0.04$ in all six comparisons for the Bochum population, Bonferroni correction not applied). Ten individuals of the Bochum population (66%) exhibited three PCR fragments for the locus SsCA25, indicating the presence of a third allele, possibly due to gene duplication. Overall observed heterozygosity in the samples from the UK ranged between 0.88 and 0.62. A third gene copy at the locus SsCA25 was not observed in those worms. We also tested the identified primers in two other cestodes, *Diphyllobothrium latum* and *Taenia solium*. However, PCR amplification was not successful. The high polymorphism of our markers in *S. solidus* suggests that they will be useful for paternity assessment, the estimation of selfing rates and the study of gene flow and population structure.

Acknowledgements

We thank Jean Mariaux, Daniel Clark and Leslie R. Noble for providing samples. The technical assistance of S. Liedtke and S. Breiholtz is gratefully acknowledged. Many thanks to M. Güntert for support. T.B., M.M., L.S. and C.W. acknowledge support from the Swiss National Foundation (31-45733.95).

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Polymerase chain reaction primers for polymorphic microsatellite loci in the invasive toad species *Bufo marinus*

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Originally from South America, the cane toad (*Bufo marinus*) was deliberately introduced to various Atlantic and Pacific islands, and to Australia, early this century as a biological control (reviewed in Easteal 1981). Because of its well known introduction history and likelihood of strong founder events and demographic flushes, the cane toad provides a good empirical model for studying the population genetics of nonequilibrium systems. Surveys of enzyme variation in the introduced populations in Australia enabled an assessment of some of the genetic consequences of colonization processes (Easteal 1988). However, low level of polymorphism observed from enzyme markers limited resolution. Thus, the development of microsatellite markers in *B. marinus* represents a significant progression in the study of this invasive species.

Two libraries enriched for microsatellites were constructed and screened for $(GT)_n$ repeats following the protocols of Armour *et al*. (1994) and Paetkau (1999). Following the former protocol, 8000 clones were probed, from which 21 positives were picked and sequenced. Eleven of those clones had flanking regions of at least 20 bp and a core sequence of at least 10 repeats, and hence selected for primer design. Following the latter protocol, 400 clones were obtained, 100 sequenced, and 18 selected for primer design. Of the 29 clones selected, preliminary genotyping tests indicated that 17 loci were not suitable as they produced dubious amplification patterns. Two *B. marinus* populations, British Guyana (source population) and Gordonvale (introduced population in Australia), were genotyped with the remaining 12 loci. The primer and core sequences of those loci are provided in Table 1.

Extractions of DNA from individuals (usually a toe section) were performed following Estoup *et al*. (1996). Polymerase chain reactions (PCRs) contained: 1–2.5 mM $MgCl_2$ (see Table 1), 0.17 mM of each dNTP, 1 × PCR buffer [100 mM Tris (pH 8.8)], 1% Triton X-100, 500 mM KCl, 160 μg/mL (BSA), 0.07–0.14 μM of fluorescent labelled primer (Table 1), 0.25 μM of unlabelled primer, and 0.2 units of DyNAzyme DNA polymerase (Finnzymes). PCRs were performed in a GeneAmp PCR System 9700 thermal cycler using the following programme: one cycle of 94 °C for 1 min 45 s; five 'touchdown' cycles: 94 °C for 15 s, 1 °C drop per cycle to a final annealing temperature of 57 °C or 52 °C (Table 1) for 20 s, 72 °C for 10 s; 27 cycles of 94 °C for 15 s, 57 °C or 52 °C for 20 s, 72 °C for 1 s; and a final hold of 72 °C for 2 min. Genotypes were scored using ABI PRISM Genotyper 2.0 software (Perkin Elmer).

All 12 loci were polymorphic with an observed number of alleles per population ranging between one and 10, and heterozygosities between zero and 0.909 (Table 1). Exact tests performed using GENEPOP 3.1 (Raymond & Rousset 1995) with a correction for multiple comparisons (sequential Bonferroni procedure, Rice 1989), detected significant deviation from Hardy–Weinberg equilibrium and linkage disequilibrium for the loci BM102 and BM128. As the sex of the individuals was known for the Gordonvale (Australia) sample, we were able to detect that both these loci were sex linked. This finding

Table 1 Primer sequences, PCR conditions and polymorphism statistics for 12 microsatellite loci identified in *Bufo marinus*. G (30 individuals) and BG (22 individuals) refer to the Gordonvale (Australia) and British Guyana populations, respectively. H_O and H_E are observed and expected (i.e. 'gene diversity'; Nei 1987) heterozygosities, respectively. The first primer listed for each locus was flourescently labelled. The repeat motif is listed 5′ to 3′ with respect to the flourescent primer, and the final concentration in a PCR also refers to this primer. T_a indicates the lower annealing temperature in a PCR. Sex linked loci are marked with an asterisk. Sequences from which primers were designed have GenBank accession nos AF273090–AF273101

Locus	Primer sequences (5′ to 3′)	Repeat motif in library	T_a (°C)	$MgCl_2$ (mM)	Primer (μM)	Size range (bp)	No. alleles		H_O		H_E	
							G	BG	G	BG	G	BG
BM102*	GATCGGAGACATCTGGCA GATCCAACTCATACATACGTACA	$(GT)_{10}$	57	2	0.07	100–124	4	5	0.800	0.455	0.693	0.515
BM121	GACCCCATTTGTGCTGAG AACCAGCATTGCTGAAGTATC	$(GT)_{21}$	57	1.5	0.07	144–169	5	8	0.600	0.909	0.761	0.821
BM128*	GCCATATTTCGTGACTGTAGC CTGGGTTATCTAATATATAAAGCTGAG	$(AC)_{23}AT(AC)_2$	57	1	0.11	115–145	3	8	0.800	0.818	0.595	0.777
BM217	AACATGACAACCCAGCCAT GCGGTGCAGATTCCTTTAGT	$(TA)_{10}$ … $(GT)_{10}$	57	1.5	0.07	149–164	1	5	0.000	0.364	0.000	0.329
BM218	GGTATGCAACTGCATGAGC GGTTGCTACTTAGTAAGTTCGC	$(CA)_{12}$	57	1.5	0.07	161–173	2	3	0.267	0.636	0.282	0.566
BM224	GGGATCTGTGCAGATGGG GCTGATCTTGCACAATCTTTG	$(TG)_5TA(TG)_5TA(TG)_2$ … $(AG)_{15}$	57	1.5	0.07	142–154	5	5	0.467	0.818	0.631	0.710
BM229	ACTAAATTTATCATGTGCGCC ACACTGTAGCCATGCTGCA	$(TG)_{14}$	52	2	0.14	84–94	2	4	0.467	0.591	0.488	0.721
BM231	GTACCTTAATGGGGAAAGATC AACAGTGCCAGTCAATAGAAG	$(AC)_3TC(AC)_6GC(AC)_2GC(AC)_{11}$	57	1.5	0.07	170–174	2	3	0.333	0.136	0.325	0.449
BM235	GAAAATGAATTGACAGGTCCTC GTGACATCACTAGTATAGAAAGAGGC	$(TA)_8(CA)_{14}$	52	2.5	0.14	228–246	3	7	0.433	0.318	0.518	0.783
BM239	AAGTAGAGTTTTGCCGCAC AGCTACGTTCCCTTCAAC	$(TG)_4CG(TG)_{16}(CG)_4$	57	1.5	0.07	100–123	4	10	0.700	0.864	0.747	0.873
BM279	GGAGAAGTTTGTATTGCGAAC ATGGAGCATATCTGATTTGTGTAG	$(TG)_{12}$	57	1.5	0.07	247–257	3	4	0.400	0.773	0.392	0.735
BM322	AATCCACTCTTTACAAGTCCG ATTGATGCCCTATCCTGAG	$(GT)_{15}$	52	2.5	0.14	228–251	2	7	0.433	0.636	0.463	0.814

Table 2 Results of cross-species amplification for each microsatellite locus using the PCR conditions standard for *Bufo marinus*. Size of products is given in bp, and number of individuals screened is indicated within parentheses. A hyphen indicates the absence of a visible fluorescent PCR amplimer

	South America		North America	South Africa		Europe
Locus	*B. paracnemis* (4)	*B. granulosis* (2)	*B. americanus* (2)	*B. gutturalis* (3)	*B. rangeri* (2)	*B. bufo* (2)
BM102	110, 112, 114	—	—	—	—	—
BM121	143, 145	—	163	125	148	—
BM128	113, 115, 119, 121	—	—	—	—	—
BM217	149, 151	—	135, 137	—	—	—
BM218	159, 169, 171, 173, 175	—	—	—	—	—
BM224	142	154, 156	146, 148, 152	140, 144, 148, 150	140, 142	136
BM229	82, 84, 88	88, 94	—	—	—	—
BM231	—	—	—	—	—	—
BM235	—	—	—	—	—	—
BM239	89, 95	89, 95, 97, 99	—	—	—	—
BM279	251, 253, 255	—	—	—	—	—
BM322	229, 231, 233	—	—	—	—	—

was confirmed with additional tests using other populations (unpublished data).

Using PCR conditions for *B. marinus*, the 12 loci were tested for interspecies priming for an additional six *Bufo* species (Table 2). BM224 was the only locus that provided an easily interpretable pattern for all species, and showed polymorphism within most species. The closely related species *B. paracnemis* (Slade & Moritz 1998) amplified successfully with nine of the 12 microsatellites and showed polymorphism at most loci. Other species could not be amplified for most loci which is not unexpected considering their more distant relationship to *B. marinus* and the deep evolutionary history of the genus *Bufo* (Goebel *et al*. 1999). These results are in agreement with the low cross-species priming found at eight microsatellites cloned from *B. calamita* (Rowe *et al*. 1997).

Acknowledgements

For specimens, we thank: M. Cunningham, A. Krupa, N. Gemmell, B. Waldman, A. Goebel, R. Crambie, V. & B. Olson. This project was supported by a Special Investigator Award from the Australian Research Council, a grant from the Institut National de Recherche Agronomique, and the Australian Department of Industry Science and Technology.

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Microsatellite markers for coral trout (*Plectropomus laevis*) and red throat emperor (*Lethrinus miniatus*) and their utility in other species of reef fish

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Keywords: *Lethrinus miniatus*, lethrinids, lutjanids, microsatellites, *Plectropomus laevis*, serranids

Received 21 March 2000; revision received 11 May 2000; accepted 10 June 2000

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There is concern about sustainable management of stocks of reef fish, particularly as we do not fully understand population dynamics and response to harvest of many species (Mapstone *et al*. 1997). Evidence to date suggests that most harvested species have restricted movement ranges (Davies 1995). Genetic diversity may be reduced if particular populations are over-harvested. To effectively manage fishing in the Great Barrier Reef (GBR), managers require information on the stock structure and gene flow among populations of harvested species along the GBR. This will allow managers to balance conservation of biodiversity with sustainable exploitation. Genetic markers are useful in determining stock structure of fish and assessing gene flow among populations (e.g. Wright & Benzen 1994). The lack of suitable genetic markers is an impediment to our understanding of the population structure of tropical reef fish.

Microsatellite markers are powerful tools for detecting intra- and interpopulation genetic diversity. Here we report the development of microsatellite markers to assess genetic diversity for populations of *Plectropomus laevis* and *Lethrinus miniatus* on the GBR. These species represent two major families of harvested reef fish. Tests against several other taxa were undertaken to ascertain the utility of these markers to differentiate between populations in a wide range of reef fish.

Partial genomic libraries were constructed using genomic DNA extracted from liver/muscle tissue of *P. laevis* and *L. miniatus*, by standard methods (Sambrook *et al*. 1989). DNA was digested with *Alu*I and *Hae*III, fragments were separated by gel electrophoresis and fragments of 400–1000 nucleotides were excised (Rassman *et al*. 1991), purified and ligated into puc18/*Sma*I BAP (Invitrogen).

Nearly 3000 clones were screened using a T4 polynucleotide kinase — [γ^{33}P]-ATP end-labelled cocktail of di-, tri- and tetranucleotide oligonucleotides [$(GT)_{15}$, $(GA)_{15}$, $(GAA)_{10}$, $(CCA)_{10}$, $(CGG)_{10}$, $(CACG)_7$, $(CCTT)_7$ and $(GATA)_7$] as described by Glenn (1996). Fifty coral trout and 112 red throat emperor clones were sequenced (DyeDeoxy terminator cycle sequencing kits, Perkin Elmer/ABI). Vector sequences were eliminated and primer pairs manually designed and optimized for 22 markers of each species according to principles described by Ehrlich (1989) (Table 1). Eight of the 22 red throat emperor markers,

Table 1 Microsatellite markers developed from *Plectropomus laevis* and *Lethrinus miniatus*, optimized PCR conditions, size of amplified fragment obtained from library clones and GenBank accession nos

Locus	Repeat motif	Primers 5′ > 3′	T_a °C	Mg mM	Size (bp)	GenBank accession no.
Bst 2.33	$(TG)_{35}(CG)_{12}$	F TAATGCCCACAAACCTGCTGG R ATGTTCCACAACGCCTGACAAACC	60	2.5	230	AF249850
19RTE	$(AC)_{18}$	F CAGCCAGGTTCCTCTTCATCC R TGTACTCTATCAATGACAGACG	58	1.5	109	AF260998
23RTE	$(GTAT)_3ATGT(GT)_8$	F AGAGTTTCAAACACCCACAGG R TCTGTGAAATATGAGCGGTGG	58	2.5	300	AF260999
67RTE	$(GT)_{15}$	F TGGCTTTGAACAGATCCAGC R TTTGGGCTATTAATGCCTTGG	56	2.5	270	AF261001
90RTE	$(TG)_{17}TATGAG(TG)_4$	F ATGCTGTCCACTTCCTCCAGC R TTTCTCAAACTCCTGCCCTTCC	62	1.5	190	AF261002
58RTE	$(TG)_9AGTG$	F TGCCAGTGTGTGGGCAGTAGG R TTCGACAAATTGTTTACTAATTTGC	52	1.5	130	AF261008
80RTE	$(GT)_{23}$	F AATGTGATTTTGTATTTACATGC R TCGTTTTCTTTCCCAGCTGG	54	2.5	195	AF261011
95TGRTE	$(TG)_{13}$	F TCTGTATTTGGTATTCCAACG R TCAGTGTCAGAGAGACAGC	54	1.5	120	AF261013
37RTE	$(TC)_4TGGT(TG)_9CC(TG)_3$	F AGCATGCACACTCATGCTGTCC R ACAGCACCAGTCAGTGCGAGG	64	2.5	170	AF261016

T_a, annealing temperature.

Table 2 *Plectropomus laevis* and *Lethrinus miniatus* microsatellite markers used to screen *Plectropomus* species, Serranids, Lethrinids, Lutjanids and a Labrid. Number of alleles detected for each locus in number of individuals for which PCR products were obtained (in parentheses) of each species. 'M' indicates that products were monomorphic. 'F' indicates that PCR failed repeatedly. H_O is the observed and H_E the expected heterozygosity obtained when 105 individuals were screened from a single population of *L. miniatus*

Group	Species	Bst 2.33	19 RTE	23 RTE	37 RTE	58 RTE	67 RTE	80 RTE	90 RTE	95TG RTE
Plectropomus	*P. laevis* (bst)	6(3)	3(3)	2(3)	2(3)	2(3)	3(2)	F(3)	3(3)	3(3)
	P. laevis (fbt)	10(5)	3(5)	5(5)	2(5)	2(5)	1(1)	F(5)	3(5)	3(5)
	P. leopardus	9(5)	4(4)	5(4)	3(4)	4(6)	2(3)	2(3)	2(5)	4(5)
	Variola louti	7(4)	M(4)	F(4)	M(4)	2(4)	2(1)	F(4)	3(4)	M(4)
Lutjanids (other)	*L. sebae*	7(4)	2(2)	3(2)	2(4)	2(4)	1(1)	F(4)	4(4)	3(4)
	L. carponotatus	5(5)	2(3)	4(5)	M(5)	3(5)	1(1)	F(5)	3(5)	3(5)
Lethrinids	*L. miniatus*	H_O = 0.93 H_E = 0.90	H_O = 0.91* H_E = 0.94	H_O = 0.90 H_E = 0.86	H_O = 0.58 H_E = 0.64	H_O = 0.73* H_E = 0.76	H_O = 0.50* H_E = 0.82	H_O = 0.85* H_E = 0.89	H_O = 0.83 H_E = 0.75	H_O = 0.72 H_E = 0.71
	L. atkinsoni	6(4)	7(4)	3(4)	4(3)	2(4)	2(2)	3(4)	5(4)	3(4)
	L.erythracanthus	3(2)	3(3)	4(3)	F(3)	2(3)	4(2)	2(3)	3(3)	2(3)
	L. nebulosus	2(2)	4(2)	4(2)	3(2)	M(2)	2(1)	2(2)	2(2)	1(2)
Serranids	*Epinephelus polyphykelodon*	3(5)	7(5)	6(5)	4(5)	M(5)	4(3)	2(1)	6(4)	4(5)
	Cephalopholis argus	2(4)	2(1)	3(4)	2(4)	2(4)	F(4)	1(1)	5(4)	3(4)
Labrid	*Cheilinus undulatus*	2(3)	4(3)	3(2)	5(3)	2(3)	4(2)	2(2)	2(3)	1(2)

Heterozygosities were calculated using Levene's correction, as implemented in GENEPOP version 3.1 (Raymond & Rousset (1999) Institut des Sciences de l'Evolution. Universite de Montpellier, France).
*Loci that had heterozygote deficiencies ($P \leq 0.05$).
RTE, Red throat emperor.

as well as the coral trout marker bst2–33, were tested on 2–7 individuals each of 12 species (Table 2). One hundred and five individuals of the target species, red throat emperor (*L. miniatus*) were also screened with the nine microsatellite markers (Table 2). The remaining 35 markers are reported elsewhere (van Herwerden *et al*. 2000). For visualization the forward primer of each pair was 5′ labelled with either FAM, TET or HEX dyes (Perkin Elmer/ABI). Polymerase

chain recation (PCR) products were sized with internal lane standards (TAMRA, Perkin Elmer/ABI). PCR reactions were carried out using 50–100 ng genomic DNA, 10 pmol of each primer, 200 μM of each dNTP, Taq Buffer (containing 2.5 mM TrisCl, 5 mM KCl $(NH_4)_2SO_4$ and 1.5 mM $MgCl_2$, pH 8.7), up to 4 mM $MgCl_2$ and 0.75 units of *Taq* DNA Polymerase (Qiagen) in 10 μL volumes. Amplifications were performed using touchdown PCR (Don *et al*. 1991) in 9600 Perkin Elmer/ABI thermal cyclers (Table 1). PCR products were screened on 2% agarose gels containing 10 and 25 ng of standard to determine dilution factors required for detection of 1 ng of each PCR product.

Heterozygosities were determined for all loci used to screen the population of red throat emperor, but not for cross-species tests, where sample sizes were small (Table 2). The coral trout locus (bst2–33) was polymorphic in all 13 species tested. Of the eight red throat emperor loci tested, five were polymorphic in 11 or 12 out of 13 species tested (Table 2). The nine markers described here can be used to investigate population structure of Serranids, Lethrinids, Lutjanids and a Labrid (*Cheilinus undulatus*). These findings indicate the potential utility of microsatellite markers developed for one species, both for other species within the genus and species of distant genera.

Acknowledgements

Tissue samples provided by the ELF Project funded by the Collaborative Research Centre Reef, Fisheries Research and Development Council (FRDC) and Great Barrier Reef Marine Park Authority. Project supported by a FRDC grant (nos 98–131). Thanks to Carolyn Smith, Beth Ballment, Gary Carlos and Robin Stewart. This is contribution 1011 from Australian Institute of Marine Science.

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Polymorphic microsatellite loci of *Oryzomys palustris*, the marsh rice rat, in South Florida detected by silver staining

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Keywords: microsatellite, *Oryzomys palustris*, polymorphic, primers, silver stain

Received 12 May 2000; revision accepted 14 June 2000

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As part of an on-going study of impacts of habitat patchiness on genetic structure, we report the characterization of microsatellite loci for the marsh rice rat (*Oryzomys palustris*). Previous studies on the population structure of this species have suggested that allozymes (Schmidt & Engstrom 1994) and mitochondrial DNA sequence data (Gaines *et al*. 1997) only reveal large-scale geographical structure. To study the species within Everglades National Park and its surrounding habitat, we need more variable loci; microsatellite loci seemed the best choice.

Our first approach was to use polymerase chain reaction (PCR) primers that amplify polymorphic microsatellite loci in the related species *Peromyscus leucopus* (Schmidt 1999) and *Mus musculus* (Moncrief *et al*. 1997). However, 15 pairs of *Peromyscus* primers and 52 pairs of *Mus* primers failed to produce polymorphic markers for *Oryzomys*.

We therefore isolated microsatellites *de novo*. A partial genomic library was created in λZAP Express (Stratagene, La Jolla, California, USA) as described by Hughes & Moralez DeLoach (1997). Approximately 150 000 clones were screened with ^{32}P-labelled oligo$(AAT)_{10}$. Fifty positive clones were purified and then sequenced using the BigDye™ Terminator Cycle Sequencing Kit and an ABI 310 genetic analyser (PE Applied Biosystems). PCR primers were developed for nine clones containing >10 uninterrupted repeats of the sequence AAT.

PCR reactions (10 μL) contained 4 mM $MgCl_2$, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 125 μM each dNTP, 500 nM forward and reverse primers, 0.25 U *Taq* DNA polymerase (Perkin Elmer) and about 10 ng DNA. Individuals had been sampled from eight tree islands <300 m apart. Reactions were cycled using the 'tube-control' function of a Hybaid thermal cycler: 1 min at 92 °C, then 30 cycles of 5 s at 92 °C, 5 s at annealing temperature (Table 1) and 10 s at 72 °C. PCR products were resolved on a 6% denaturing polyacrylamide gel and stained

Table 1 Characteristics of polymorphic microsatellite loci from *Oryzomys palustris*

Locus	Primer sequence (5′ → 3′)	Annealing temp. (°C)	GenBank accession no.	Number of (AAT) repeats	Number of alleles/ number of samples	Allele size range (bp)	H_O	H_E
OryAAT03	TGGCTTCAGTGGGTATTTATTAT TGCGCACATGTATATTAAAGAA	55	AF227630	16	6/18	125–143	0.440	0.758*
OryAAT10	TTGGGTTGGCTCTAATAGAA TTTGCTATTGTTCACTTTCTAA	53	AF259064	14	7/15	124–148	0.530	0.838*
OryAAT16	CTTCCATGAGTATCTGTAATAATA CAGCCAGGACTGTTACAC	50	AF259065	11	10/16	94–125	0.875	0.910
OryAAT21	GCCTCCTACTGTTGTTTTGTTA GCATTATCTGTTCTCTATCCACTAC	55	AF259066	16	6/15	184–199	0.800	0.830
OryAAT26	CAATTGCTATTTTTCTGTAAA CCAGCTGTTTTTTATTGTACT	50	AF259067	13	12/17	97–141	0.880	0.927
OryAAT28	TCGGGAATATAGAAAGAAGTA TGCCACTCAAAAATGATTTAAC	55	AF227631	16	8/17	70–112	0.705	0.814*
OryAAT40	GGGTGTTCCAGAGAATGAATCTA TCCCAACATCCTCACAAATTTAT	55	AF259068	13	9/17	148–175	0.588	0.802
OryAAT60	AAGGCAGCTAAAAAAATCTT ATCGTCTCTGTTTTTATATCAG	53	AF259069	11	13/16	130–181	0.750	0.918
OryAAT64	TTTCCTAAGAGCTAGAAATAA TGGAAAACCCTTAGAAATTCT	50	AF259070	11	8/20	74–95	0.350	0.828*

H_O and H_E are the observed and expected heterozygosities, respectively. *Loci whose H_O are significantly different from H_E ($P < 0.05$) using the exact Hardy–Weinberg test (GENEPOP 3.1, Raymond & Rousset 1995).

with Silver Sequence™ (Promega Corp), according to the manufacturer's instructions.

The nine AAT loci were highly polymorphic (Table 1). The average number of alleles per locus is 8.7, and the average observed heterozygosity is 0.67. The polymorphic nature of these loci makes them highly informative tools for future studies of population genetic structure and relatedness in *O. palustris*. There was a trend over all loci with the expected heterozygosity being greater than the observed heterozygosity. This may indicate population subdivisions among tree islands, or the presence of null alleles or some combination of these two factors.

The utility of these nine primer pairs was examined in another *Oryzomys* species, *O. megacephalus*, and in *Sigmodon hispidus*, *Neotoma floridana* and *Rattus rattus*. Annealing temperature was decreased to 5 °C below that used in *O. palustris*. Loci were either monomorphic, or failed to amplify in these species. This result mirrored our initial unsuccessful attempt at identifying polymorphic loci for *O. palustris* with primers from *Peromyscus* and *Mus*. Together, these results indicate that heterospecific primers will be of less value in small mammals than in taxa such as birds (Primmer *et al*. 1996).

Acknowledgements

This work was supported by the University of Miami. Edwin. A. Gines-Candelaria was supported by grants from National Institute of General Medical Sciences 1R25GM 50083-02 and Howard Hughes Medical Institute 71199-514104.

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Microsatellites in the subterranean, mound-building termite *Coptotermes lacteus* (Isoptera: Rhinotermitidae)

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Received 16 March 2000; revision accepted 25 June 2000

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The conceptual framework for the evolution of social life and colony organization is provided by kin selection theory, with predictions often tested through direct estimates of colony and population genetic structure (Pamilo *et al*. 1997). All termite species are eusocial yet, despite their significance as potential falsifiers to this theory, have received comparatively little attention in this respect (Reilly 1987; Husseneder *et al*. 1999). Colonies of the subterranean, mound-building species *Coptotermes lacteus* (Rhinotermitidae) are normally founded by a single reproductive pair which remain the sole progenitors for as long as 20 years (Gay & Calaby 1970). Upon failure of one or more of these primary reproductives, however, colonies will recruit secondary reproductives of either sex through differentiation of nymphal instars. This in turn promotes the formation of polygamous reproductive associations, a shift in alate production from seasonal to perennial, and extremely male-biased alate sex ratios (Lenz & Runko 1993). Thus, in addition to the application of variable markers for inferring colony structure of normal *C. lacteus* populations, they will make it possible to monitor how shifts in genetic heterogeneity affect reproductive decisions of orphaned colonies. In this note we characterize six polymorphic microsatellite markers from *C. lacteus*; member of the family Rhinotermitidae (~200 sp.). Four microsatellite markers have recently been characterized for the Termitidae (Kaib *et al*. 2000).

Partial genomic libraries were constructed from DNA extracted from the heads of 500 individual termites pooled from five different mounds (South Brooma State Forest, NSW Australia). Approximately 6 μg of genomic DNA was restricted with double-digest combinations of either *Sau*3AI/*Rsa*I or *Sau*3AI/*Alu*I and fragments ranging between 300 and 800 bp were purified and ligated into *Hin*cII/*Bam*HI sites of pUC19 plasmid vector. Transformations were carried out by electroporation (GenePulser, BioRad) into *Escherichia coli* JM109 cells, then plated onto nutrient agar for white/blue selection of recombinant/nonrecombinant transformants (Ausubel *et al*. 1995). Approximately 4000 positive clones were isolated in this manner, transferred onto Hybond N + (Amersham) nylon membranes, and subsequently hybridized with $(AG)_{10}$ and $(GT)_{10}$ dinucleotide probes previously end-labelled with [γ^{33}P]-ATP. On the basis of positive hybridization signal, 30 putative microsatellite-containing clones were identified and sequenced. Polymerase chain reaction (PCR) primer pairs were designed for 10 of these loci using PRIMER (Lincoln *et al*. 1991).

Genomic DNA for microsatellite analysis was extracted from the heads of single individuals using the Chelex® protocol (Walsh *et al*. 1991). Each locus was PCR-amplified using [γ^{33}P]-ATP end-labelled PCR primers. PCRs were carried out in a final volume of 10 μL containing 20–50 μg genomic DNA, 10 μg bovine serum albumin, 0.08 U *Taq* polymerase (Promega), and a final concentration of 2.5 mM dNTPs, 0.1 mM unlabelled forward primer, 0.4 mM unlabelled reverse primer, 0.03 μM of radioactively labelled forward primer, and 1 μL 1× Promega Buffer (with 1.5 mM $MgCl_2$). PCR cycling profiles for each of the six markers began with an initial denaturation at 94 °C for 3 min, followed by 30 cycles consisting of 30 s at 94 °C, 30 s at the appropriate annealing temperature (Table 1) and 30 s at 72 °C, with a final extension phase of 72 °C for 10 min. Amplification products were electrophoresed on 5% denaturing polyacrylamide gels at a constant power of 65 W for 3–4 h and then scored after exposing electrophoretic profiles to film (Fuji RX). Six of the 10 loci examined gave scorable and polymorphic banding patterns. Table 1 gives estimates of the variability of each locus determined using a sample of 12 workers obtained from different colonies. The expected heterozygosities were calculated as in Nei (1987) and ranged from 0.30 to 0.90, the highest diversity being observed from an imperfect microsatellite with the highest number of alleles ($N_A = 7$).

The primers presented here are sufficiently variable to investigate population and colony genetic structure of *C. lacteus* and possibly of other termite species.

Table 1 Characteristics of microsatellite loci from *Coptotermes lacteus*. N_A is the number of alleles observed, H_O and H_E are observed and expected heterozygosities, respectively, and T_a is the annealing temperature; 12 workers were screened per locus

Locus	Repeat motif	N_A	Size range (bp)	H_O	H_E	Primer sequence (5′–3′)	T_a	Accession nos
Clac1	$(AG)_9AA(AG)_4$	5	180–188	0.82	0.70	F-CAGAGGTGACATCAGAAATTG R-GCACATAACAGTAAACCTGCTG	52	AF275658
Clac2	$(GA)_7C(AG)_7$	3	206–212	0.64	0.53	F-GTGATAGTGAAGCGTCGG R-CTGTCATCAATGAGCGGC	50	AF275659
Clac3	$(TA)_7(CA)_{11}$	3	206–212	0.33	0.30	F-CATATTGGTAGCGAATGACGTG R-ATAAGCAGAGTTGCAGCTATGC	48	AF275660
Clac4	$(GT)_{21}(GC)_4(GT)_3(GA)_{10}(GT)_3$	3	102–109	0.50	0.63	F-CCAAGTCTGTCTGGTATAAAC R-CGTCTTGCATGTGCCTTTAC	52	AF275661
Clac6	$(AG)_{16}AA(G)_{10}$	7	180–192	0.83	0.90	F-GCTTATTGGATACAGTATGG R-CTATAATAGAGACACAGCGG	56	AF275662
Clac8	$(GA)_{10}$	3	249–258	0.33	0.56	F-CAAATCTACAGGCGAGACCAC R-GCTGGTAGTTTAGTTGTGTTTC	54	AF275663

Acknowledgements

J. Painter, C. Crozier and M. Carew for technical advice; P. Gleeson and S. Runko for termite collections.

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Characterization of microsatellites in capercaillie *Tetrao urogallus* (AVES)

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Keywords: capercaillie, gene flow, microsatellites, population structure, *Tetrao*

Received 12 May 2000; revision accepted 25 June 2000

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The Capercaillie (*Tetrao urogallus*) is listed in the red-data books of threatened species of most central European countries (Storch 2000). Its range is fragmented both at the continental (central Europe) and the regional (e.g. Alps) scale, and the distribution of populations resembles a metapopulation pattern (Storch 1997). In this context, information on (sub) population connectivity is highly relevant for species conservation. To assess the extent of gene flow in relation to geographical distance in Capercaillie we developed species-specific microsatellites. As the abundance of microsatellites seems to be much lower in birds than in other vertebrates (Primmer *et al*. 1997), we used an enrichment method for establishing a microsatellite-rich partial genomic library (Piertney *et al*. 1998a). DNA was extracted from tissue of an adult capercaillie female using standard proteinase K digestion and phenol–chloroform procedures (Sambrook *et al*. 1989). DNA was digested using *Sau*3AI restriction endonuclease and a 300–900 bp fraction isolated. Fragments were ligated to a SAU linker molecule (Piertney *et al*. 1998b). Resultant fragments were denatured and hybridized to a 1-cm² piece of Hybond N + membrane (Amersham International Ltd) to which a synthetic polymer, either $(CA)_n$ or $(GATA)_n$, was bound. Hybridization took place at 60 °C overnight in 2× SSC, 0.1% SDS for $(CA)_n$ and in 5× SSC, 0.1% SDS for $(GATA)_n$. After three low stringency washes of 2× SSC, 0.1% SDS for $(CA)_n$/5× SSC, 0.1% SDS for $(GATA)_n$ the enriched fraction was removed by heating to 95° for 5 min in sterile water. The enriched fraction was precipitated and complementary strands were reformed in a polymerase chain reaction (PCR) (30 cycles at 94 °C denaturation, 55 °C annealing and 72 °C extension) using the SAU-L-A oligonucleotide as a primer. SAU linkers were removed by restriction with *Sau*3AI and fragments were ligated into a phosphorylated, *Bam*HI-cut pUC18 vector (Pharmacia Ltd). Vector molecules were transformed into INVαF'One-Shot™ *Escherichia coli* (Invitrogen) which were then grown overnight on Luria-Bertani (LB) medium with ampicillin. Colony lifts were made from the plates onto Hybond N + membranes and screened for the presence of microsatellites following Estoup & Turgeon (1996) in 5× SSC, 0.1% SDS at 55 °C. Positive clones were sequenced on an ABI 373 sequencer using Big Dye terminator chemistry (Perkin Elmer Applied Biosystems) and primers were designed on sequences flanking the repeat elements (Whitehead Institute Primer 3.0). Genetic variability was examined in ≈ 20 individuals from the European Alps. DNA was extracted from capercaillie feathers using a silica-based column method (Qiagen) according to the manufacturer's protocol. PCR-amplifications were performed in 10 μL reactions in an Eppendorf Gradient thermal cycler. Individual mixes contained approximately 10 ng template, 0.2 mM of each nucleotide, 10 pmoles of each primer, 2.5 mM $MgCl_2$, 0.25 units of *Taq* DNA polymerase (Gibco BRL), 20 mM Tris-HCl (pH 8.4) and 50 mM KCl. PCR profiles consisted of 3 min denaturation at 94 °C, 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at the specified temperature and 45 s extension at 72 °C with a final 5 min 72 °C step. PCR fragments were resolved by electrophoresis on 6% denaturing polyacrylamide gels and afterwards stained with silver. High levels of variability could be detected in the analysed loci (Table 1), making them useful for paternity analysis and population genetic studies.

Acknowledgements

The work was funded by the DFG (STO 230/4–2). We thank all

Table 1 Description of microsatellite loci for *Tetrao urogallus*; sequences and repeat type, PCR fragment length (based on the clone sequence), number of resolved alleles (number of analysed birds are in parentheses), optimal annealing temperature, heterozygosity, and GenBank accession nos of the clone sequences. Significant differences between the observed and expected heterozygosity are marked with an asterisk

Locus	Primer sequences (5′–3′)	Repeat type	Size (bp)	No. of alleles	Annealing temp.	H_O	H_E	Accession no.
TUD1	F: ATTTGCCAGGAAACTTGCTC R: AACTACCTGCTTGTTGCTTGG	$(CA)_{14}$	209	8 (19)	59	0.70	0.82	AF254644
TUD2	F: GTGACAACTCAGCCCCTGTC R: AATAAGGGTGCGCATACACC	$(CA)_{13}$	200	10 (19)	59	0.83	0.89	AF254645
TUD3	F: TCCAAGGGGAAAATATGTGTG R: TTCTTCCAGCCCTAGCTTTG	$(TG)_{12}$	192	11 (19)	60	0.66	0.82	AF254646
TUD4	F: TTAGCAACCGCAGTGATGTG R: GGGAGGACTGTGTAGGAGAGC	$(CA)_{21}$	167	11 (19)	60	0.61*	0.88	AF254647
TUD5	F: CCTTGCTGCACATTTTCTCC R: GGTGCTGAGCATGTACTAGGG	$(GT)_{23}$	193	12 (19)	57	0.72	0.88	AF254648
TUD6	F: GGTGAGCAAGCCACAAATAAC R: GAGGACTGCAGAACCCACTG	$(CA)_{21}$	210	13 (16)	58	0.69*	0.88	AF254649
TUD7	F: TGACACTGGGGTCATTAGGC R: AACATGGGCAGGAGGAGAC	$(CA)_{11}$	200	5 (19)	59	0.58	0.63	AF254650
TUD8	F: TGCAGCCTCCTCTAATTTCG R: CTGGACATCAGCAATCATGC	$(GT)_{15}$	187	11 (17)	59	0.70*	0.85	AF254651
TUT1	F: GGTCTACATTTGGCTCTGACC R: ATATGGCATCCCAGCTATGG	$(CTAT)_{12}$	217	8 (20)	60	0.60	0.83	AF254653
TUT2	F: CCGTGTCAAGTTCTCCAAAC R: TTCAAAGCTGTGTTTCATTAGTTG	$(GATA)_{12}$	160	9 (20)	60	0.70	0.76	AF254654
TUT3	F: CAGGAGGCCTCAACTAATCACC R: CGATGCTGGACAGAAGTGAC	$(TATC)_{11}$	154	8 (20)	60	0.50	0.80	AF254655
TUT4	F: GAGCATCTCCCAGAGTCAGC R: TGTGAACCAGCAATCTGAGC	$(TATC)_8$	179	7 (20)	60	0.80*	0.77	AF254656

people who provided feather samples, D. Begerow for sequencing, and S. Piertney for technical advice.

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Identification of polymorphic microsatellite loci in the Formosan subterranean termite *Coptotermes formosanus* Shiraki

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Keywords: *Coptotermes formosanus*, genetic variability, microsatellites, population structure

Received 3 April 2000; revision received 4 June 2000; accepted 29 June 2000

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The Formosan subterranean termite *Coptotermes formosanus* Shiraki (Rhinotermitidae) is thought to have originated in China, but has spread to many areas around the world where it is a highly destructive pest of wood structures (Su & Tamashiro 1987). In the USA, this species is abundant in Hawaii, and has become established in many places on the mainland, particularly in the south-eastern and south central regions (Wang & Grace 1999).

Table 1 Primer sequences and characteristics of 15 microsatellite loci of *Coptotermes formosanus*

Locus	Core repeat*	Size (bp)	Concentration of primer (nM)	Primer sequence (5′ → 3′)	GenBank accession no.
Cf 4:1A2-4	$(AAG)_{14}$	175	100	F: CTGTTTCTCGTAATCGGGGA†	AF247458
			200	R: TTCTTCAACGTCTTCGCCTT	
Cf 4:1A2-5	$(CAA)_{10}$	141	200	F: TCGGACTCCAGGTACTACCAA	AF247459
			100	R: GATTGCCGTTCCTTCCTTCT†	
Cf 1-1	$(TTA)_7(GTA)_{11}$	255	100	F: TTGCTCAGGATAGGGACAGG†	AF247460
			200	R: TGGTTAATCGTGCCCACATA	
Cf 2-5	$(CAT)_7$	175	100	F: CCTCAAATCCCTCACACACA†	AF247462
			200	R: GGACATTTTGCTTCCTCCAA	
Cf 4-4	$(ACT)_{20}$	214	100	F: GCATAAACGAACCCTGGAAA†	AF247463
			200	R: TGCCAAACATGTGTTGCTTT	
Cf 4-9A	$(TCA)_{11}$	283	200	F: GTGTGGGATTTGAGGTGGAC	AF247464
			200	R: GAAAAACAGCGACTGCTTCC†	
Cf 4-10	$(CAT)_{11}$	229	200	F: GCAAGTTTTGCCCTGTCAGT†	AF247465
			200	R: GAAAAACAGCGACTGCTTCC	
Cf 8-4	$(CTA)_9(CTC)_{15}$	221	200	F: TCTGTGGAACGTGGTGTGAT†	AF247468
			200	R: CCTCTCTGTGCCTGCTTAGG	
Cf 10-4	$(AGT)_{22}$	154	200	F: GCGCATGTGGACTGTAAAAA	AF247469
			200	R: TCCAAGTATGCTGATCGGGT†	
Cf 10-5	$(GAT)_8$	295	200	F: CAGCTATATTGGGCACAGCA†	AF247470
			200	R: CACGACGGACTGAAGTGGTT	
Cf 11-1	$(GTA)_{10}$	204	200	F: CGTTCCTTCGAAACTTCTGC	AF247471
			200	R: TACCATCACCACCACCACC†	
Cf 12-4	$(TAG)_{21}$	172	200	F: AGCGTCTAGCCTTGCACTTC†	AF247472
			200	R: CTCCTCACCAAAATCCGGTA	

*Sequenced allele. †Primer to which M13 forward tail is attached. F, forward; R, reverse.

Polymorphic genetic markers for *C. formosanus* would be useful in elucidating details of colony organization, population structure, and relationships among introduced and native populations. Previous studies employing allozymes have found limited genetic variation in this species (Korman & Pashley 1991; Strong & Grace 1993). To provide variable genetic markers for examining colony and population structure, we developed microsatellite markers for *C. formosanus*.

Microsatellite loci were isolated following the protocol of Glenn (1996) with modifications as described by Vargo (2000). The partial genomic library was constructed from pooled samples of heads of workers from eight colonies originating from Gretna, Lake Charles and New Orleans, Louisiana, USA. After grinding the tissue in liquid nitrogen, the DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). Fragments (300–700 bp) of *Sau*3AI-digested genomic DNA were ligated into pZErO-2 plasmids (Invitrogen), cut with *Bam*HI, and transformed into *Escherichia coli* TOP10 cells (Invitrogen). Approximately 20 000 clones were plated and lifted onto nylon membranes. The membranes were probed with the oligonucleotides $(AAT)_{10}$, $(AAC)_8$, $(ATC)_8$, $(AAG)_8$ and $(ACT)_8$, which were end-labelled with [γ^{32}P]-dATP. Southern blots confirmed 42 positive clones. Sequencing of these clones yielded 25 sequences containing five or more tandem repeats. We designed 22 primer pairs.

Microsatellite analysis was performed by fluorescent labelling according to the methods of Oetting *et al*. (1995). Labelling was achieved by attaching the first 19 bp of the M13 forward sequencing primer (CACGACGTTGTAAAACGAC) to the 5′ end of one of the specific primers in each pair as indicated in Table 1. A fluorescent-labelled M13 primer (M13F-29/IRD 800, Li-Cor) was included in the polymerase chain reaction (PCR), yielding labelled product which was detected in a Li-Cor 4000 automated sequencer.

Whole bodies of workers, either frozen alive and stored at –80 °C or killed and preserved in 95% ethanol, were pulverized in 1.5 mL Eppendorf tubes with a plastic pestle. DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). The PCR amplifications were carried out in a total volume of 10 μL, containing 4 μL (about 20 ng) template DNA, 10 mM Tris–HCl, 50 mM KCl and 0.1% Triton®X-100, 2 mM $MgCl_2$, 0.2 μg/μL BSA, 200 μM each dNTP, 32 nM labelled M13F primer, 0.4 U *Taq* DNA polymerase (Gibco BRL) and variable amounts of the specific primers (Table 1). All loci were amplified on a PTC-100 thermal cycler (MJ Research Inc., Littletown, Massachusetts, USA) using the following touch-down programme: initial denaturation step at 94 °C (30 s), followed by six cycles at 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s), ramping down the annealing temperature 1 °C per cycle, and then 30 cycles at 94 °C (30 s), 54 °C (30 s) and 72 °C (30 s), with a final extension step at 72 °C (5 min). PCR products were separated by electrophoresis on polyacrylamide sequencing gels (8% Long Ranger, 7 M urea).

Table 2 Allele frequencies and heterozygosity levels for *Coptotermes formosanus* microsatellites for introduced and native populations

Locus	Origin	*n*	Number of alleles	Size* (bp) of most common allele	Frequency of most common allele	H_O	H_E	*P*
Cf 4:1A2-4	US and China	17	5	188	0.50	0.41	0.66	0.047
	US only	15	4	188	0.47	0.40	0.70	0.071
Cf 4:1A2-5	US and China	17	4	160	0.65	0.35	0.49	0.325
	US only	15	3	160	0.63	0.33	0.49	0.232
Cf 1-1	US and China	17	5	274	0.35	0.35	0.75	0.0001
	US only	15	4	274	0.33	0.40	0.73	0.0001
Cf 2-5	US and China	17	2	188	0.97	0.06	0.06	1.0
	US only	15	1	188				
Cf 4-4	US and China	17	6	245	0.40	0.59	0.78	0.190
	US only	15	6	245	0.40	0.53	0.77	0.059
Cf 4-9A	US and China	17	8	278	0.38	0.71	0.77	0.023
	US only	15	8	278	0.43	0.67	0.74	0.014
Cf 4-10	US and China	17	4	233, 242	0.41	0.53	0.64	0.070
	US only	15	3	233, 242	0.47	0.47	0.56	0.789
Cf 8-4	US and China	17	6	240	0.30	0.65	0.78	0.032
	US only	15	5	240	0.30	0.67	0.76	0.077
Cf 10-4	US and China	17	6	170	0.50	0.50	0.66	0.093
	US only	15	4	170	0.60	0.43	0.62	0.093
Cf 10-5	US and China	17	10	293	0.26	0.53	0.84	0.001
	US only	15	9	293	0.30	0.47	0.80	0.001
Cf 11-1	US and China	17	3	220	0.82	0.12	0.30	0.003
	US only	15	2	220	0.87	0.00	0.23	0.004
Cf 12-4	US and China	17	4	146	0.56	0.41	0.57	0.048
	US only	15	4	146	0.53	0.40	0.59	0.045

*Size includes the 19-mer M13F sequence. *n*, number of individuals examined (only one individual per colony was genotyped); H_O, observed heterozygosity; H_E, expected heterozyogosity; *P*, probability by HW exact test in GENEPOP version 3.1b (Raymond & Rousset 1995).

Individuals from 17 different colonies were screened for variability. Colonies originated from the following locations: 11 from Louisiana, three from Florida, one from North Carolina and two from mainland China. Of the 22 primer pairs tested, 15 gave scorable products. Twelve of the 15 loci showed variation among the entire study population, with 2–10 alleles per locus (Table 2). One locus, Cf 2-5, was variable only in the samples from China. Among all 12 polymorphic loci, observed heterozygosity was less than the expected in all but one locus (Cf 2-5; Table 2), with significant deviations ($P < 0.05$, HW exact test in GenPop version 3.1b; Raymond & Rousset 1995) at Cf 4:1A2-4, Cf 1-1, Cf 4-9A, Cf 8-4, Cf 10-5, Cf 11-1 and Cf 12-4. Reduced heterozygosity is expected in subterranean termites which commonly form inbred colonies (Thorne *et al*. 1999).

The present set of microsatellite markers, with numerous polymorphic loci, provides a sensitive tool for investigating the colony and population genetic structure of both native and introduced populations of *C. formosanus*.

Acknowledgements

N.-Y. Su (University of Florida) kindly provided samples from Florida, and J. Powell (USDA, Mississippi State University, Mississippi, USA) generously donated samples from China. We thank the Forest Biotech Group at North Carolina State University (NCSU) for their generous assistance and support, especially D. O'Malley, D.M. Johnson, B.C. Lee, A. Myburg, and R. Whetten. K. Patel gave technical assistance. K. Nix coordinated shipment of termites from Louisiana to North Carolina. E.L.V. was supported by a Faculty Research and Professional Development Grant from NCSU and the W.M. Keck Program for Behavioural Biology. Funds from the Louisiana Department of Agriculture and Forestry supported G.H.

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Isolation and characterization of polymorphic microsatellite loci in the common frog, *Rana temporaria*

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Received 3 May 2000; revision accepted 29 June 2000

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The common frog, *Rana temporaria*, is the most widespread amphibian species in Europe, and in many areas of northern and central Europe it is also the most common anuran species (Gasc *et al*. 1997). Consequently, it is a frequently studied organism used in ecological and evolutionary investigations (Beebee 1995; Miaud *et al*. 1999), as well as in conservation genetics studies (Reh & Seitz 1990; Hitchings & Beebee 1997; Seppä & Laurila 1999). However, the lack of polymorphic microsatellite markers developed for the common frog has restricted its usage as a model organism in evolutionary studies, which generally require highly variable loci for estimation of relatedness among individuals (e.g. Lynch & Ritland 1999). With this in mind, we describe here the first microsatellite markers for the common frog.

Genomic DNA was digested with *Bam*HI and fragments between 400 and 1200 bp were purified from agarose gels using a QiaEx II gel extraction kit (QiaGen). Subsequently, DNA fragments were ligated to pUC18 plasmid vector and electroporated into *Escherichia coli* DH5α cells. Transformed cells were plated and white colonies were transferred to new plates. Lifts were screened with ^{32}P-labelled $(CA)_{15}$ (12 000 colonies) and $(CT)_{15}$ (8000 colonies) probes, plus a cocktail including $(AAAT)_7$, $(GATA)_7$, $(GAAT)_7$ and $(AAAG)_7$, and one including $(GGAA)_7$, $(GGAT)_7$, $(GACA)_7$ and $(AAAC)_7$ oligonucleotide probes (4000 colonies), according to Sambrook *et al*. (1989). Another genomic library was also constructed using *Hin*dIII-digested DNA, ligated to pUC19 vector. Approximately 4000 colonies were screened with a DIG-labelled $(CA)_{15}$ probe according to instructions in the manufacturer's manual (Roche Molecular Biochemicals). Positive clones were sequenced with Big Dye terminator chemistry using M13 forward and reverse primers, and sequences were recorded on an ABI Prism 377 sequencer (Perkin-Elmer). Primers were designed using Oligo (Primer Analysis Software, MBI).

DNA was prepared from muscle tissue by proteinase K digestion and two phenol/chloroform and one chloroform extraction, followed by ethanol precipitation and resuspension in 20 μL of water. The basic thermal cycling on a PTC-100 (MJ Research Inc.) comprised 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at the selected annealing temperature, 45 s at 72 °C, and finally 7 min at 72 °C. Two types of 'touch-down' programs were also applied. One set of primers (RtSB3) started with an annealing temperature of 60 °C which decreased by 0.5 °C per cycle for 10 cycles followed by 20 more cycles at 55 °C. The second set (RtSB14) started at 65 °C and then decreased by 1 °C per cycle for 10 cycles followed by 25 cycles at 55 °C.

Table 1 Characteristics of *Rana temporaria* microsatellites. Polymorphism was estimated from the genotypes of 19–44 individuals from one population

Locus	Repeat motif	Primers (5′ → 3′)	Number of alleles	*n*†	Allele size (bp)	H_E	H_O	T_a (°C)	GenBank accession no.
RtU4	$(GT)_{23}(T)_{13}$	F: GGCTTCAAAGTAGAATAAAG R: AATCTTTTCCTTACTGTAGC	10	31	75–108	0.71	0.39	50	AF257481
RtU7	$(GATA)_{37}$	F: GCATTATTACAGCATTCTGGAT R: TTAATGGCTGGATAGATTTACC	22	46	152–295	0.89	0.91	50	AF257482
RtSB3	$(GT)_{14}(CT)(GT)_2$	F: GAGATCCATGTGTATTTATCG R: CCACTGCGATTCTGACCTGTC	7	44	172–205	0.75	0.91	60–55*	AF257479
RtSB14	$(GT)_{12}(GTT)_{16}$	F: TGTGTCCAGCAATGAATGTTA R: GCAGAGTTACAGCCAAGGAA	4	19	174–191	0.71	0.63	65–55*	AF257478
RtSB80	$(TA)_{13}$	F: ACAGCTATATCCGACCAC R: GGAGGACATAAGTTCAATAA	9	45	199–250	0.75	0.78	58°	AF257480

†Number of individuals genotyped; T_a, annealing temperature; *Initial annealing temperature for RtSB3 and RtSB14).

Amplifications were performed in 20 μL reaction volumes using 50 ng of genomic DNA (35 ng for RtSB30). Reaction mixtures contained 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 10 pmol of each primer, 50 μM of each dNTP (RtSB14 75 μM), 1.0 U of AmpliTaq DNA Polymerase (Perkin-Elmer) and 10 μg BSA (Pharmacia Biotech). For two sets of primers (RtSB3 and RtSB80), 1.0 U of AmpliTaq Gold DNA Polymerase (Perkin-Elmer) was used together with 2.5 mM $MgCl_2$, 50 mM KCl and 5 mM Tris–HCl (pH 8.0). Products were run on an ABI Prism 377 instrument (Perkin-Elmer) with fluorescent-labelled primers.

Of the 70 positive clones (out of 16 000 totally screened), we developed primers for 20 loci, 13 of which were subsequently discarded because of non-specific amplification. Two further loci turned out to be monomorphic. The five remaining primer pairs amplified five polymorphic microsatellite loci (Table 1). The number of alleles per locus ranged from 4 to 22, and the observed heterozygoties ranged from 0.34 to 0.91, suggesting a high degree of intra-population variation in isolated loci (Table 1). For RtU4, the observed heterozygosity was significantly lower than expected ($\chi^2 = 4.55$, $P < 0.05$), which may reflect the presence of null alleles. For the other loci, we found no significant deviations between observed and expected heterozygosities ($\chi^2 \leq 1.48$, $P > 0.05$). We also tested the primers for cross-amplification in a related species, the moor frog (*R. arvalis*), and found that all loci markers also amplified products in this species. The microsatellite loci identified in the study exhibit much more intra-population variation than has been detected using allozymes in *R. temporaria* (Hitchings & Beebee 1997; Laurila & Seppä 1998; Seppä & Laurila 1999), and should prove useful in further genetic and evolutionary investigations of this common model organism.

Acknowledgements

We thank Jesper Brohede for help and advice with the laboratory work, and Pierre-André Crochet and Anssi Laurila for help and advice in the course of the work. Our research was supported by the Swedish Natural Science Research Council and the Maj and Tor Nessling Foundation.

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Locus-specific microsatellite markers for the fungal chickpea pathogen *Didymella rabiei* (anamorph) *Ascochyta rabiei*

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Keywords: *Ascochyta blight*, chickpea, microsatellites, plant pathogenic fungi

Received 31 May 2000; revision accepted 29 June 2000

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The genetic basis of the interaction between the fungal pathogen *Ascochyta rabiei* and its main host chickpea (*Cicer arietinum* L.) has not been satisfactorily clarified. Pathogen populations show considerable variation in aggressiveness, but 'races' or 'pathotypes' are poorly defined (Udupa *et al*. 1998). The design of appropriate disease control strategies would benefit from studies addressing (1) the genetic definition of pathotypes, (2) analysis of the population structure, gene flow, and the epidemiology of the pathogen, and (3) the characterization of fungal pathogenicity genes.

Suitable genetic markers are prerequisites for such studies. Among the marker types applied to *A. rabiei*, only little variation was revealed by random amplified polymorphic DNAs (RAPDs, Udupa *et al*. 1998). Higher sensitivities were achieved by hybridization fingerprinting (Geistlinger *et al*. 1997), but this procedure is time-consuming and requires microgram amounts of DNA (Weising & Kahl 1997). Microsatellites constitute a locus-specific, polymerase chain reaction (PCR)-based marker system that has become an important tool for genome analysis in many organisms including plant-pathogenic fungi (e.g. Neu *et al*. 1999; Tenzer *et al*. 1999). Here we report the development and characterization of 20 polymorphic microsatellite markers for *Ascochyta rabiei*.

Fungal DNA was isolated according to Geistlinger *et al*. (1997). To avoid sampling bias, four separate genomic libraries were constructed from the US-American *A. rabiei* isolate ATCC76501 using the restriction enzymes *Alu*I, *Rsa*I, *Hae*I and *Sau*IIIA, respectively. After digestion, DNA aliquots

were electrophoresed on agarose gels, fragments of 300–750 bp were excised, purified on glassmilk (Bio101) and ligated into the *Eco*RV or *Bam*HI site of a dephosphorylated pBluescript IISK phagemid (Stratagene). Ligation products were electroporated into competent *Escherichia coli* cells following standard procedures. Bacterial clones were plated, and screened by colony hybridization with pools of ^{32}P-labelled oligonucleotides ('dinucleotide pool': $(CA)_{10}$ plus $(GA)_{10}$; 'trinucleotide pool': $(CAA)_{10}$ plus $(GAA)_{10}$). End-labelling of probes, (pre)hybridization and stringent washing steps were performed as described by Weising & Kahl (1997). Positive colonies were verified by a second round of hybridization. Plasmids were isolated by standard procedures (Sambrook *et al*. 1989) and sequenced by the chain termination method using the BigDye kit (PE Biosystems). Sequencing products were analysed on an ABI PRISM 373 automated sequencer (PE Biosystems). Of 50 clones sequenced, 47 contained a repeat.

Table 1 Primer sequences and characteristics of *Ascochyta rabiei* microsatellites

Locus	Repeat of cloned allele	Primer sequence (5′ → 3′)	Expected size (bp)	*n*	H_E	Product(s) in *A. fabae*	Accession no.
*Ar*A02T	$GAA(N)_9(GAA)_8$	GATCACATGCAACTAGGGTATC ATGCAGACGTAGAAGTCCATAC	152	2	0.30	(+)	AJ246947
*Ar*A03T	$(GAA)_{31}$	TAGGTGGCTAAATCTGTAGG CAGCAATGGCAACGAGCACG	379	11	0.84	(+)	AJ246948
*Ar*A06T	$(CAACAC)_7(N)_9(CAC)_3$	CTCGAAACACATTCCTGTGC GGTAGAAACGACGAATAGGG	162	3	0.43	(+)	AJ246951
*Ar*A08T	$(CTTCCT)_4(CTT)_6$	CAGAGGGGAATTGTTGTTC ACGACGAGGATGAGGACTTC	249	3	0.24	(–)	AJ246952
*Ar*A11T	$(GAA)_8$	GATGCGAATCTGTTCTAGGAGT GGTGTCGAGGTTGTTACAATG	141	3	0.24	(+)	AJ246954
*Ar*R08T	$(GAA)_{13}$	GTGAGCTACTTAGCACCTCTGT GCTGTGTCGGGTTGAGTAAC	166	3	0.46	(+)	AJ246961
*Ar*R10T	$(CAGCAA)_2(CAA)_8$	GAAGGCTTAAGAGAACATCTCC GCTTGGCTCATGTAATGCTG	170	3	0.58	(–)	AJ246962
*Ar*H02T	$(GAA)_{58}(GTA)_6$	CTGTATAGCGTTACTGTGTG TCCATCCGTCTTGACATCCG	365	5	0.50	(+)	AJ246964
*Ar*H04T	$(CTT)_{10}$	CACCTTGCGATGCAATACAC CCATTCAACGCACATAGCAG	208	4	0.56	(–)	AJ246965
*Ar*H05T	$(CTT)_{18}$	CATTGTGGCATCTGACATCAC TGGATGGGAGGTTTTTGGTA	197	14	0.90	(+)	AJ246966
*Ar*H06T	$(CAA)_9(CAG)_7(CAA)_{21}$	CTGTCACAGTAACGACAACG ATTCCAGAGAGCCTTGATTG	167	9	0.86	(+)	AJ246967
*Ar*S03T	$(GTT)_5(GCT)_3(GTT)_5$	ATGGAGAAGTCGAGGTCCAT CTCTTGCGTGGCCTAGAAGG	152	3	0.31	(+)	AJ246970
*Ar*R01D	$(GTGTGTGG)_6$	CAGAGGGGAGTCACAAGTATC GAGTTACAGCTGCAAGACATTC	194	3	0.65	(–)	AJ246971
*Ar*R04D	$(GTGTGTAT)_2(N)_8(GT)_{10}$	GCTTAGTTGGGCTTGTTACTT CACACCTCTCTACCAATGAGAC	160	3	0.54	(+)	AJ246972
*Ar*R12D	$(CA)_{15}$	ATACACCCAAACCGGGTATC GTATGGAATGTGCGATAGGA	158	5	0.45	(–)	AJ246975
*Ar*A02D	$(CACACAA)_5$	CTATCACCATGCCTCCATCA TGTTCCCTTTGAGTTGAAGAG	150	3	0.24	(–)	AJ246976
*Ar*H02D	$(CA)_4N(CA)_5(N)_2(CA)_4$	AGAAAGGGGAGATTCGAGAC AGGTCAGCACGAGATAGCAC	141	3	0.17	(+)	AJ246977
*Ar*H07D	$(GT)_{23}$	GAGATCCGTGTGAAGCATGA CCATGTGGACAGATTACATTCC	184	6	0.74	(–)	AJ246979
*Ar*H08D	$(CA)_3(CT)_8(CA)_8(CT)_{10}$	ACTTTGACTTCGACTTCGACT GTGGAAGAGAAGTGGATTGAC	177	4	0.25	(+)	AJ246980
*Ar*H11D	$(GACA)_6$	GACTCTCTCAGAGTGGGACAC CTTGTGATCGTTTCCTAAACTC	152	2	0.17	(–)	AJ246982

Loci are abbreviated as follows: species name (*Ar*, *Ascochyta rabiei*); restriction enzyme used for library generation (A, *Alu*I; R, *Rsa*I; H, *Hae*II; S, *Sau*IIIA), clone number, hybridization cocktail (T, trinucleotide pool; D, dinucleotide pool). Expected allele sizes were derived from the sequenced clone. Annealing temperatures were 53 °C throughout. *n*, observed number of alleles among 22 *A. rabiei* isolates of a worldwide collection. H_E, genetic diversity (Nei 1987; Tenzer *et al*. 1999). Transferability of primer pairs to *A. fabae* is indicated by (+).

Using the Primer 3 computer program (Rozen & Skaletsky 1997), primers could be designed to microsatellite-flanking regions of 37 clones. PCR was performed in 25 μL volumes containing 1.6 mM $MgCl_2$, 0.2 mM dCTP, dGTP and dTTP, 0.02 mM dATP, 0.06 μL [α^{32}P]dATP, 75 mM Tris–HCl (pH 9), 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 5 pmol of each primer, 0.5 units *Taq* DNA polymerase (Eurogentec) and 10 ng of template DNA. After initial denaturation (95 °C, 20 s), PCR was run for 35 cycles (94 °C for 20 s, 53 °C for 25 s, 67 °C for 23 s) in a Perkin-Elmer 2400 thermocycler. Products were separated on 6% sequencing gels and autoradiographed (Sambrook *et al*. 1989).

Primer functionality was initially tested on three *A. rabiei* isolates, and one isolate of *A. fabae*. Using an annealing temperature of 53 °C, single bands of the expected size were obtained for 26 loci. Twenty-four primer pairs also amplified one or more products from *A. fabae*. Whether these fragments contain microsatellites and/or are polymorphic within *A. fabae* remains to be examined. Twenty marker loci were selected to genotype 22 *A. rabiei* isolates from the USA, Pakistan, Syria, Turkey and Tunisia. Each of these constituted a unique clonal lineage as defined by its fingerprint haplotype (Geistlinger *et al*. 1997). The Popgene computer package (Yeh & Boyle 1997) was used to calculate allele frequencies and to determine Nei's expected genetic diversity valves for haploid organisms (Nei 1987; Tenzer *et al*. 1999; Table 1). All loci were polymorphic, with 2–14 alleles and genetic diversity values of 0.17–0.90.

With up to 14 alleles among 22 isolates, these microsatellites are more variable than other types of molecular markers applied to *A. rabiei*. Locus-specific primers not only ensure a high reproducibility of results, but also allow the analysis of mixed samples. Groppe & Boller (1997) have specifically amplified a microsatellite locus from the fungal endophyte *Epichloe* in the presence of contaminating host DNA. Following a similar strategy, microsatellite markers could be used to type *A. rabiei* isolates directly from individual lesions. The distribution of *A. rabiei* pathotypes in the field could then be monitored more efficiently, avoiding the lengthy procedures of single-sporing and culturing.

Acknowledgements

We acknowledge the support from the Gesellschaft für Technische Zusammenarbeit (GTZ) (grant 89.7860.3-01.130).

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Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbird families

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Received 31 March 2000; revision accepted 2 July 2000

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The blue tit, *Parus caeruleus*, is a hole-nesting woodland bird that has been the focus of numerous ecological and behavioural studies. Nine polymorphic blue tit minisatellite loci have previously been cloned in our laboratory (Verheyen *et al*. 1994). We have now cloned a series of polymorphic microsatellite loci in this species and also tested their utility in a wide range of other oscine passerine birds.

DNA was extracted according to the method described by Bruford *et al*. (1998). A genomic library was prepared, enriched for $(CA)_n$, $(GA)_n$ and $(AT)_n$, as described by Armour *et al*. (1994) using modifications suggested by Gibbs *et al*. (1997). Details of the eight characterized loci are provided in Table 1.

Each 15-μL PCR reaction contained 20–50 ng of DNA, 0.5 μM of each primer, 2.5 mM $MgCl_2$ (except *Pca3* and *Pca8* which required 3.0 mM $MgCl_2$), 0.1 mM of each dNTP and 0.25 units of *Taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) in the PCR buffer (final concentration: 20 mM $(NH_4)_2SO_4$, 75 mM Tris–HCl pH 8.8, 0.15 mg/mL DNAase-free

Table 1 Characterization of eight blue tit microsatellite loci

Locus	EMBL accession no.	Repeat motif*	Primer sequence (5′ → 3′)	Predicted annealing temperature (°C)	Expected length of PCR product (bp)*	Number of alleles†	H_E†	H_O†
Pca1	AJ279803	$(CAG)_8$	GAT CGC TGT GCT CCT TGT CAG CTG GCC CAT TTT GCT GTG C	63	125	1	0.00	0.00
Pca2	AJ279804	$(CCAT)_{12}$	GTT GGC CTT CTT GGC CCC TGT TGG AGG TTA GGA GGC CTC T	64	291	6	0.85	0.63
Pca3	AJ279805	$(GT)_6CT(GT)_3CT(GT)_5CT(GT)_3CT(GT)_{13}$	GGT GTT TGT GAG CCG GGG TGT TAC AAC CAA AGC GGT CAT TTG	65	191	7	0.88	0.75
Pca4	AJ279806	$(AC)_{11}AT(AC)_{10}$	AAT GTC TTA CAG GCA AAG TCC CCA AAC TTG AAG CTT CTG GCC TGA ATG	65	191	7	0.88	1.00
Pca5	AJ279807	$(CA)_{17}$	TTG GCT GGG AGC AGA GCT G CCA GCC TGT CCT CAG CAG C	65	132	4	0.75	0.75
Pca7	AJ279809	$(TG)_{24}$	TGA GCA TCG TAG CCC AGC AG GGT TCA GGA CAC CTG CAC AAT G	57‡	127	6	0.78	0.63
Pca8	AJ279810	$(GATA)_{16}(TG)_{25}$	ACT TCT GAA ACA AAG ATG AAA TCA TGC CAT CAG TGT CAA ACC TG	57	295	14	0.98	0.88
Pca9	AJ279811	$(GT)_{21}$	ACC CAC TGT CCA GAG CAG GG AGG ACT GCA GCA GTT TGT GGG	65	131	8	0.90	1.00

*In cloned allele; †in eight unrelated individuals from Belgium; H_E, expected heterozygosity; H_O, observed heterozygosity; ‡65 °C produced a cleaner product.

Table 2 Cross-utility of eight blue tit primer pairs in 28 other passerine species

Family	Species	Number of alleles / number of individuals tested							
		Pca1	*Pca2*	*Pca3*	*Pca4*	*Pca5*	*Pca7*	*Pca8*	*Pca9*
Climacteridae	Brown treecreeper, *Climacteris picumnus*	1/1	1/6	0	0	1/1	1/1	—	0
Maluridae	Superb fairy-wren, *Malurus cyaneus*	1/1	0	1/1	1/1	1/1	1/1	—	1/1
Meliphagidae	Bell miner, *Manorina melanophrys*	1/1	3/6	0	0	1/1	1/1	—	0
Pardalotidae	White-browed scrub wren, *Sericornis frontalis*	0	0	1/1	0	1/1	1/1	—	1/1
Pomatostomidae	White-browed babbler, *Pomatostomus superciliosus*	1/1	0	0	0	0	1/1	—	1/1
Laniidae	Loggerhead shrike, *Lanius ludovicianus*	1/1	0	0	1/6	1/6	2/6	—	1/6
Corvidae	Black-billed magpie, *Pica pica*	1/1	1/1	0	2/6	1/6	1/1	0	0
Corvidae	Azure-winged magpie, *Cyanopica cyana*	1/1	1/10	—	—	11/28	1/10	—	—
Corvidae	Madagascar paradise-flycatcher, *Terpsiphone mutata*	—	—	1/1	—	—	1/1	—	—
Cinclidae	White-throated dipper, *Cinclus cinclus*	0	2/6	—	2/6	1/6	1/1	—	2/6
Muscicapidae	Eastern bluebird, *Sialia sialis*	1/1	1/1	0	0	0	1/6	1/1	1/1
Muscicapidae	European pied flycatcher, *Ficedula hypoleuca*	—	0	0	—	—	2/6	0	—
Muscicapidae	Bluethroat, *Luscinia svecica*	0	4/6	0	2/6	1/1	1/6	—	3/6
Sturnidae	Common starling, *Sturnus vulgaris*	1/1	1/6	1/6	0	0	2/6	0	1/1
Paridae	Great tit, *Parus major*	2/8	1/8	1/8	2/6	1/1	3/6	12/24	3/6
Aegithalidae	Long-tailed tit, *Aegithalos caudatus*	0	1/15	7/700	10/700	1/15	2/15	1/15	1/15
Hirundinidae	Sand martin, *Riparia riparia*	0	1/1	0	6/6	0	1/6	—	1/1
Pycnonotidae	White-spectacled bulbul, *Pycnonotus xanthopygos*	0	1/6	0	0	1/6	1/6	0	1/1
Zosteropidae	Silver-eye, *Zosterops lateralis*	0	1/1	0	1/1	1/1	1/1	—	—
Sylviidae	Seychelles warbler, *Acrocephalus sechellensis*	0	1/6	0	2/6	1/6	1/1	1/1	1/1
Sylviidae	Sedge warbler, *Acrocephalus schoenobaenus*	0	1/12	17/400	7/40	10/400	1/12	0	1/1
Sylviidae	Willow warbler, *Phylloscopus trochilus*	1/7	1/7	40/300	—	—	—	7/7	—
Alaudidae	Lesser short-toed lark, *Calandrella rufescens*	0	1/1	0	1/1	1/1	1/1	0	—
Nectariniidae	Palestine sunbird, *Nectarinia osea*	—	1/1	0	2/6	1/6	1/1	—	1/1
Passeridae	House sparrow, *Passer domesticus*	1/1	1/6	0	0	0	1/1	0	1/1
Passeridae	Sociable weaver, *Philetarius socius*	—	—	1/1	—	—	1/1	—	1/1
Passeridae	Red-billed buffalo-weaver, *Bubalornis niger*	—	—	1/1	—	—	1/1	—	1/1
Fringillidae	European greenfinch, *Carduelis chloris*	—	0	0	1/6	0	1/1	—	1/1
Number of species tested for amplification		22	25	26	22	23	27	12	22
Percentage amplifying a PCR product in at least one test		55	76	38	64	74	100	42	86
Number of species tested for variability using six unrelated individuals		2	12	5	11	9	10	3	5
Percentage with ≥3 alleles using six unrelated individuals*		0	17	60	27	22	10	67	20
Percentage with ≥5 alleles using six unrelated individuals*		0	0	60	27	22	0	67	0

0, no PCR product; —, not tested. *Based on first six analysed.

BSA, 10 mM β-mercaptoethanol). PCR amplification was performed in a Hybaid Touchdown™ thermal cycler. The reaction profile was 95 °C for 3 min, then 94 °C for 30 s, *X* °C for 45 s, 72 °C for 45 s for two cycles each at *X* = 60 °C, 57 °C, 54 °C and 51 °C, then 25 cycles at *X* = 48 °C, followed by 72 °C for 5 min (except for *Pca*7 and *Pca*9 which had two cycles each at *X* = 67 °C, 64 °C, 61 °C and 58 °C, then 25 cycles at *X* = 55 °C).

Seven loci displayed four or more alleles in our test panel of eight unrelated blue tit individuals (Table 1), and many more alleles have been found for several loci used in a larger sample of individuals (D. I. Leech and I. R. Hartley, personal communication).

The primers were tested in 28 other passerine species (Table 2) representing 20 passerine families (following Sibley & Monroe 1990). One high-quality DNA sample from each species was tested for amplification, with the products visualized on agarose gels stained with ethidium bromide. When testing for polymorphism, the PCR products were run on 6% polyacrylamide gels and visualized with silver staining (Promega) or by autoradiography after PCR with one of the primers end-labelled with [γ^{33}P]-dATP (Sambrook *et al*. 1989).

Each primer pair amplified a product in 38–100% of the passerine species (Table 2). *Pca8* was highly polymorphic (five or more alleles) in 67% of the species tested (Table 2).

The proportion of tested species that displayed polymorphism was inversely correlated with the proportion that amplified successfully (taking those six loci where at least five species were tested for polymorphism, Fig. 1). This result is consistent with a hypothesis that the mutation rate varies among loci but that the probability of a strand-slippage mutation in a microsatellite array (so producing polymorphism) is correlated with the probability of a point mutation in the adjacent flanking DNA (tending to lead to non-amplification). This hypothesis is consistent with observations that mutation rates vary in the genome (Wolfe *et al*. 1989) and that the DNA flanking microsatellites is apparently neutral (Brohede & Ellegren 1999).

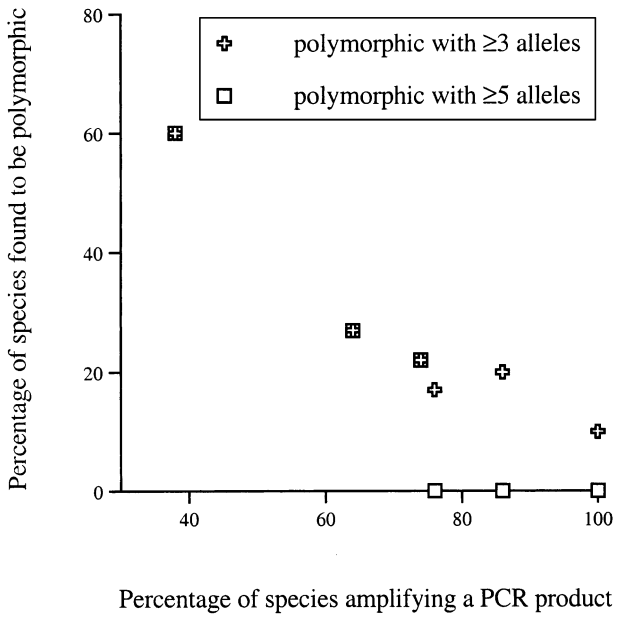

Fig. 1 The relationship between polymorphism and amplification for six blue tit loci. Spearman rank correlation corrected for ties: ≥3 alleles, $r_s = -0.943$, $P < 0.05$; ≥5 alleles, $r_s = -0.941$, $P < 0.05$.

At least four primers detect highly polymorphic loci in other passerine species (Table 2). Although few passerine species have been tested with *Pca3* and *Pca8*, ≥60% were highly polymorphic. *Pca4* and *Pca5* were tested for polymorphism in more species and were highly polymorphic in >20% of those tested.

Acknowledgements

We thank M. Carter for technical assistance and N. Chaline, R. Marshall, J.G. Martinez, D.J. Ross and M. Wilson for contributing data on the cross-utility of primers. D. Bryant, M. Clarke, S. Degnan, E. Doerr, M.C. Double, P.A. Gowaty, I.R. Hartley, B. Hatchwell, J. Komdeur, R. Marsden, J.G. Martinez, R.A. Mulder, N. Mundy, P. Taberlet, G. Verheyen, J. Wetton, M. Winterbottom, J. Wright, S. Yezerinac and R. Zilberman kindly supplied blood and DNA samples. This work was supported by the European Commission and the Natural Environment Research Council.

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Microsatellite markers for the European tree frog *Hyla arborea*

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Received 8 June 2000; revision received 5 July 2000; accepted 13 July 2000

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The rapid decline of frog populations all over the world has raised much concern (Houlahan *et al.* 2000). Fragmentation and loss of habitat are thought to be important causes for this rapid decline. The slow-moving frog species, conservative in their choice of breeding grounds, are probably more sensitive to habitat fragmentation compared to mammals and birds. Genetic studies can provide valuable information on the effects of habitat loss and fragmentation on frog population dynamics and long-term survival. Microsatellites are suitable markers for this type of study, as they are highly polymorphic, co-dominant, easy to use and very amenable to automation. Handled with care, they can be used to answer a variety of ecological and population genetic questions. Here we report on the development of a set of microsatellite primers for the European tree frog (*Hyla arborea*), a species protected under the European Union habitat directive. These markers will be used in an ongoing study to quantify the effects of habitat fragmentation on genetic diversity and exchange between populations of tree frogs in the Netherlands.

The procedure for microsatellite enrichment by selective hybridization was modified from Karagyozov *et al.* (1993) by Van de Wiel *et al.* (1999) and Van der Schoot *et al.* (2000). DNA was extracted from a single tadpole; digested with *Rsa*I, and size-fractionated by agarose gel electrophoresis. DNA fragments between 350 and 1000 bp were recovered by electro-elution, enriched by hybridization to three pools of oligonucleotides (pool 1 GA/GT; pool 2 TCT/GTG/GAG/CGT/TGA/GCT/AGT/TGT; pool 3 TGTT/GTAT/GATA) and subsequently ligated in pBluescript SK+ (Stratagene) and

Table 1 Characterization of *Hyla arborea* microsatellite markers and their products

Microsatellite locus	Repeat*	Predicted product length (bp)	Primer sequences (forward, reverse)	PCR conditions†	Number of alleles (bp)	Allelic range	H_O/H_E	EMBL number
WHA1–9§	$(CA)_{20}$	144	5′-CGTTTGGACGTGATGCTG-3′ 5′-GAGGAGTTTCTTCACAAGGGG-3′	60NP	5	126–136	0.75/0.76	AJ403985
WHA1–20§	$(GT)_{18}$	194	5′-GTCCCTTCCTGAATAAGTGTCG-3′ 5′-CCATTCCCTCCTGGCTTT-3′	55NP	3	189–193	0.67/0.57	AJ403986
WHA1–25§	$(GT)_{20}$	109	5′-AAGAATCTGCCGCAAAGAAG-3′ 5′-TAGGAAGGGACAGGAGGTCA -3′	55NP	4	111–117	0.83/0.74	AJ403987
WHA1–54	$(CA)_{19}$	144	5′-CCTGGTCATGCTACACGCTA-3′ 5′-GACAACAACCCCCAATAATCC -3′	60NP	2‡	129–145	0.00/0.21	AJ403988
WHA1–60	$(GT)_{22}$	165	5′-TAGGTCATGTATAGCCTGTT-3′ 5′-TCTGTTTACTTCAGGGGT-3′	55LP	7	153–169	0.67/0.85	AJ403989
WHA1–61	$(TG)_7$ ... $(AT)_6$	149	5′-CAGGTCCAAGCTCTCTCCC-3′ 5′-GCACATTCACATTATATAGACAACACA-3′	60LP	2‡	142–150	0.08/0.16	AJ403990
WHA1–67§	$(CA)_{21}$	214	5′-GCTTTACACATGGGGGTAT-3′ 5′-CACTCCTTTTAGAGTATGTTGTTG-3′	55LP	6	202–226	0.50/0.78	AJ403991
WHA1–103§	$(GT)_{21}$	234	5′-CAAAGTGACAATGTGGGGTCTCAT-3′ 5′-ATAGCATCAAATCCAGCCGTAGG-3′	60NP	4	230–244	1.00/0.80	AJ403992
WHA1–104	$(GT)_{22}$	291	5′-ACTTGGGACAGCCAGTATGTTTT-3′ 5′-TGAGCTGGTGGGTATAACCTAAC-3′	60NP	5	264–292	0.50/0.74	AJ403993
WHA1–133	$(CA)_{24-1}$	185	5′-ATGCCCCTCATAGAACACATACAA-3′ 5′-GGGCTGCGGGTACAGTAGTG -3′	55LP	2‡	145–157	0.08/0.08	AJ403994
WHA1–140§	$(GT)_{25}$	123	5′-ATGTGCCATAGAAATGAAGG-3′ 5′-AGGCTTGCTGCTATTATGTC -3′	55NP	6	112–132	0.67/0.76	AJ403995
WHA5–22A	$(CAA)_6$ ... $(CAG)_6$	244	5′-TTACAGCAACAGCAAATGG-3′ 5′-ATCAGGGACTGGGTCTGT-3′	50NP	2	235–241	0.42/0.34	AJ403996
WHA5–29	$(ACC)_{74-20}$	332	5′-TTCATCCATTCTCATCTCTTCTCA-3′ 5′-ACATGGGGCCCTTCTACC-3′	60LP	2‡	330–336	0.00/0.16	AJ403997
WHA5–57	$(GGT)_6$	260	5′-TTGTCCTGACATGCACACCT -3′ 5′-CGTGTCTAACCCCAGCTCAT-3′	60LP	2	262–332	0.00/0.29	AJ403998
WHA5–201	$(CAC)_{13-1}$ ... $(CAC)_{8-1}$	248	5′-TCATGGACTGTCGTCATGGT-3′ 5′-AGGTAAATGGAATCTGGGTGTG-3′	60LP	4	238–247	0.42/0.56	AJ403999

*Stretches shorter than five repeats have not been taken into account; '—' denotes mismatch. †50, 55 or 60: annealing temperature; NP (normal programme): 3 min at 94 °C, 35 cycles (15 s at 94 °C, 45 s at the annealing temperature, 60 s at 72 °C), then 20 min at 72 °C or LP (long programme): 3 min at 94 °C, 35 cycles (45 s at 94 °C, 45 s at the annealing temperature, 105 s at 72 °C), then 20 min at 72 °C, using a Hybaid Touchdown cycler. ‡Only polymorphic between Dutch and Croatian samples. §Label on forward primer is recommended to simplify patterns and thus scorability.

transformed to *E. coli* DH5α (Life Technologies). Colonies were transferred onto Hybond N+ membranes and screened by hybridization to a mixture of the appropriate oligonucleotides. Two hundred positive clones (25% of the total number) were obtained, of which 130 were sequenced using a Taq DyeDeoxy Terminator Cycle Sequencing Kit and an ABI 377 sequencer (PE Biosystems). About 30% of the sequences contained long compound microsatellites interspersed with stretches of non-repeat DNA forming mini-satellite types of arrays. Similar results were also obtained for *Rana arvalis* microsatellites (Arens *et al*., unpublished data); this contrasts to other animal species (*Chorthippus parallelus* and *Metrioptera brachyptera*) and plant species (*Populus nigra*, *Ilex aquifolium* and *Maianthemum bifolium*) where microsatellites were isolated in a similar way (unpublished data). In particular, (GAT) motifs were found in these kinds of arrays, forming 16% of the trinucleotide-enriched clones (for typical examples, see EMBL accession numbers AJ400838 and AJ400839). Primers were designed using the software package Primer 0.5 (Whitehead Institute for Biomedical Research). In total, 37 primer pairs were tested on a set of 12 randomly chosen individuals from different populations from the east of the Netherlands and one individual from Croatia. Initial amplification was performed in 20 μL containing 75 mM Tris–HCl pH 9.0,1.5 mM $MgCl_2$, 20 mM $(NH4)_2SO_4$, 0.01% (w/v) Tween-20, 100 μM of each dNTP, 200 nM of each primer, 16 ng template DNA and 0.1 unit Goldstar DNA polymerase (Eurogentec). Raising the $MgCl_2$ concentration to 1.75 mM was necessary to prevent frequent sample drop-out for most microsatellite loci. Products were separated on a 6% denaturing acrylamide gel. After visualization with silver staining (Silver Sequence, Promega), the patterns were analysed for the presence of polymorphisms and the quality of the banding pattern according to Smulders *et al*. (1997). Only patterns of quality 1 (weak or no stutter bands, well-scorable) or quality 2 ('stutter' bands present, but product still scorable) are amenable for use in population studies. In total, 15 *H. arborea* microsatellite loci were found to produce unambiguously scorable products (Table 1). Only 11 loci were found to be polymorphic among the 12 Dutch samples. Animals from the test set belong to Dutch populations that may have gone through a bottleneck, as low numbers of animals within these populations were counted some 30–40 years ago. Higher numbers of alleles per locus might be expected in populations elsewhere. Since inter-species amplification of microsatellites within *Hyla* and *Rana* species is very limited (Call & Hallett 1998; our personal observations), development of microsatellite markers for individual species is necessary. This set of microsatellite loci will provide valuable markers for population genetic and ecological studies in the European tree frog.

Acknowledgements

We would like to thank Eduard Kletečki (Croatian Natural History Museum, Zagreb) for providing the Croatian sample. The Ministry of Agriculture, Nature Management and Fisheries funded this work through a Wageningen University and Research Centre expertise development program.

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Isolation and characterization of microsatellite loci in smallmouth bass, *Micropterus dolomieu* (Teleostei: Centrarchidae), and cross-species amplification in spotted bass, *M. punctulatus*

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Keywords: microsatellites, *Micropterus dolomieu*, PCR, primers, smallmouth bass

Received 23 May 2000; revision accepted 7 July 2000

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Members of the genus *Micropterus* are endemic to eastern and central North America. They have high economic importance in these areas as game fish, which has resulted in extensive introduction of species and within-species stocks outside their native ranges. Allozyme data indicated that most of the genetic diversity within smallmouth bass, *Micropterus dolomieu*, is centred in the Interior Highlands region of North America and that populations in the remainder of the native range of the species, northward into Canada and eastward into the Eastern Highlands, contributed little to the genetic diversity of the species (Stark & Echelle 1998). Recent introductions of non-native *M. dolomieu* into reservoirs in the Interior Highlands region have created the need for multiple intraspecific markers to assess effects of introductions on native populations. We report here the isolation and characterization of 11 polymorphic microsatellite loci in *M. dolomieu* and the successful

Table 1 Microsatellite primer sequences, size of cloned allele, and other characteristics of 11 polymorphic microsatellite loci in collections of *Micropterus dolomieu* from Broken Bow reservoir. Accession numbers (GenBank) refer to clone sequences from which the primers were designed

Locus	Primer Sequence (5′–3′)	Size (bp)	Core Repeat	*n*	No. of alleles	H_O	H_E	Accession no.
Mdo 1	GCTCTTCCCAGTGGTGAGTC* ATCTCAGCCCATACCGTCAC	210	$(GT)_{14}$	117	6	0.811	0.744	AF294489
Mdo 2	GCCCTTTCATATTGGGACAA* CTGCTCTGGCGTACATTTCA	197	$(GT)_{14}$	157	6	0.643	0.673	AF294490
Mdo 3	AGGTGCTTTGCGCTACAAGT* CTGCATGGCTGTTATGTTGG	135	$(CA)_{20}$	137	6	0.562	0.571	AF294491
Mdo 4	TCTGAACAACTGCATTTAGACTG* CTAATCCCAGGGCAAGACTG	142	$(CA)_{11}$	18	3	0.611	0.656	AF294492
Mdo 5	CAGGTTCCCTCTCACCTTCA* ATGGTCTCACCAGGGACAAA	200	$(CT)_9CC(CA)_{10}GA(CA)_3TA(CA)_2$	19	2	0.105	0.102	AF294493
Mdo 6	TGAAATGTACGCCAGAGCAG* TGTGTGGGTGTTTATGTGGG	150	$(CA)_7(TA)_4$	18	3	0.611	0.541	AF294494
Mdo 7	TCAAACGCACCTTCACTGAC* GTCACTCCCATCATGCTCCT	172	$(CA)_{12}$	19	2	0.105	0.102	AF294495
Mdo 8	GTGAGGACCAGCCAAAATGT* GGAAGATTGAGGTCCCAACA	220	$(CA)_{19}$	18	8	0.722	0.771	AF294496
Mdo 9	TTTGATGGGCGTTTTGTGTA* GACCGGTCCTGCATATGATT	126	$(GT)_{10}$	19	5	0.842	0.691	AF294497
Mdo 10	GTGTCTCCGTGTGTTGATGG* AACACCAGAGGCAAACAAGC	101	$(GT)_{10}$	21	2	0.095	0.093	AF294498
Mdo 11	TTGTGGAGAGGGGCATAAAC* GCATCCTCCCACGTTACCTA	174	$(GT)_{11}GA(GT)_3$	17	3	0.176	0.355	AF294499

*radiolabelled primer; *n*, number of individuals genotyped for each locus.

application of the resulting primers in amplifying microsatellite loci in spotted bass, *M. punctulatus*.

Cloning and characterization of microsatellite sequences was performed following Strassman *et al*. (1996). Genomic DNA from the liver of a single specimen of *M. dolomieu* (New River, West Virginia) was extracted following Longmire *et al*. (1997) and digested to completion with *Alu*1, *Hae*III, and *Rsa*I. Fragments ranging from 200 to 600 bp were purified from a 1% agarose gel with the QIAquick gel extraction kit (Qiagen) and subjected to a phosphatase treatment. A partial genomic library was developed in a Bluescript II SK + vector (Stratagene). The vector was cut at the *Eco*RV site and the ends were dephosphorylated. Competent JM109 cells of *Escherichia coli* (Promega) were transformed with the ligated vector and grown on agar plates. Colonies were lifted from plates using Hybond N + nylon membranes (Amersham) and subsequently probed with a radioactively labelled $(GT)_n$ oligonucleotide. Thirty-seven colonies screened positive for the $(GT)_n$ probe. Big Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer) was used to generate fluorescently labelled sequence fragments from 35 of those colonies. Order and size of labelled fragments was detected with a Perkin-Elmer, ABI Prism 373 automated sequencer and primers flanking microsatellite repeats were identified using software available at the Primer 3 website (http://waldo.wi.mit.edu/cgi-bin/primer/primer3.cgi). Polymerase chain reaction (PCR) conditions were optimized for 11 loci.

Variation at each locus was assessed in a collection of *M. dolomieu* from Broken Bow reservoir, Oklahoma, USA (Table 1). PCR was performed in 15-μL volumes using approximately 60 ng of genomic DNA, extracted (Longmire *et al*. 1997) from muscle or liver tissue, Perkin-Elmer's True Allele premix, and primers end-labelled with $\gamma^{32}P$. PCR reactions were carried out in a Perkin-Elmer 9600 thermal cycler using the following profile: an initial 12-min denaturation at 95 °C, followed by 10 cycles at 94 °C for 15 s, 55 °C for 60 s, 72 °C for 30 s, followed by 25 cycles at 89 °C for 15 s, 55 °C for 60 s, and 72 °C for 30 s. The profile ended with a 30-min elongation at 72 °C. PCR products were separated by electrophoresis in 4% denaturing polyacrylamide gels and bands were visualized using autoradiography. Number of alleles per locus ranged from 2–8, and observed heterozygosity ranged from 0.095–0.811. GENEPOP software (Raymond & Rousset 1995; http//:wbiomed.curtin.edu.au/genepop/) was used to perform an exact *U*-test (Rousset & Raymond 1995) for differences between observed and expected heterozygosities. Significant heterozygote deficiencies were detected at loci Mdo 3 ($P = 0.018$) and Mdo 11 ($P = 0.031$).

All 11 of the primer sets developed for *M. dolomieu* allowed amplification of microsatellite loci in a collection ($n = 31$) of *M. punctulatus* from Lake Tenkiller, Oklahoma, USA. Three loci were polymorphic. The number of alleles and observed heterozygosity (H_O) per locus in Mdo 3, Mdo 5, and Mdo 11, were, 2(0.323), 3(0.452), and 3(0.484), respectively. A significant

heterozygote deficiency was detected at locus Mdo 11 ($P = 0.015$). Our results indicate that microsatellite loci identified from *M. dolomieu* may be useful with other species of *Micropterus*.

Acknowledgements

The authors thank K. Brown, A.F. Echelle, J. Long, M. Scott, and D. Rutledge for assistance with tissue collection and S. Hoofer for technical laboratory assistance. We also thank personnel at the Recombinant DNA/Protein Resource Facility at Oklahoma State University for DNA sequencing and primer synthesis. Funding was provided by the Federal Aid in Sport Fish Restoration Act under Project F-41-R of the Oklahoma Department of Wildlife Conservation. The Oklahoma Cooperative Fish and Wildlife Research Unit is a cooperative program of the United States Geological Survey (Biological Resources Division), the Oklahoma Department of Wildlife Conservation, and Oklahoma State University.

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Isolation of microsatellite markers from the Adonis blue butterfly (*Lysandra bellargus*)

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Keywords: Lycaenidae, *Lysandra bellargus*, microsatellites, PCR

Received 25 June 2000; revision accepted 18 July 2000

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The adonis blue butterfly, *Lysandra bellargus*, is at the northern edge of its range in the UK. A series of discontinuous metapopulations are found in Southern England on south-facing calcareous grassland. The butterfly is extremely sensitive to habitat and climatic change, as exemplified by its severe decline following a drought in 1976 (Thomas 1983). Details concerning the population dynamics of the butterfly are available from various UK conservation bodies, making the butterfly ideal for studies investigating the relationships between spatial and temporal population structure and genetic diversity.

Microsatellites are currently the favoured marker for studies of intra-specific genetic variation. However, their isolation from Lepidoptera is unusually difficult, and to date very few studies have successfully used microsatellite markers in this insect group (e.g. Meglecz *et al*. 1998; Saccheri *et al*. 1998; Keyghobadi *et al*. 1999).

Other studies have applied a genomic library approach to the isolation of lepidopteran microsatellites, with limited success (e.g. Keyghobadi *et al*. 1999; Meglecz & Solignac 1998). Here we describe use of an enriched genomic library constructed for CA/GT dinucleotide repeats. Fragments of 300–700 bp were isolated from *Mbo*I-digested genomic DNA, of which 3 μg was ligated to 9 μg of *Mbo*I adaptor molecule (see Kandpal *et al*. 1994). The ligated product was PCR-amplified in a 50 μL reaction, using a single adaptor oligonucleotide as a primer. The reaction mixture contained 10 ng of template DNA, 2.5 U *Taq* DNA polymerase (ABgene), 1.25 mM of primer, 20 mM $(NH_4)SO_4$, 75 mM Tris–HCl, pH 8.8, 0.01% (v/v) Tween®-20, 1.5 mM $MgCl_2$, 0.25 mM dNTPs (ABgene). The PCR conditions were: 94 °C for 4 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 120 s, and a final period at 72 °C for 10 min. PCR product (2 μg) was hybridized to a biotin-labelled $(CA)_{15}$ probe (1 μg) (Genosys) by incubation overnight at 50 °C in a 100 μL volume containing 0.5 M sodium phosphate, pH 7.4, plus 0.5% SDS. Enrichment was carried out using streptavidin-coated magnetic beads (Promega). A magnetic particle concentrator (Promega) was used to capture hybridized fragments through the biotin–streptavidin bead bond. Enriched fragments were then released through increasingly stringent washes (see Mundy & Woodruff 1996). DNA purified from all washes was subsequently amplified by PCR using an adaptor molecule as a primer, and probed using a ^{32}P-labelled CA/GT polynucleotide (Pharmacia) to check for levels of enrichment. Fragments in the most enriched PCR product were ligated into pGEM®-T Easy (Promega) and used to transform JM109 cells (Promega) to construct an enriched library. Recombinant colonies were screened for $(CA)_n$ repeats using a colony-PCR-based approach (PIMA) (see Lunt *et al*. 1999). The DNA from each colony was amplified using three primers: M13 forward and M13 reverse primers, plus a $(CA)_n$-specific oligonucleotide (5′-TGTGGCGGCCGC$(TG)_8$V-3′). Extra amplification products were present in 43 clones and these were sequenced using Big Dye Terminators (PE-Applied Biosystems) on a Perkin-Elmer ABI 377 automated sequencer. Regions containing distinct microsatellites (<6 CA/GT) were found in 13 clones, of which four were too close to the linker to allow for primer design and were discarded.

Primers were designed for nine microsatellites and tested on DNA from 36 *Lysandra bellargus* extracted as described by Alijanabi & Martinez (1997). Of these, five proved to be polymorphic (Table 1), one was monomorphic, and another three await optimization. All PCR amplifications were performed

Table 1 Characteristics of five microsatellite loci from *Lysandra bellargus* with core repeat, primer sequences, size range of PCR product, optimal annealing temperature (T_a), number of alleles, observed (H_O) and expected (H_E) heterozygosity

GenBank	Microsatellite	Primer sequence (5′ → 3′)	T_a (°C)	Number of alleles	Size range	H_O	H_E	GenBank accession no.
Lb1/57	$(CA)_{13}$	F: TGTATCAGCAACAGCTCGGT R: GGAAGCGTTCATCGGTAAAA	46	4	172–224	0.281	0.487	AF276049
Lb4/18	$(CA)_8GGCCCCGCCGC(T)_9$	F: GATACCTATGCCAGGCTCCA R: CGAATGTCATACAGGTTGCG	50	5	146–153	0.500	0.742	AF276050
Lb4/19	$(GACGGT)_{15}$	F: AGATTAGGCATTGGCGTGTC R: CTGCGATCCATTTCCGTTA	50	8	180–260	0.778	0.854	AF276051
Lb1/41	$(GT)_7AGGTGA(GT)_5ATGAGTGC(GT)_4$	F: CGTGTCTGTCACCCCCTTTA R: ATGACGGGTAGGGATTAGGG	50	5	170–236	0.485	0.653	AF276048
LbG2	$(CA)_{10}GTC(CGCA)_3ATGC(CAGC)_6$ $(GC)_3TCT(CA)_{12}(GA)_4$	F: ATCAAGGTCCGCACAGCA R: CGCTCGCTTATCAGACAACA	50	4	164–182	0.622	0.681	AF276052

in a Hybaid 'Omnigene' Thermal Cycler, in 12.5 μL final volume. The reaction mixture contained 10–50 ng of template DNA, 0.15 U *Taq* DNA polymerase (ABgene), 0.2 pmol of each primer (the forward primer of each pair was 5′ end-labelled with fluorescent phosphoramidites FAM, HEX or TET), 20 mM $(NH_4)SO_4$, 75 mM Tris–HCl, pH 8.8, 0.01% (v/v) Tween®-20; 1.5 mM $MgCl_2$, 0.25 mM dNTPs (ABgene); in the case of LbG2, 1 × Q solution (Qiagen) was used. The following conditions were used for amplification: 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at annealing temperature, and 30 s at 72 °C, followed by 30 min at 72 °C. Microsatellites were scored using an ABI 377 automated sequencer running Genescan software (PE-Applied Biosystems).

The observed heterozygosity (H_O) was calculated for each locus using Arlequin (Schneider *et al*. 2000). H_O values were consistently lower than H_E (Table 1). Inbreeding within these small populations may cause this reduction in heterozygosity, and is currently under investigation.

Acknowledgements

The University of Southampton funded this research. We thank Dr Federica Pannacciulli, Dr Arati Iyengar, Sue Thomas and Dr Mark Dixon for their help and advice.

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Five new polymorphic microsatellite loci in the European hedgehog *Erinaceus europaeus*

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Keywords: *Erinaceus europaeus*, European hedgehog, microsatellite, primers

Received 23 June 2000; revision accepted 18 July 2000

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The European hedgehog (*Erinaceus europaeus*) inhabits an environment increasingly fragmented by urban and agricultural development throughout Europe (Becher 1994; Becher & Griffiths 1997; Becher & Griffiths 1998). Populations occur in rural, suburban and urban areas, with each type of environment being subject to different levels of fragmentation. Barriers to the flow of genes between local populations include habitat features such as roads (Mulder 1996; Richardson *et al*. 1997), rivers (Stewart *et al*. 1999), arable fields and the

Table 1 Primer sequences for five microsatellite loci in *Erinaceus europaeus*

Locus	GenBank accession no.	Primer sequence (5′ → 3′)	Repeat type	T_a (°C)	Size (bp)	Number of alleles	H_O
EEU12H	AF276820	F: CTGCATGTACCTCTCCTCTACCTC R: TTTTCTTTTTCCACCGGTGTTATC	$d(CT)_{15}$	65.2	100	7	0.62
EEU36H	AF276822	F: GACTCTGGAACTCAAAACCAGG R: GGTAGACAGAGAGATCAAAAGGGA	$d(CT)_{21}$	64.0	150	5	0.15
EEU37H	AF276823	F: ATGAGGTGAGGCTTACCAAAAA R: GGAATCTCACAGATGTAAAGTTCTAGC	$d(GT)_{23}$	64.0	260	6	0.71
EEU43H	AF276824	F: CCATGTACAGTGGATTTACCTGC R: ACCCTAGGAGCAACTTGGAGAT	$d(GT)_{25}(GA)_6$	65.2	159	9	0.84
EEU54H	AF276825	F: CATCGGCTCAGCATTCTCTT R: ATCCCCAGAGTTGTGTTTGC	$d(GA)_{31}$	56.2	284	8	0.70*

The number of microsatellite loci available in this species now totals 11. Allele numbers were calculated from a sample of 132 individuals, and observed heterozygosity values from a population of 39 individuals. All samples were drawn from populations within Southampton. *Denotes significant deviation from H_E.
T_a, annealing temperature.

presence of predators (Doncaster 1993; Micol *et al*. 1994). The margins of these habitats can define wildlife corridors and potential dispersal routes (Doncaster *et al*., unpublised). This makes the European hedgehog an ideal model for studying the genetic effects of habitat fragmentation at various spatial scales. However, the study of hedgehog populations within highly fragmented areas, covering small spatial scales, requires a high level of definition from the chosen molecular marker. Previous microsatellite studies have detected significant fine-scale population sub-structure using six polymorphic loci, but were unable to correlate this with linear geographical distance or with other geographical features (Becher & Griffiths 1998). The addition of new polymorphic microsatellite loci may elucidate previously unseen sub-structure within study populations, and increase the robustness of the statistical analyses.

This report describes five new polymorphic microsatellite loci to complement the existing six (Becher & Griffiths 1997), and could bring a higher degree of genetic definition to future studies of hedgehog populations.

These primers were based on sequences derived from a genomic library created by S.A.B. Details of its construction can be found in Becher & Griffiths (1997). Six sets of primers were designed initially. They were designed using the online primer designer PRIMER 3.0 (Rozen & Skaletsky 1998), except the primers for locus EEU33B which were designed using OLIGO 2.0 (Rychlik & Rhoads 1989). The primers were tested for locus polymorphism in 20 or more populations gathered from within a 20-mile radius of Southampton, Hampshire. The forward primer of each pair was labelled with either 6-FAM, HEX or TET ABI fluorescent dyes, and PCR was carried out under the following conditions. Each reaction contained 1 × Qiagen PCR buffer (contents Tris–Cl, KCl, $(NH_4)_2SO_4$ at unspecified concentrations, 1.5 mM $MgCl_2$, pH 8.7 at 20 °C), ABgene dNTPs (1.0 mM), $MgCl_2$ solution (2.5 mM), 1 × Qiagen Q solution (list of ingredients unavailable), 0.2 μM of each primer, 1.25 units of *Taq* DNA polymerase (Qiagen), and was made up to a final volume of 12.5 μL with HPLC water. PCR took place at 94 °C for 4 min 30 s (1 cycle), 28 cycles of 94 °C for 30 s, annealing temperature (see Table 1) for 2 min and 72 °C for 30 s, and finally 72 °C for 10 min (1 cycle).

The resulting PCR products were typed using 5% denaturing polyacrylamide gel by vertical elecrophoresis at 20–60 mA for 2 h on an ABI 377 automated sequencer, employing GENESCAN and GENOTYPER software.

Linkage disequilibrium analysis between loci was performed using the computer program GENEPOP version 3.1d (Raymond & Rousset 1995). The analysis was performed on the six new loci as well as the six existing loci, for six hedgehog populations within the Southampton area (population size ranges $9 < n < 39$, the total number of individuals was 132). Co-segregation was found to be significant in 29 out of a possible 396 combinations of populations and loci. A Bonferroni sequential correction was performed on the results of the linkage disequilibrium analysis, and all but four of the previously significant results were found to be non-significant. However, one of the newly designed loci (EEU33B, GenBank accession number AF276821) was found to consistently significantly co-segregate with one of the existing loci (EEU3, GenBank accession number U63912), and was consequently excluded from further population analyses.

The observed heterozygosity (H_O) was calculated for each locus from an urban population of 39 individuals using the computer program ARLEQUIN (Schneider *et al*. 2000; http://anthropologie.unige.ch/arlequin/software/). Values ranged from 0.15 to 0.84 (see Table 1), with an average observed heterozygosity of 0.60, and are consistent with the H_O values of the existing microsatellite loci. No sigificant deviation from H_E values were detected except for locus EEU54H ($H_O = 0.70$, $H_E = 0.65$).

Acknowledgements

We wish to thank Andrew Davey and Tiawanna Taylor for the collection of a large proportion of the samples used in this study.

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Polymorphic microsatellite loci from the lettuce root aphid, *Pemphigus bursarius*

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Keywords: Aphididae, microsatellites, *Pemphigus bursarius*

Received 20 May 2000; revision received 5 July 2000; accepted 24 July 2000

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Pemphigus bursarius is an occasional pest of agriculture with an annual life cycle that alternates between sexual reproduction on black poplars (*Populus nigra*) and parthenogenetic reproduction on the root systems of various Compositae, including lettuce. In general, aphids exhibit low allozyme variability (e.g. Tomiuk & Wöhrmann 1980), possibly as a consequence of the response of parthenogenetic lineages to natural selection (Hales *et al*. 1997). Microsatellite loci are typically more variable than allozymes (Tautz 1989) and are likely to provide a more informative system for the study of aphid populations.

A microsatellite-enriched *P. bursarius* genomic library was constructed according to the methods of Edwards *et al*. (1996). Simultaneous enrichment was carried out for a variety of microsatellite motifs by using oligonucleotides with the following sequences: $(AT)_{15}$, $(GT)_{15}$, $(GA)_{15}$, $(GC)_{15}$, $(GCC)_{10}$, $(CAA)_{10}$, $(ATT)_{10}$, $(CATA)_{10}$ and $(ATAG)_{10}$. Enriched DNA was digested with *Mlu*I, cloned into the *Bss*HII site of pJV1 (Edwards *et al*. 1996) and used to transform *E. coli* DH10B (Life Technologies). Plasmid DNA was extracted from 96 positive clones, digested with *Xho*I and separated on agarose gels to determine the size of the cloned fragment. Thirty-six clones with inserts >100 bp were chosen at random for sequencing. Clones were sequenced using a Big Dye dye-terminator kit (Applied Biosystems), following the manufacturer's instructions, with pUC19 universal forward primer. Sequences were detected using an ABI 377 DNA sequencer (Applied Biosystems).

Genomic DNA was extracted from 27 *P. bursarius* fundatrices (stem mothers, foundresses) collected from Lombardy poplars (*P. nigra* var. *italica*) growing at Horticulture Research International using a Puregene kit (Gentra Systems) according to the manufacturer's instructions. The use of fundatrices as a source of DNA ensured that all individuals were from different clones. Microsatellite loci were amplified in a 20 μL PCR reaction containing 10–50 ng of DNA, 1 unit of *Taq* DNA polymerase (Boehringer), 200 μM each dNTP, 0.25 μM each primer, 10 mM Tris, 50 mM KCl and 2.0–3.5 mM $MgCl_2$, overlaid with 30 μL of mineral oil (Sigma). Amplification was carried out in a Hybaid TR1 thermal cycler using a cycle programme of 3 min at 95 °C followed by 35 cycles of 95 °C for 1 min, the primer annealing temperature for 1 min and 72 °C for 1 min, followed by a final incubation at 72 °C for 30 min. The forward member of each primer pair was labelled at the 5′ end with one of the fluorescent dyes 6-FAM, TET or HEX by the supplier (Genosys). Amplified loci were separated through 4% polyacrylamide gels at 3 kV for approximately 1 h in an ABI 377 DNA sequencer. Amplified fragment sizes were estimated by comparison to TAMRA-labelled GS350 size standards (Applied Biosystems) co-loaded with each sample, using GENESCAN 2.1 (Applied Biosystems). Unbiased allele frequencies and expected heterozygosities (H_E) were calculated using the GENEPOP 3.1 computer program (Raymond & Rousset 1995).

Sequencing of 36 clones yielded 32 different sequences, 15 of which (50%) contained a microsatellite. Although enrichment was carried out for several repeat motifs, the majority of cloned microsatellites had a $(GCC)_n/(CCG)_n$ motif. It is unclear whether this is an artefact of the enrichment process or reflects the distribution of repeat types in the *P. bursarius* genome. Primers were designed to amplify seven of the microsatellites and each primer pair was tested at annealing temperatures ranging from 50 to 64 °C in 2 °C increments and at $MgCl_2$ concentrations from 0.5 to 4.0 mM in 0.5 mM increments. Five primer sets successfully amplified the target locus at their optimum annealing temperatures and $MgCl_2$ concentrations. However, primer set Pb 29 required the inclusion of a proprietary

Table 1 Properties of five *Pemphigus bursarius* microsatellites. Variation was assessed on a sample of 27 individuals collected from a single stand of Lombardy poplar growing in Warwickshire, UK

Locus	Cloned repeat	Primer sequences (5′ → 3′)	GenBank accession no.	T_a (°C)	[$MgCl_2$] (mM)	Size range (bp)	Number of alleles	H_O	H_E
Pb 02	$(CCG)_7$	HEX-ATTCAGACCGTCCGGCGTTC TGGCAGTCCGTCTCGACTTG	AF267192	62	2.0	84–96	4	0.44	0.55
Pb 10	$(CGG)_7$	6FAM-CTCTCGGGAGGGATTTGA GTAACGCCACGCCAAGAT	AF267193	58	3.0	104–110	3	0.19	0.17
Pb 16	$(GGC)_6$	HEX-CTGGTCGTGTAGTAAGTC AACGCTAACTCCTCTGTC	AF267194	50	3.5	150–168	5	0.23	0.37
Pb 23	$(GGC)_5$	TET-GACAGACTTCGGTATGTG ACTGCCAACACCGTCACT	AF267195	54	3.0	92–110	7	0.89	0.79
Pb 29	$(GCC)_5ACC(GCC)_2$	6FAM-TTTAACGGACGGCCATTG CGTAGAGACCGAAGGTGA	AF267196	54	3.5	182–194	5	0.54	0.81

product, 'Q solution' (Qiagen), at 1 × concentration in the PCR reaction to suppress the amplification of non-target sequences.

The variability of the five loci in a population sample is summarized in Table 1. All were found to be polymorphic, with 3–7 alleles and observed heterozygosities (H_O) ranging from 0.19 to 0.89. These markers are currently being employed to investigate aspects of population structure and aphid–host plant relationships in *P. bursarius*.

Acknowledgements

We thank Keith Edwards for the plasmid vector pJV1. Microsatellite sequencing was conducted by Alta Bioscience, Birmingham, UK. This work was funded by the Biotechnology and Biological Sciences Research Council.

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