Molecular Ecology (1999) **8**, 1075–1092

PRIMER NOTES

Characterization of polymorphic gophers (Geomyidae: *Thomomys*)

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Keywords: microsatellites, museum skins, pocket gophers, *Thomomys*

Received 6 November 1998; revision accepted 20 January 1999

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Pocket gophers are Geomyid rodents located predominantly in western and southern North America. These subterranean rodents are known for exceptional degrees of morphological and genetic differentiation over short geographical scales and for their propensity to hybridize at contact zones (Patton 1990). These attributes have inspired the wide use of pocket gopher systems to investigate influences of population genetic processes on evolutionary dynamics (reviewed in Steinberg & Patton 1999). The addition of microsatellites to the array of tools applicable to pocket gopher systems will offer means of improving the resolution and scope of these investigations. In particular, if the microsatellites can be used on the large collections of skins available in research museums (*Thomomys mazama*) and presents methods and results from experiments applying these markers to museum skins dating back to 1940. In addition, transferability of these markers to other species of *Thomomys* is addressed.

To develop microsatellite markers, *T. mazama* DNA was prepared from ethanol-preserved liver using phenol–chloroform methods (Hillis *et al*. 1990). A partial, size-selected (300–700 bp) genomic library was constructed using a pGEM plasmid vector (Promega) and screened at high stringency for $(GT/AC)_n$ and $(CT/AG)_n$ tandem repeats using probes prepared with Amersham's ECL 3′-oligolabelling and detection kit. From this library, 22 clones were sequenced and 12 primer pairs were synthesized (Integrated DNA Technologies). After initial testing, seven of these primers were resynthesized with fluorescent labels for genotyping reactions. To extract DNA from frozen and ethanol-preserved material, small amounts (< 10 mg) of liver or kidney were incubated in 500 µL of 5% Chelex (Bio-Rad) with 500 µg/mL proteinase K on a rocking platform at 55 °C overnight. The tubes were then heated to 95 °C for 15 min, vortexed, and centrifuged for 5 min. The supernatant was removed from the chelex

Table 1 Description of *Thomomys mazama* microsatellites including repeat motif, cloned allele size, number of alleles detected (*A*), annealing temperature (AT), primer sequence, GenBank accession number, observed and expected Hardy–Weinberg heterozygosities (H_O/H_E), and success analysing loci from museum skins of different age classes

Locus	Repeat motif*	Size*	*A*†	AT (°C)	Primer sequence (5′–3′)	GenBank Accession no.	H_O‡	H_E‡	Proportion of skins successfully amplified§ 1940–41 (*n* = 15)	1953–56 (*n* = 15)	1974–76 (*n* = 10)
Tm1	$(GT)_{28}$	181	14	65	TCACATACTAGCCCAAAGTCCTC GTGGTAGAGCAAAAGAAGCTGAA	AF106025	0.48	0.83	0.60	0.73	1.00
Tm2	$(GT)_{18}$	177	10	55	CATGATAACACATTTCTCAGACCC ACGTGAGACCAGTATTTCTTCAAA	AF106026	0.69	0.69	0.60	1.00	1.00
Tm3	$(GT)_{24}$	100	14	55	GTCCTCAGGCCATCCTCAGT TGGCTATGTCTGTGCATGTAGAAT	AF106027	0.69	0.74	0.87	0.93	1.00
Tm4	$(GT)_{29}$	317	12	50	GACCTAAGTCCAAAACCTAGCAGT ATCTGAAAACCAAGTATCAAAGCC	AF106028	0.86	0.84	0.00	0.00	0.30
Tm5	$(GA)_{33}$	249	21	55	GCTTTTGTTTTGAGGGCAAC GGGACTGGTGAACAGCAAAT	AF106029	0.38	0.71	0.53	0.87	1.00
Tm6	$(GT)_{17}$	173	6	55	CCGGATCTTGGATTAGGCAT GGCTGTTTTAATTTCCTTCATGT	AF106030	0.21	0.36	0.67	0.93	1.00
Tm7	$(CA)_{10...}$ $(GT)_9$	288	13	55	TCTACTGAACCACCAGAAAATCAA AGCACTGGACTTGAACACAAATAC	AF106031	0.52	0.61	0.47	0.93	1.00

*Determined from sequenced clone.
†From 195 *T. mazama* individuals sampled from 14 northern Oregon and western Washington (USA) populations.
‡Estimated for the population represented by the largest number of samples (*n* = 29).
§From a sample of 40 *T. mazama* museum skins collected since 1940 from three sampling locations in western Washington.

Table 2 Success amplifying loci across species within the genus *Thomomys* as assessed by generation of one or two PCR products of the expected size (determined by agarose gel electrophoresis using ethidium bromide staining)

	Tm1	Tm2	Tm3	Tm4	Tm5	Tm6	Tm7
T. talpoides douglasi	+	+	+	−	+	+	+
T. talpoides quadratus	+	+	+	+	+	+	+
T. talpoides yakimensis	+	+	+	−	+	+	+
T. talpoides shawi	+	+	+	−	+	+	+
T. talpoides monoensis	+	+	+	+	+	+	+
T. monticola	+	+	+	−	+	+	−
T. bottae saxatalis	+	+	−	−	+	+	−
T. bottae operarius	+	−	−	−	−	−	−
T. bottae modicus	+	+	+	−	−	+	+
T. umbrinus intermedius	+	+	+	−	+	+	+
T. townsendii relictus	+	−	−	−	−	+	+

beads and diluted 1:1 in water for use in PCR. For preparation of DNA from museum skins, small skin patches ($\approx 1 \times 1 \times 5$ mm) were excised from the region of the ventral incision, minimizing damage to the specimens. The same extraction protocol was applied except that prior to incubation the patches were rinsed once in 10% bleach, 5–10-times in water, then macerated with sterile disposable scalpels. Each round of extractions was conducted on a freshly bleached surface in a laboratory maintained free of PCR products, and included two negative controls.

Amplifications were carried out in 12.5 μL following the program: 5 min at 94 °C; 30 cycles (35 for skins) of 30 s at 94 °C, 30 s at X °C, 45 s at 72 °C; followed by 5 min at 72 °C, where X corresponds to annealing temperature (AT) in Table 1. Reactions contained 2.5 μL of DNA, 4 pmol of each primer, 0.5 or 0.75 U (0.75 for skins) of *Taq* polymerase (Boehringer Mannheim), 200 μM dNTP, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, and 50 mM KCl. PCR products were visualized on an ABI 377 automated sequencer (Applied Biosystems) after electrophoresis on 5% Sequagel (National Diagnostics). Assignment of allele size was achieved with reference to size standards present in each lane (TAMRA 350, Applied Biosystems) using the program Genotyper (Applied Biosystems).

Seven of the 12 loci amplified products of the expected size range in *T. mazama* and at least two other species, and all were polymorphic in *T. mazama* (Tables 1 and 2). Only one locus (Tm4) could not be readily amplified from skin extracts, perhaps due to its larger size. Specimen age clearly influenced whether skins could be genotyped; however, most loci from skins < 25-years-old could be amplified (Table 1). Six individuals accounted for ≈ 80% of the failed amplifications from material from the 1940s, suggesting inconsistent DNA quality among specimens. Artefacts due to low DNA concentration such as allele-dropout or replacement (e.g. Goosens *et al*. 1998) were not evident in comparisons of allele frequencies from individuals sampled from one location in the 1940s, 1970s and 1990s (E. Steinberg, unpublished data). Reduced amplification success from skins > 50-years-old suggests that a large number of specimens from older populations might be required for accurate gene frequency estimates. Nonetheless these results are promising for the utility of these new markers for both spatial and temporal assessments of genetic variation in pocket gophers.

Acknowledgements

I thank Mary Ashley for help cloning microsatellites in her laboratory, Jim Patton and Peg Smith for inviting me to complete this project in the MVZ Evolutionary Genetics Laboratory, Bev Dow, Mike Webster, and Kelly Zamudio for technical assistance, and Phillip England for input on the manuscript. Hopi Hoekstra and Peg Smith provided DNA for transferability assessments and the MVZ and Burke Museums provided museum skins for extraction experiments. This work was supported by The Foundation for Northwestern Natural History, The Nature Conservancy, and the Molecular and Cellular Biology Program of the University of Washington/Fred Hutchinson Cancer Research Center.

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Dinucleotide microsatellite loci reveal a high selfing rate in the freshwater snail *Physa acuta*

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Keywords: microsatellites, *Physa acuta*, population genetics, population structure

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Pulmonate snails, like many other molluscs, are simultaneous hermaphrodites that generally retain the ability to self-fertilize (Duncan 1975). They provide an excellent model system for studies on mating systems and inbreeding

depression, which has been virtually unexplored in animal hermaphrodites (Jarne & Städler 1995). The mating system of the freshwater snail *Physa heterostropha* was studied using recessive alleles at two complementary albino loci (Wethington & Dillon 1993; Dillon & Wethington 1994). Recent estimates of the inbreeding depression and selfing rates were obtained using the same system (Wethington & Dillon 1997), which has the disadvantage of enabling studies of laboratory lines only. The use of highly polymorphic markers such as microsatellites, which are assumed to be neutral, provides a much better tool for genetic studies. Indeed, it has been shown in another basommatophoran, *Bulinus truncatus*, that microsatellite markers were polymorphic despite the high selfing rate (Viard *et al*. 1997). This prompted us to develop microsatellite markers in order to proceed to similar studies in the freshwater snail *P. acuta* (Basommatophora: Pulmonata).

The protocol of Rassmann *et al*. (1991) was slightly modified as following to develop the microsatellites. The partial genomic library was constructed with genomic DNA purified from several snails, all originating from the same population. The genomic DNA was digested with *Alu*I, *Rsa*I and *Hae*III and fragments between 300 and 600 bp were selected for cloning in a pBluescript-II KS+ vector (Stratagene). A mixture of synthetic oligonucleotides $(TG)_{25}$-$(CT)_{25}$ $(CTT)_8$-$(GATA)_8$, and $(AAAT)_8$, end-labelled following the manufacturer's protocols with the DIG system (DIG Oligonucleotide Tailing Kit, Boehringer Mannheim), were used as probes to score approximately 3500 recombinant clones. Hybridization took place at the following temperatures: 70 °C for the dinucleotides, 56 °C for $(CTT)_8$-$(GATA)_8$ and 46 °C for $(AAAT)_8$. The DIG-labelled oligonucleotides were detected using the DIG Luminescent Detection Kit (Boehringer Mannheim). For the dinucleotide mixture 122 positive clones were obtained, two for the di-trinucleotides, and 16 for the tetranucleotides alone, all of them representing 4% of the clones scored. Sixty-four inserts were sequenced and primers flanking each repeat sequence were designed for 20 loci (only among the dinucleotide clones) using PRIMER 1.0 (Lincoln *et al*. 1993). Of these, 10 were polymorphic (at least two alleles on 10 individuals from 10 different populations) seven were monomorphic, and the rest showed unspecific extra bands.

The whole high-molecular-weight DNA extraction was performed following the protocol for molluscs (Winnepenninckx *et al*. 1993). An individual snail foot was homogenized in a 1.5-mL reaction tube with a small plastic pistil. Five-hundred microlitres of CTAB (hexadecyltrimethyl-ammonium bromide) extraction buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA (Titriplex III, ethylenedinitrilo tetraacetic acid disodium salt dihydrate), 100 mM Tris-HCl pH 8.0, 0.2% v/v β-mercaptoethanol and 0.3 mg/mL proteinase K were added and the digestion took place overnight at 60 °C. Following a phenol–chloroform–isoamylalcohol extraction and re-extraction of the aqueous phase with chloroform–isoamylalcohol, the DNA was precipitated by adding 2/3 vols of isopropanol. The pellet was rinsed with 70% ethanol, dried and resuspended in 500 µL of distilled water.

The PCR reactions were set up in a 10-µL reaction mixture containing: 1× PCR buffer (10× buffer supplied with *Taq*: 100 mM Tris-HCl pH 8.8, 500 mM KCl, 1% Triton X-100), 0.6–1.2 mM $MgCl_2$, 85 µM dCTP, dGTP and dTTP, 8.5 µM dATP, 0.2 µg/µL BSA, 0.5 µM of each primer, 1 µL (about 50 ng) of template DNA, 0.02 µL of ^{33}P-dATP (100 µL stock activity: 1 mCi) and 0.5 U of *Taq* polymerase (Eurobio EXTRA-POL II). Samples were overlaid with light mineral oil (Sigma) and amplified on Biometra UNO-Thermoblock and PTC-100 MJ Research, Inc. thermocyclers using the cycling profile: initial denaturation step at 92 °C (2 min), followed by 29–35 cycles at 92 °C (50 s), 47–64 °C (50 s), 72 °C (1 min), and one final elongation step 72 °C (5 min). Samples of the PCR products were resolved on polyacrylamide sequencing gel (6% acrylbisacrylamide, 8 M urea) together with an amplified clone as size marker. Exposure took place overnight at room temperature on a Fuji Medical X-ray New RX film.

Table 1 Primer sequence and characteristics of seven polymorphic microsatellite loci of the snail *Physa acuta*

Locus	Core repeat	Size (bp)	*T* °C	$MgCl_2$ mM	Cycles	Primers (5′–3′) forward/reverse	GenBank Accession nos
32-B	$(GA)_{13}$	147	62	1.2	29	ACAAAGATGGAGAGGGAGAGG CAACCGGATGTGACCTTTG	AF108758
61	$(CA)_{14}$	135	50	1.2	33	CCTTCAATCCTACACTTCTAGCC TTCAAAGCCCTAGTGTGATTTC	AF108763
19	$(AC)_{20}$	181	53	1.2	32	AACAGAGGGTGAGTTTGTTGA CGTTCGTTTGATTACGGTGT	AF108759
83	$(GT)_{14}$	144	55	0.6	29	GTGGGGGACTGAGTTGACAT CAGGGAGCCAGACCATTG	AF108761
27	$(TG)_{28}$	151	55	0.6	33	GAGAAAAAGAAAGTCGGTGTGC GTCCAGCCCTCACATACCAC	AF108764
59-B	$(GT)_{18}$	161	64	0.6	35	CGTCCTTTGGGTGTTGGTCA AAAGGCTCACGTTAGTTTGAGTCC	AF108762
9	$(AC)_{23}$	156	47	0.6	30	ATCGTGTTTACAGACAGACAGA CGAATCAAGTGCGTATATAGG	AF108760

T °C is the optimal annealing temperature.

Table 2 Observed (H_O) and expected (H_E) levels of heterozygosity and heterozygote deficit within populations: $F_{IS} = (H_E - H_O)/H_E$

	Lake (n = 16)			Pond (n = 16)		
Locus	H_O	H_E	F_{IS}	H_O	H_E	F_{IS}
32-B	0.438	0.604	0.276	0.286	0.712	0.598
61	0.133	0.357	0.627	0.313	0.507	0.383
19	0.500	0.813	0.385	0.000	0.635	1.000
83	0.000	0.362	1.000	0.200	0.856	0.766
27	0.250	0.760	0.671	0.286	0.708	0.596
59-B	0.250	0.492	0.492	0.313	0.544	0.425
9	0.000	0.250	1.000	0.000	0.179	1.000

Specimens of snails were hand-collected from a lake (Geneva Lake, Versoix) and a pond in its close vicinity (Marion) located in western Switzerland. Sampling was performed in each case in a small area (a few square metres) in order to minimize the effect of possible population substructure. Sixteen individuals per population were used to score seven polymorphic loci (out of 10 designed, the rest not having been analysed yet) displaying two to eight alleles over all populations (Table 1).

Details of the expected and observed levels of heterozygosity in each population are indicated in Table 2.

Expected heterozygosity is similar for lake and pond populations, and in both cases much larger than observed heterozygosity. These heterozygote deficits yield extremely high estimates of F_{IS}, over all loci. Null alleles might be partly responsible: one individual (tested several times) never amplified for locus 83, although it did for all the others. However, even though sample sizes leave little room for detecting null alleles unless very pronounced, the nature of the target species and the consistent heterozygote deficit over localities and loci makes null alleles unlikely as the main reason for this. The possibility that high F_{IS} values result from local structure appears unlikely as well, given the small spatial scale of sampling. These values seem best explained by a high selfing rate. Our estimate of 79%, calculated as $s = 2F_{IS}/(1 + F_{IS})$ (Hartl & Clark 1989), where F_{IS} is averaged over the two localities and seven loci, is much higher than the 36% obtained by Wethington & Dillon (1997).

Acknowledgements

We thank A. Wyttenbach and M. Krieger for their valuable assistance during the development of the microsatellites. M. Glaettli, H. Freymond and J. Goudet helped with the analysis. Financial support was provided by the Société Académique Vaudoise (grant to A.M.) and by the Swiss National Science Foundation (grant no. 3100–040347 to N.P.).

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Low polymorphism at 19 microsatellite loci in a French population of Argentine ants (*Linepithema humile*)

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Keywords: *Linepithema humile*, *microsatellites*, *population structure*, *social insects*

Received 7 December 1998; revision received 22 January 1999; accepted 25 January 1999

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The Argentine ant, *Linepithema humile*, has been accidentally introduced from South America to many areas with Mediterranean-like climate such as South Africa, California (USA), southern Europe and Australia. It is considered a significant pest species in many parts of the world either because they tend herbivorous aphids, invade human habitations or displace indigenous species (Visser *et al*. 1996; Human & Gordon 1997). On the other hand, the Argentine ant has attracted considerable interest from evolutionary biologists because 90% of all queens are executed by workers at the beginning of the reproductive season (Keller *et al*. 1989). This largely unexplained phenomenon and the existence of supposedly genetically derived cues to avoid sib matings (Keller & Passera 1993) could be further enlightened with the availability of

polymorphic genetic markers. Unfortunately, the markers that have been used so far exhibit a low level of polymorphism (Kaufmann *et al*. 1992). The high rates of mutation commonly observed at microsatellite loci suggest that this class of loci may have the potential to address the above objectives.

Specimens of *L. humile* were collected in Port-Leucate, near Perpignan, France and near Buenos Aires, Argentina. One-hundred workers (from 10 different locations, 10 per site) and 32 workers (from two different sites, 16 per site) were sampled from the French and Argentinean populations, respectively. Isolation of genomic DNA for genetic analysis followed the methods outlined in Krieger & Keller (1997). The partial genomic library to develop the microsatellite primers was constructed from workers of the French population.

Microsatellite loci were isolated using the protocol described by Rassman *et al*. (1991) with a few modifications. Genomic DNA from 50 workers was digested with *Alu*I, *Rsa*I and *Hae*III, and fragments of the selected size (300–600 bp) were ligated into a pBluescript-II KS+ vector (Stratagene). Oligonucleotide probes $(TC)_{10}$ and $(TG)_{10}$ labelled with the DIG system (Boehringer Mannheim) were used to screen about 2500 recombinant clones. Plasmid DNAs of the putative positive clones were digested with *Hin*dIII and *Bam*HI to excise the ant DNA. The digests were electrophoresed on a 0.8% agarose gel to further validate the presence of microsatellites by Southern blotting. Forty-one clones generated hybridization signals, from which 25 inserts were sequenced. Twenty-one primer pairs flanking the microsatellite sequences were designed using the program PRIMER 1.0 (Lincoln *et al*. 1993), from which 19 gave satisfactory amplification results.

Polymerase chain reaction (PCR) amplifications were carried out in a total volume of 10 μL, which contained 1 μL (about 10 ng) of template DNA, 10 mM Tris-HCl, 50 mM KCl, 1 mM $MgCl_2$, 75 μM dCTP, dGTP and dTTP, 7 μM dATP, 0.02 μL of ^{33}P-dATP, 0.2 μg/μL BSA, 5 pmol of each primer

Table 1 Primer sequences and characteristics of 14 Argentine ant (*Linepithema humile*) microsatellite loci

Locus	Core repeat*	Size* (bp)	No. of alleles†	H_O†	H_E†	Primers (5′–3′)	GenBank accession no.
Lhum-3	$(TC)_{12}TT(TC)_5$	197	1/5	0.00/0.66	0.00/0.77	ATAATCGTTCGTTGTTCATTCG TTCATACTATTTACTTTACACGGATACAC	AF093514
Lhum-4	$(TG)_{10}$	141	1/2	0.00/0.03	0.00/0.03	AGATTGGAACGTAGATTGGTGG TGCGAAGAATGATTCAGTGC	AF093515
Lhum-11	$(TC)_{18}$	125	7/7	0.44/0.78	0.49/0.70	GACGCGATGGGAACCAAC TCAACAATGGGGTCCAACAT	AF093517
Lhum-13	$(TC)_{19}$	168	5/8	0.49/0.69	0.49/0.79	TTACCGCGAGTGAAACAGC GCGCGATCTCATAATGTTGG	AF093519
Lhum-14	$(TG)_9$	146	1/2	0.00/0.16	0.00/0.15	TTGCGAATGACATCTTACACG CGGTTACGTGTAAATTCTCTCG	AF093520
Lhum-18	$(TG)_{16}$	121	1/1‡	0.00/0.00	0.00/0.00	CACCAACACACGCAACATG TTTCGCTAATAGCATGCACG	AF093521
Lhum-19	$(TC)_{16}$	160	7/7	0.64/0.66	0.64/0.74	CTCTTAAAGCAATTGCATGTGG ACGATCGCGTCCTTTGAG	AF093522
Lhum-28	$(TC)_{17}$	185	3/8	0.30/0.81	0.30/0.77	GAAATCGAGCAGAGAGGGC TCGACCACCAACCTAACCTC	AF093524
Lhum-33	$(TC)_{12}$	105	1/4	0.00/0.60	0.00/0.60	ATTATATCACAGCAAGATAAATTGCC GTCACGATGCGAAATTGTTG	AF093525
Lhum-35	$(TC)_{11}TG(TC)_{18}$	130	10/10	0.36/0.78	0.38/0.82	TGAGTGCCATTCACTTGCAT GATTTTGCATTGCGTGGAAT	AF093526
Lhum-39	$(TC)_{11}$	156	5/5	0.11/0.25	0.11/0.23	CAAAAAGTCAGTCGACTAGGGC GTTTGTGCTGTCCCGCTG	AF093527
Lhum-40	$(TC)_7$	200	1/1‡	0.00/0.00	0.00/0.00	GCACGTGTATGCTTCGGAC GGATGTATGAAAAGATTAGGGGG	AF093528
Lhum-52	$(TC)_{12}$	108	2/3	0.46/0.59	0.42/0.58	CTCTTATGCAATGTTTGCGG ATGACCTGCACGCGAAAG	AF093531
Lhum-62	$(ATT)_5$	152	2/2	0.49/0.50	0.50/0.41	CAGCCTTGTAGTTCAAGTATGACG GACTGTCAAATTATACAAATATGCTTTG	AF093533

*Sequenced allele.
†From a sample of 100 and 32 individuals from the French and Argentinean population, respectively. Number of alleles observed (H_O) and expected (H_E) heterozygosity are given for both, the French (first value) and the Argentinean (second value) population.
‡Monomorphic in both populations but differs in allele size.
Primer sequences of the five other monomorphic loci (*Lhum-2*, *Lhum-10*, *Lhum-12*, *Lhum-47*, *Lhum-57*) can be obtained from GenBank with accession numbers of AF093513, AF093516, AF093518, AF093529 and AF093532.

and 0.5 U of *Taq* DNA polymerase (Eurobio). All PCR reactions were performed using Perkin-Elmer and Biometra thermocyclers with the following cycle parameters: initial denaturation step at 92 °C (2 min), followed by 35 cycles at 92 °C (50 s), 57 °C (50 s) and 72 °C (1 min), and one final elongation step at 72 °C (5 min). PCR products were separated by electrophoresis using a denaturing polyacrylamide sequencing gel (6% acryl-bisacrylamide, 8 M urea).

Eight out of the 19 microsatellite loci were polymorphic in the French population. The number of alleles at the polymorphic loci ranged from two to 10 (Table 1). Expected heterozygosity (H_E) for the eight polymorphic loci varied between 0.107 and 0.638 (Table 1). Preliminary data from the population in Argentina (the native habitat of this species) show an increased level of heterozygosity and some of the loci that were monomorphic loci in the French population (*Lhum-3*, *Lhum-4*, *Lhum-14*, *Lhum-33*) were polymorphic in the Argentine population (Table 1). Furthermore, two of the monomorphic loci (*Lhum-18*, *Lhum-40*) were also monomorphic in the Argentine population but for different alleles.

The detected degree of polymorphism in the French population was rather low, with 11 out of 19 loci being monomorphic and the remainder having a moderate number of alleles. The preliminary data from the native habitat of this species show greater genetic diversity.

Acknowledgements

We thank L. Favre, A. Wyttenbach and M. Chapuisat for assistance during the development of the microsatellites.

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Characterization of microsatellite loci from a New Zealand freshwater fish (*Galaxias vulgaris*) and their potential for analysis of hybridization in Galaxiidae

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Keywords: fish, *Galaxias vulgaris*, galaxiid, hybridization, microsatellite

Received 23 December 1998; revision received 22 January 1999; accepted 25 January 1999

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The Galaxiidae is the most speciose freshwater fish family in New Zealand, comprising 19 species in two genera. Isozyme analysis of *Galaxias vulgaris*, endemic to South Island, has revealed high genetic structuring (Allibone & Wallis 1993), best interpreted as a species complex (Allibone *et al*. 1996). Morphological analyses subsequently led to the identification of four new species and the redescription and reinstatement of *G. anomalus* (McDowall & Wallis 1996; McDowall 1997).

Studies of natural hybridization can shed light on the evolutionary status of taxa with respect to speciation and reproductive isolation (Harrison 1993). Genetic studies of contact zones rely on diagnostic loci that discriminate taxa. Isozyme analyses have revealed loci diagnostic for comparisons of *G. anomalus*, *G. depressiceps*, *G. vulgaris* and *G. paucispondylus* (Allibone & Wallis 1993; Allibone *et al*. 1996), but some galaxiid species are not readily distinguished by allozymes (McDowall 1997). Microsatellites are highly polymorphic and have been applied to questions concerning levels of population variation, gene flow and hybridization (e.g. Roy *et al*. 1994; Moulin *et al*. 1996). Here we describe six variable microsatellite loci in *G. vulgaris* and examine the utility of the primers in related species.

Methods used in the isolation of microsatellites were generally based on Rassmann *et al*. (1991). Genomic DNA was extracted from muscle tissue of *G. vulgaris* (Rubicon River, Waimakariri River) using CTAB (Grewe *et al*. 1993). Total DNA was digested with *Mbo*I and 500–800 bp fragments were size-selected by agarose gel electrophoresis and ligated into dephosphorylated pUC19 digested with *Bam*HI. Ligation products were transformed with *Escherichia coli* DH5α competent cells (Gibco BRL) and plated overnight on Luria–Bertaini (LB) media. Recombinant clones were hybridized to synthetic oligonucleotides: $(AAG)_8$, $(AAC)_8$, $(AAT)_8$ and $(AAAG)_8$ each end-labelled with [γ^{32}P]-ATP. A total of 30 positive clones was obtained and plasmid DNA was isolated by cell lysis with 0.5% SDS and precipitation with polyethylene glycol. Inserts were sequenced by cycle sequencing (ABI Prism BigDye kit) and the reaction products separated and identified on an ABI 377 automated sequencer. Primers were designed from microsatellite flanking sequences and are summarised in Table 1.

Table 1 Primer sequences and characteristics of variable microsatellite loci in *Galaxias vulgaris*. Observed and expected heterozygosity are shown for each locus, based on six individuals. The clone sequences from which the primers were designed have GenBank accession numbers AF110470–AF110475

Locus	Repeat sequence	Primer sequence (5′–3′) (F = Forward, R = Reverse)	Anneal temp. (°C)	Expected size (bp)	No. of alleles	H_O	H_E
Gvu4	$(GAAA)_9$	F: TGTCTGCTGAGTCACCTTC	50	160	3	0.33	0.32
		R: AATGGCAAGTGGGTTTA					
Gvu5	$(GTT)_7(GCT)_3$	F: ATAGGGCTGAAATAAGTGTTGT	50	128	9	1.00	0.94
		R: ATCTCTATGTCCCTCAGTTGTTA					
Gvu6	$(NNGA)_{19}(GGGA)_4$	F: AAGGATGAAAGAGGGAGGAAGAGA	57	175	3	0.83	0.68
		R: GGTCGCCATCACAACACT					
Gvu7	$(GA)_{24}$	F: ATAAAGCAGGATTGGGAGTGTC	50	102	6	1.00	0.85
		R: TGGTCATTCCTTTTCAGTATCATC					
Gvu8	$(TG)_{23}$	F: ACAGCTTTCAACAGGGGAATCATA	50	121	5	0.67	0.79
		R: TCACCCTGATCTCTTGACATAATC					
Gvu10	$(AT/GT)_{15}$	F: AATACCATAGTTTCTTTA	41	81	2	0.50	0.53
		R: CAGGTGGTCGTGATGTG					

Table 2 Cross-species amplification of *Galaxias vulgaris* microsatellite primers in galaxiids and salmonids. Expected heterozygosity is given for variable loci

Species (*n*)	Gvu3	Gvu4	Gvu5	Gvu6	Gvu7	Gvu8	Gvu10
Galaxias							
anomalus (5)	+ (0.53)	++	++ (0.73)	++	++ (0.51)	++ (0.45)	++ (0.50)
depressiceps (5)	+	++	++ (0.77)	++ (.056)	++	++ (0.25)	++
pullus (5)	+ (0.53)	++	++ (0.84)	++ (0.69)	++ (0.64)	++ (0.71)	+ (0.65)
eldoni (5)	+ (0.69)	++ (0.53)	++ (0.61)	++ (0.80)	++ (0.79)	++ (0.62)	–
Sp. nov. (5)	+	++ (0.65)	++ (0.93)	++	++ (0.93)	++ (0.89)	–
paucispondylus (5)	+ (0.42)	+	++ (0.43)	+ (0.85)	++	++ (0.96)	–
fasciatus (5)	+ (0.36)	+	++ (0.69)	++	++ (0.95)	+ (0.43)	–
maculatus (5)	+	+ (0.50)	+	–	–	+ (1.00)	–
Salmo							
trutta (2)	+	+	++	+	–+	–	
Oncorhynchus							
tshawytscha (1)	–	–	++	+	–	–	–

++, amplified successfully at the specified annealing temperature; +, faint amplification or multiple bands; –, smear bands or no product; *n*, number of fish analysed.

Genotyping of microsatellite loci was performed by polymerase chain reaction (PCR) amplifications on a PTC-100 thermal cycler (MJ Research). Reactions (15 µL) contained 0.5 U of Taq DNA polymerase (Qiagen), 200 µm of each dNTP, 10 pmoles of each primer, 1.5 mm MgCl2 and 1–10 ng of extracted DNA. Thermal cycling involved 35 cycles of 94 °C (30 s), 41 °C–57 °C (see Table 1) (30 s) and 72 °C (15 s). Amplification products were resolved in 8–9% nondenaturing polyacrylamide gels, visualised by SYBR Green I (Roche) staining, and sized with a 20-bp ladder (Bio-Rad).

Of the 10 microsatellite loci amplified, six were polymorphic in *G. vulgaris* (Table 1). We tested all primer sets on eight additional galaxiid species and two salmonids (brown trout, *Salmo trutta*; quinnat salmon, *Oncorhynchus tshawytscha*) (Table 2). Within *Galaxias*, some of the primer sets yielded products that varied in size substantially from those seen in *G. vulgaris*. We detected several diagnostic loci, for various species pairs, that have the potential for future hybridization studies (e.g. at least two loci were diagnostic for *G. anomalus* and *G. depressiceps*).

Most of the primer sets yielded unambiguous amplification products in closely related species, such as *G. anomalus*, *G. depressiceps*, *G. pullus*, *G. eldoni* and *G. Sp. nov.*, and reasonably unambiguous amplification products in *G. paucispondylus*

and *G. fasciatus*, whereas only ambiguous or faint amplifications were detected in the more distant *G. maculatus*. Five primer sets yielded amplification products in brown trout but only two amplified in quinnat salmon (Table 2). Overall, microsatellite loci characterized from *G. vulgaris* amplified successfully in many congeners and have potential for genetic studies of these and related species.

Acknowledgements

We thank Grant Montgomery, Mike Tate and Graham Young for providing facilities for molecular cloning procedures.

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Primers for genotyping single nucleotide polymorphisms and microsatellites in the pathogenic fungus *Coccidioides immitis*

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Received 7 December 1998; revision received 28 January 1999; accepted 17 February 1999

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The pathogenic fungus *Coccidioides immitis* is endemic to semi-arid soils of southwestern United States, Central and South America (Pappagianis 1988). Until recently, *C. immitis* had been considered a single species; however, recent molecular analysis has shown that *C. immitis* consists of a species pair, now referred to as the Californian and non-California types (Zimmermann *et al*. 1994; Burt *et al*. 1997; Koufopanou *et al*. 1997). These species are estimated to have been reproductively isolated from one another for the past 11 million years (Koufopanou *et al*. 1997; Koufopanou *et al*. 1998). A study by Burt *et al*. (1997) used a panel of single nucleotide polymorphisms (SNPs) to infer the mode of reproduction and the amounts of gene flow between populations of the non-Californian species of *C. immitis*. However, due to the time of separation between the two species, these SNP markers had all gone to fixation within the California species and were uninformative in population genetic studies.

In this study, we describe the characterization and isolation of two classes of polymorphic genetic markers from the California species of *C. immitis*: (i) SNPs and (ii) microsatellites. Due to their low mutation rates, SNP markers were isolated to test hypotheses about the mode of reproduction in California *C. immitis*. The multi-allelic nature of microsatellites lends them to clinical diagnostic uses in both species of *C. immitis* simultaneously, and were isolated to this end.

To search for SNPs, genomic DNA was amplified from seven tester strains of Californian *C. immitis* using pairwise combinations of arbitrary primers and low-stringency conditions as described by Burt *et al*. (1994). A second amplication was performed with the exception that 0.1 μL of [α^{35}S]-thio-dATP (12.5 mCi/mL, 1 mCi = 37 Mbq) was added and the amplicons electrophoresed on a polyacrylamide gel (AT Biochem, Malvern, PA, USA) to reveal single-strand conformational polymorphisms (SSCPs). Polymorphic amplicons were cycle sequenced using fluorescent dNTPs (Applied Biosystems) to determine the genetic basis of the variation and specific primers designed to amplify the locus (Table 1). We also used this technique to screen *C. immitis* sequences from GenBank: (i) *CTS1*; (ii) *pyrG* (*OR*); (iii) *ITS*; (iv) *bl* and unpublished sequences to *HSP 60* (*HSP*) and *BGL2* kindly provided by G. Cole (Medical College of Ohio, Toledo). For each locus, a single restriction endonuclease was chosen to score each polymorphic site in a restriction assay (Table 1). If no suitable restriction site existed, then SSCP was used to score the polymorphism (locus *BGL2*).

Microsatellite loci were isolated as described by Rassmann *et al*. (1991). Libraries containing 300–500 bp inserts in the vector pGEM 4Z (Promega) were made for both *C. immitis* species. Colony lifts and replicas were fixed to Hybond-N (Amersham) following the manufacturer's instructions. $(AC)_{15}$ and $(GA)_{15}$ oligonucleotides (Operon) were end-labelled using T4 polynucleotide kinase (Promega) and [γ^{32}P]-ATP (5000 Ci/mmol, Amersham), and used independently to screen the libraries. Filters were hybridized in 10%

Table 1 Single nucleotide polymorphisms at 13 California *Coccidioides immitis* loci. Accession numbers of novel sequences in GenBank are: BOR, AF091613; BGL2, AF091614; OR13/6, AF091615; Rand1, AF091616; VK, AF091617; VL, AF091618; 621.2, AF091611

Locus	Primers (5′–3′ direction)		T_a (°C)	Site (nucleotide polymorphism)*	Enzyme	H_O§
1. VL	VL1	TTCCTAATACGGTCAGACC	52	70 (t/c)	*Hin*fI	0.50
	VL2	CCTTATTGTTGACAAAGGG				
2. CTS1	CT11	GCCGAGTACATTACGAAGAA	58	1441 (g/c)	*Mnl*I	0.94
	CT12	AGATACAACGGGAAAGGGGC				
3. OR	OR13	CTCGGTCCCTACATGGTCG	57	69 (a/g)	*Mae*III	0.73
	OR6	CGTGAAGACGACAAAATCCTC				
4. ITS	ITS4	TCCTCCGCTTATTGATATGC	58	461 (t/c)	*Hae*III	0.58
	ITS5	GGAAGTAAAAGTCGTAACAAGG				
5. VK	VK1	ATCACTAGACTATGGAAATG	54	290 (g/c)	*Mnl*I	0.85
	VK2	CGCTGGTTATTTATTATACG				
6. bl†	BLA	GTAATCCAGAACGCCGCAGG	55	215 (–/a)	*Hin*fI	0.85
	BLB	GCAATTCCCCCACTACCTAC				
7. CAG	CAG.1	ATGTCTCGCCTGGAGCAC	56	377 (t/g)	*Hae*III	0.94
	CAG.2	GCTGTTCCACGTTTCGAC				
8. CNS†	CNS19A	TTTCTCAGGCTCCCTCTCCGGAGTTG	53	64 (c/t)	*Msp*I	0.94
	ITS4A	TCCTCCGCTTATTGATATGCGAGAG				
9. HSP	HSP1	CATGGAAACTCGTAGCTCGC	56	252 (t/c)	*Eco*RV	0.73
	HSP2	TTACAAGAACTGGCGTGG				
10. BOR	BOR.1	CTCAGTGTCGGAGGTGTC	58	135 (t/c)	*Taq*I	0.69
	BOR.2	CGTCGCCAGGGTTGTGCC				
11. BGL2	BGL2.1	GCGAAACGGACTGAAGAC	55	253 (t/c)	(SSCP)	0.90
	BGL2.2	GACGACTGTCAATAGAGG				
12. RAN1	RAND1A	CCGCAAGCCATTTCAAAAAC	56	192 (t/c)	*Nru*I	0.90
	RAND1B	GGGTCTCGGGATTCGTGCTC				
13. 621.2‡	621.2U	ACAATGAACGAGCAGCAAGG	64	255 (g/a)	*Sau*96I	0.69
	621.2L	TGAAAGATGTGTAGACCCGA				

*Position of the polymorphic nucleotide in GenEMBL sequence.
–, Signifies an indel; T_a, annealing temperature of the PCR.
†Original primers developed by A. Burt.
‡Original primers developed by D. Carter.
§H_O = sum of the squared allele frequencies in Californian *C. immitis*.

w/v Dextran sulphate (Sigma), 1% w/v sodium dodecyl sulphate (SDS, Sigma), 1 M NaCl at 60 °C, with the probe at a final concentration of 10 ng/ml and washed twice in 2× SSPE (0.36 M NaCl, 20 mM Na_2HP0_4, 2 mM ethylenediaminetetraacetic acid), 0.1% w/v SDS at 50 °C. Putative positive clones were rescreened in the same way and the inserts amplified using M13 forward and reverse primers. These amplified inserts were then cycle sequenced using the same primers.

Using this method 12 simple sequence repeat motifs were identified, seven from the Californian *C. immitis* library and five from the non-Californian library. Primers to eight of these loci were designed (Table 2) and the forward primers labelled with either FAM, ROX or HEX fluorescent dyes. PCR amplifications were performed for 30 cycles of (94 °C, 30 s; annealing temperature (Ta °C), 30 s; 72 °C, 45 s) followed by a final extension for 7 min at 72 °C. PCR products were electrophoresed through a 6% denaturing polyacrylamide gel with a TAMRA-labelled internal size standard using an automated sequencer (Applied Biosystems).

All microsatellite-containing loci amplified successfully and were polymorphic within the *C. immitis* species from which they were developed. Furthermore, all loci could be amplified from their sister taxa showing that the flanking sequences of these loci are sufficiently conserved to allow amplification from both the known taxa of *C. immitis*. Only a single allele was found within each *C. immitis* strain, confirming that the fungus is haploid. The numbers of alleles at each polymorphic locus and their range in allele size are shown in Table 2.

Here, we have described the isolation of two sets of polymorphic genetic markers, one based on SNPs and the other on microsatellites, from the pathogenic fungus *C. immitis*. We are currently using these markers to generate data sets from panels of *C. immitis* that were isolated from clinical and environmental sources from a range of geographical locations spanning the entire distribution of this pathogen. Used together, these data will enable recent epidemiological patterns to be characterized, as well as analysis of deeper evolutionary processes.

Table 2 Primer sequences of nine *Coccidioides immitis* microsatellite loci. Loci 1–5 and 6–9 were isolated from Californian and non-Californian *C. immitis* genomic DNA, respectively. The numbers of alleles are based on samples of 25 chromosomes from the Californian *C. immitis* (loci 1–5) and 29 chromosomes from the non-Californian *C. immitis* (loci 6–9). Accession numbers of the cloned sequences deposited in Genbank are 621.2, AF091611; ACJ, AF108439; CA1, AF108440; GA37, AF108441; GAC2, AF108442; K09, AF108443; K01, AF108444; K03, AF108445; K07, AF108446

Locus	Repeat motif	Primers (fluorescent label)*		T_a (°C)	Allele size (bp)	No. of alleles	H_O
1. 621.2†	$(GT)_{13}$	621.2 U(FAM)	ACAATGAACGAGCAGCAAGG	64	397–426	10	0.19
		621.2 L	TGAAAGATGTGTAGACCCGA				
2. ACJ	$(GA)_6(GT)_{19}$	ACJ.F2(FAM)	CAGGCATCTTCAGTCCTCTC	58	186–227	9	0.20
		ACJ.R	TGGCTGCCCGAAGATTCA				
3. GA1	$(CT)_{13}$	GA1.F(ROX)	ACCTATTTGACTATGTCCAG	58	252–264	10	0.44
		GA1.R	ATGCTTTTAGGGGAAGATGC				
4. GA37	$(CT)_{11}$	GA37.3F(HEX)	CGCTGATATGAATTGGGACC	58	214–249	14	0.33
		GA37.4R	GGTCTGAGGTTCGACATT				
5. GAC2	$(CT)_{18}$	GAC2.1(FAM)	AGGATGCGACCGTCCGAGC	58	206–228	8	0.29
		GAC2.2	TTTGCGGGAGAATGGCTTCC				
6. KO9	$(GT)_{12}$	KO9.F2(FAM)	GCTGTCGATGACGGCTTGC	58	143–166	13	0.41
		KO9.R	CCAATCCCAAGCATTTACTC				
7. KO1	$(TG)_{14}(GA)_{10}$	KO1.F(HEX)	GCCAATCCCTACAAAATATG	58	231–247	10	0.28
		KO1.R	GAGGTCACTGTTGATCTTGC				
8. KO3	$(CT)_7(GT)_{11}$	KO3.F(HEX)	ACCTCAAAAGGCGAGACTAC	58	239–255	7	0.50
		KO3.R	TGCCGAGTGTTTGACCACAG				
9. KO7	$(GT)_{11}$	KO7.F(ROX)	ACTATTCCTTGCCCTACACC	58	292–301	8	0.35
		KO7.R	CCCCTGATACCCCTGTTTAG				

*Primers are in the 5′–3′ direction.
†Original primers developed by D. Carter.
T_a, annealing temperature; H_O, sum of squared allele frequencies in California (loci 1–5) and non-California (6–9).

Acknowledgements

We would like to thank Ron Talbot for supplying the isolates used in this study, and D. Greene and S. Kroken for helpful discussion and comments. This work was supported by grants from the National Institutes of Health.

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Isolation and characterization of microsatellites from carnivorous marsupials (Dasyuridae: Marsupialia)

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Keywords: carnivorous marsupials, conservation genetics, *Dasyurus*, microsatellites

Received 15 January 1999; revision accepted 11 February 1999

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Quolls (*Dasyurus* spp.) are among the largest remaining carnivorous marsupials in Australia and New Guinea; as such, they occupy an important ecological niche. All six species of quolls are threatened to some degree (Flannery 1995; Maxwell *et al*. 1996), but the reasons for the observed declines in each species are varied and poorly understood at present. Management for conservation purposes is necessary to prevent further declines or loss of these species; however, the lack of knowledge of the genetic variation and differentiation within members of this genus poses a serious stumbling block to effective conservation measures.

Microsatellites have a broad range of applications in conservation and evolutionary genetics and are widely used in population, parentage, and phylogeographic studies (Bruford *et al*. 1996). As a first step in the genetic management

Table 1 Six primer pairs used to characterize genetic variability and differentiation within *Dasyurus* species

Locus	Primer sequences (5′–3′)	GenBank Accession no.	Annealing temp. (°C)*	[$MgCl_2$] mM†	Repeat array	Individuals examined	Size range (bp)	No. of alleles
1.3‡	F: ATTGATGAACAAGACATAGCG R: TCATATAAGTCTTACTGTGCA	AF124211	55–45	2.0	$(CA)_{15}$	270	80–110	15
3.1.2‡	F: AGGAAACTTCACAAGTGTCGA R: ATTAATGACTCATCTGTTGTTGG	AF124212	63–53	1.0	$(CA)_{18}$	253	143–169	14
3.3.1	F: CAGCCCTTGAGTCTTGAGATT R: CATACCACCCCAGGAGTTTC	AF124213	60–50	1.5	$(CA)_{20}$	276	91–145	23
3.3.2	F: AATAGCAGAGACTCGATCC R: AGCCTTTATTACCTGGGAAG	AF124214	55–45	1.0	$(CA)_{21}$	263	108–148	19
4.4.2	F: GAAATCCAAGCTCATTTTAG R: AATCAACTCTGGAATGCATC	AF124215	50	0.625	$(CA)_{19}$	293	70–110	17
4.4.10	F: AATGCTAGATTTCACTCCC R: CCTCACATTTCTGGAACTG	AF124216	55	2.5	$(CA)_{29}$	248	179–217	19

*PCRs were run at the higher temperature for the first cycle and then in decreasing increments by 2 °C until the lower temperature was reached, then run for 35–40 cycles at the lower temperature or for 35–40 cycles at the single temperature indicated.
†Indicates final concentration in 10 μL reaction volume.
‡Amplifies successfully with *Antechinus minimus*.

of quolls, six highly polymorphic microsatellite loci have been isolated and characterized (Rassmann *et al*. 1991) and are described here.

Microsatellites were isolated from two partial genomic libraries. The first library was developed from the DNA of a Tasmanian eastern quoll. Approximately 20 μg of DNA was restricted to completion with *Alu*I and *Hae*III. The resultant fragments were electrophoresed on a 2.0% agarose gel, and fragments approximately 80–250 bp in length were cut out, purified, and ligated into a pBS K/S + vector at the *Sma*I site. Competent DH5α *Escherichia coli* cells were transformed with the ligation products and plated onto selective Luria broth (LB) media with ampicillin, X-Gal, and IPTG (LB-AXI plates; Sambrook *et al*. 1989) and grown overnight.

The second library was derived from approximately 100 μg of DNA pooled from a Tasmanian eastern quoll and a mainland tiger quoll restricted to completion with *Eco*RV, *Hpa*I, *Sma*I, and *Bsu*RI. The restricted DNA was run on a 2.0% agarose gel and fragments approximately 180–400 bp in length were cut out and purified. The fragments were ligated into pCR-Script SK+ cloning vector restricted with the *Srf*I restriction enzyme (Stratagene). Epicurian-coli XLI-Blue MRF′ Kan supercompetent *E. coli* cells (Stratagene) were transformed and plated onto LB–MAXI agar plates (equivalent to LB–AXI plates with the addition of 1× methicillin) and grown overnight.

Colonies from both libraries were blotted onto Hybond-N nitrocellulose membranes (Amersham) and fixed according to manufacturer's recommendations. The membranes were prehybridized for 2 h in a solution of 50% 6× SSC/0.05× Blotto (5% nonfat dried milk, 0.02% sodium azide) and 50% formamide and hybridized overnight at 42 °C with a [γ^{32}P]-ATP labelled synthetic $(CA)_{200-500}$ probe. Membranes were washed twice in 2× SSC/0.1% SDS at room temperature for 5 min and once in 1× SSC/0.1% SDS at 68 °C for 1.5 h. Membranes were exposed to X-ray film for up to 1 week at –70 °C.

Sixty-eight hybridizing colonies were isolated and cultured in LB broth; plasmid DNA was obtained by the TENS miniprep method (Zhou *et al*. 1990). The clones were sequenced in both the forward and reverse directions using the dideoxy chain termination method (Sanger *et al*. 1977) and Sequenase 2 enzyme (USB). Twenty-nine clones had microsatellites present. Primer sites were identified for 13 of these loci using PRIMER 0.5 (Lincoln *et al*. 1991).

Optimal polymerase chain reaction (PCR) conditions varied for each set of primers; six of these primer pairs produced unambiguous and polymorphic amplification products (Table 1). Optimization PCRs (nonradioactively labelled reactions) were performed over a range of annealing temperatures and $MgCl_2$ concentrations. PCRs were carried out using approximately 50 ng of DNA, 0.5 μM of each primer, 0.5–8.0 μM $MgCl_2$, 0.3 units of *Tth* DNA polymerase (Biotech), 67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL gelatin, and 0.2 mM of each dNTP in a total volume of 10 μL. Once optimal PCR conditions were determined (Table 1), radioactively labelled PCRs were performed to screen populations for variation. Radioactive PCR conditions were identical to optimized conditions, except that 0.08 μM forward primer end-labelled with [α^{33}P]-ATP using T4PNK (Promega) and 0.08 μM unlabelled reverse primer was used in a 10 μL reaction. Initial denaturation for 2 min at 92 °C was followed by 35–40 cycles of 0.5 min at 92 °C, 0.5 min at the optimal annealing temperature (Table 1), and 1 min at 72 °C with a final cycle of 10 min at 72 °C. Radioactive PCR products were resolved on 6% urea polyacrylamide gels with sequenced M13 as a size marker. PCR products were visualized after autoradiography for 24–72 h.

The five species of quolls analysed with these primers produced amplification products within a relatively narrow size range of each other. In addition, two primer pairs have produced polymorphic banding patterns in *Antechinus minimus*, a small carnivorous marsupial within the same family. All loci displayed high numbers of alleles (Table 1). These primers are proving useful in elucidating many aspects of

the conservation genetics of *Dasyurus* species (K. B. Firestone *et al*. unpublished).

Acknowledgements

This research was funded by Sydney City Council, Cassegrain Wines, the Winifred Scott Foundation, and Taronga Zoo. I thank P. England for assistance with manufacturing and screening the original genomic library. B. Houlden and W. Greville provided technical assistance and advice through various stages of this work. S. Burnett, G. Hall, M. Jones, D. Moyle, M. Oakwood, and M. Westerman kindly provided tissue samples used in this study. I also thank D. Edwards who trialled these primers on *Antechinus* species.

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Microsatellite markers for the hake *Macruronus magellanicus* amplify other gadoid fish

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Keywords: gadoids, *Macruronus magellanicus*, microsatellites

Received 2 February 1999; revision accepted 25 February 1999

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The long-tail hake *Macruronus magellanicus* is a demersal–pelagic species that constitutes the most abundant fish resource in the southwestern Atlantic (Prenski *et al*. 1996). However, the fisheries have only recently developed and stock identification is required before large-scale exploitation occurs. However, ecological (R. Gonzalez, B. Prenski, personal communication) and morphological (A. Giussi, personal communication) data suggest stock differentiation in Patagonian gulfs. Mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP), the only genetic data available, detected no differentiation among coastal and offshore samples (M. E. D'Amato & G. R. Carvalho, unpublished), indicating a need to develop more polymorphic markers such as microsatellites.

DNA was extracted from fresh muscle tissue according to Corach (1991) or from ethanol-preserved samples by saltation (Brufford *et al*. 1992). Prior to microsatellite isolation, an enrichment procedure using random amplified polymorphic DNA (RAPD)–PCR similar to that of Cifarelli *et al*. (1995) was employed. Amplification of 20–100 ng of DNA was performed in a 15 μL final volume with 0.2 mM each dNTP, 2 mM $MgCl_2$, 0.5 U *Taq* polymerase (BioLine), and 5 pmols of one or two RAPD primers (Kit A, Operon Technologies Inc). Reactions were run in a Hybaid Omnigene Thermocycler, at 94 °C for 3 min, 40 cycles at 93 °C for 50 s, 37 °C for 1 min, 72 °C for 1 min; 72 °C for 10 min. Half-volume (7.5 mL) of all 60 RAPD reactions was run in 1% agarose gels in TBE buffer, and transferred to nylon membranes by Southern blot (Sambrook *et al*. 1989). Repetitive elements were detected by hybridization to [γ^{32}P]-dATP 5′ end-labelled $(AC)_{12}$ $(AG)_{12}$ $(CAG)_7$ $(TAC)_8$ $(AAC)_8$ $(GTG)_8$ $(AAG)_8$ $(AAT)_8$ $(AAAT)_6$ $(AAAC)_6$ $(GATA)_{10}$ $(GACT)_6$ $(GACA)_6$ and $(GTAT)_6$. After autoradiography, 1 μL of the remaining PCR reactions which showed positive bands was cloned into pGEM -T Easy Vector (Promega) and INVa F′ (Invitrogen® *Escherichia coli* cells were transformed by the TFB–DMSO method as described in Sambrook *et al*. (1989). Approximately 2200 colonies were transferred to nylon membranes and probed with the same [γ^{32}P]-labelled oligos. Depending on the RAPD primers used, the ratio of positive to negative colonies ranged between 1:9 and 1:33. A total of 38 colonies was sequenced with universal M13 Cy5-labelled primers (T7 Sequenase version 2.0, Amersham) on an ALF*express*™ automatic sequencer (Pharmacia). Microsatellites were found in 30 clones. Ten of these microsatellites, however, were complex or long (150 bp or more) dinucleotide repeats that were discarded because these primers are also intended for ancient DNA analysis, where long DNA tracts are more difficult to amplify. Two clones were found to contain two different microsatellites (Mm 4–2, and Mm 19–1 g), while Mm 14–1 contains three.

PCR reactions were performed in 10 μL volumes containing 0.2 mM dNTPs, 2 mM $MgCl_2$ (except Mm 9–2, 1.5 mM $MgCl_2$), 0.12 μM each primer. Extensive optimization was carried out for all primer pairs on *M. magellanicus* and several other gadoid species. Optimal conditions are described in Tables 1 and 2 by the following numerical system: (1) initial denaturing 97 °C for 30 s, followed by five cycles at 96 °C for 30 s, 60 °C for 40 s, 10 cycles at 96 °C for 20 s, 55 °C for 40 s, 25 cycles at 96 °C for 20 s, 50 °C for 30 s, and a final extension step at 72 °C for 1 min; (2) as (1), but five cycles at 65 °C followed by 30 at 60 °C; (3) 94 °C for 1 min, followed by five cycles at 94 °C for 45 s, 52 °C for 45 s, 72 °C for 45 s, followed by 30 cycles at 94 °C for 45 s, 50 °C for 45 s, 72 °C for 45 s, final extension at 72 °C for 3 min; (4) as (3), annealing temperatures: 54–52 °C; (5) as (3), annealing temperatures 46–48 °C.

Table 1 Microsatellite loci from *Macruronus magellanicus*. PCR conditions are indicated in the text. GeneBank Accession nos are indicated below each locus

Locus	Primers 5′–3′	Repeat sequence	T_m (PCR condition)	Alleles	Size range (bp)	H_O (no. of individuals)	H_E
Mm 110–8	F: CATGGTCGGGGAAATAGAGGG	$(CA)_{12}$	60 °C (1)	15	111–155	0.59 (41)	0.59
AF121788	R: CTACCAACGGATGAGCCAAAC						
Mm 110–13	F: CATGGAAGTGATTCATCTCTG	$(CA)_6CT(CA)_9$	60 °C (1)	22	101–151	0.56 (41)	0.65
AF121787	R: TTCGGCATGTACTCACTTTGC						
Mm 5–4	F: AACTCAAGTAACCCACAAAC	$(TGTA)_{11}$	60 °C (1)	12	207–257	0.61 (41)	0.83
AF121794	R: GAACCGCTCATCCAAACAAC						
Mm 14–1(T3)	F: ATCTAGTCTCTGTGCTGGCA	$(TAA)_8$	46–48 °C (5)	9	98–125	0.825 (38)	0.815
AF121786	R: TTTTCACTATTCCTTGTCCCA						
Mm 14–1(T4)	F: ACTGCACTCTGGACTGGGAC	$(GATA)_7$	60 °C (1)	9	99–127	0.625 (40)	0.64
AF121786	R: TTGAACTGAACCATAAATGCC						
Mm 18–1	F: GTTATGGAAACAAAATGTGCG	$(TTC)_4$	65 °C (2)	3	104–110	0.10 (39)	0.099
AF121790	R: TAGAGGTGACCGTCTTTTAT						
Mm 18–5	F: AGAGTTCATCCGATTTCACCG	$(CAG)_4$	60 °C (1)	2	96–99	0.395 (38)	0.447
AF121791	R: AATCTTCCCATTAGTATTCACGC						
Mm 17–4	F: CCATCAAGATATAATTACGCTG	$(ATCT)_{15}$	60 °C (1)	24	192–272	0.973 (37)	0.955
AF121789	R: GCTACTGTATGTATATGTAGCCGT	$ATAT(ATC)_{14}$ $(AT)_3CTATGT$ $ATCT(A)_6$					
Mm 19–1 g-1	F: TTAATGGCGGTAAGCGTGGC	TAAATACA	60 °C (1)	2	138–146	0.09 (22)	0.34
AF121792	R: GCCCGCATTTTACATTTCCC	$(TAAA)_5GCACA$ $(TAAA)_2$ TATATAAA					
Mm 19–1 g-2	F: AAGAAGAAGAGATGGGAGCG	$(GA)_{11}$	60 °C (1)	7	94–110	0.468 (32)	0.75
AF121792	R: CTTTATTCTGGCGGAGGACG						
Mm 422	F: TGCTCCTTAGTACTAAAACGC	$(TA)_9$	60 °C (1)	5	132–140	0.28 (39)	0.356
AF121793	R: TATTTTCATTTCAATACAGAGG						
Mm 9–2	F: GGTGAGTTCACCGATGTCGC	$(CA)_{20}$	65 °C (2)	8	122–142	0.72 (39)	0.878
AF121795	R: TGGCTAGCTAGACGCGATGT						

T_m = temperature of melting; H_O = observed heterozygosity; H_E = expected heterozygosity.

Table 2 Cross-species amplification with *Macruronus magellanicus* microsatellite primers. The table shows only results with clear bands and no stuttering. PCR conditions are indicated in the text

Primer	Species	PCR conditions	Allele size range in bp (no. of alleles)	*N*
Mm 110–8	*M. novozelandiae*	1	122–146 (6)	10
	Melanogrammus aeglifinus	4	160 (1)	3
	Merlangius merlangus	4	108 (1)	6
	Micromesistius poutasou	3	108–114 (3)	3
Mm 110–13	*M. novozelandiae*	1	103–119 (4)	10
	Gadus morhua	5	162 (1)	6
	Pollachius virens	3	109–123 (2)	3
	Trisopterus esmarkii	3	109–111 (2)	2
	M. merlangus	3	109–111 (2)	5
	Gadiculus argenteus	3	107–109 (2)	3
Mm 14–1(T4)	*M. novozelandiae*	1	100–124 (4)	6
	Molva molva	3	236 (1)	5
Mm 18–1	*M. novozelandiae*	1	107 (1)	4
	T. esmarkii	3	109–111 (2)	2
Mm 18–5	*M. novozelandiae*	1	91–94 (2)	4
Mm 9–2	*M. novozelandiae*	1	130–142 (6)	10
	M. merlangus	3	102 (1)	6
Mm 19-g1	*M. novozelandiae*	1	138–146 (2)	4
Mm 19–1 g2	*M. aeglifinus*	3	135 (1)	6

T_m = temperature of melting; *N* = sample size.

PCR products were run in ALF*express*™ along with size markers obtained as in Van Oppen *et al*. (1997).

Polymorphism was tested in a sample of 40 individuals from San Matias Gulf, northern Patagonia (Table 1). Only loci 19–1 g1 and 19–1 g2 deviated from Hardy–Weinberg (H–W) expectations (Fisher's exact test $P = 0.0004$ and 0.0007, respectively) after sequential Bonferroni correction (Rice 1989), arising possibly from population substructure, selection, inbreeding, sampling, or null alleles. No linkage disequilibrium between loci was detected after applying sequential Bonferroni (Rice 1989) correction.

Primers were also tested on other gadoid species listed in Table 2. The hoki *Macruronus novozelandiae* showed the highest level of polymorphism among the tested gadoids. Although sample sizes are low, some of these primers (Mm 110–8, 110–13 and 9–2) seem promising as population markers in several other gadoid species, as well as for evolutionary studies in this family.

Acknowledgements

We specially thank to R. Gonzalez, A. Pettovello, B. Prenski, G. Lovrich, P. Smith and P. Galvin for providing the samples.

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Identification and characterization of microsatellite loci in ash (*Fraxinus excelsior* L.) and their conservation in the olive family (Oleaceae)

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Received 6 February 1999; revision accepted 28 February 1999

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Fraxinus excelsior (L.) is an anemophilous (wind-pollinated) deciduous tree, distributed throughout Europe and Asia Minor. Its wood is still favoured by European foresters, as indicated by improvement and plantation programmes. These programmes rely increasingly on identification tools such as molecular markers in order to reduce ambiguities and to identify accurately elite material to be propagated.

To complete the few existing microsatellite loci (Brachet *et al*. 1999), this study reports the characterization of 10 new microsatellite markers in *Fraxinus excelsior* (L.) and shows their potential for further use in various species of the *Oleaceae* family, which contains the important forest genus *Fraxinus* as well as the olive genus *Olea* and many ornamentals.

Sixteen elite trees, from seven Irish and one French provenance, have been used for this study. Fourteen species from the genus *Fraxinus* and 11 *Oleaceae* species (one to six individuals of each species) were tested for cross-specific and cross-genera amplification. DNA extraction was performed on mature leaves of *Fraxinus excelsior* according to a rapid protocol of DNA extraction (Lefort & Douglas 1999). Young leaves from other *Fraxinus* and *Oleaceae* species were processed with the DNeasy Plant Mini Kit 50 (Qiagen).

A microsatellite-enriched library of *Fraxinus excelsior* was produced with a method described previously (Edwards *et al*. 1996). Eighteen single clones chosen at random were purified (Wizard Plus Miniprep DNA Purification System, Promega, Madison, WI, USA) and sequenced with the use of the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin-Elmer Applied Biosystems). Sequence analysis was performed with an automated DNA sequencing system (Applied Biosystems 373 DNA Sequencer). Twenty-one microsatellite loci were obtained from these 18 clones but not all sequences were suitable for design of amplification primers. Twelve primer sets for PCR were designed empirically from the DNA sequences of 12 loci and 10 are shown here (Table 1). PCR amplifications for population screenings were conducted under the following conditions: 50 (one reaction included 75 mM Tris-HCl pH 9.0, 50 mM KCl, 1.3–2.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.001% gelatine, 62.5 μM dNTPs each (Biofinex, Praroman, Switzerland), 0.4–1 μM forward and reverse primer, 1.5 U AmpliTaq polymerase (Perkin-Elmer, Foster City, CA, USA) and 5–50 ng of DNA template. After an initial denaturation step of 5 min at 96 °C, the PCR consisted of 28–35 cycles with 1 min at 94 °C, 1 min at the appropriate annealing temperature (Table 1), and 30 s at 72 °C, followed by a final elongation step of 8–10 min at 72 °C. PCR products were analysed on CastAway 6% polyacrylamid 7 M urea precast gels (Stratagene Cloning Systems, La Jolla, CA, USA)

Table 1 Ten *Fraxinus excelsior* microsatellite primer pairs, including locus name, Genbank Accession no., repeat motif, primer sequences, specific annealing temperature (T_a), number of alleles (n = 16 trees) and observed heterozygosity (H_O)

Locus	GenBank Accession no.	Repeat motif	Primer sequence(5′–3′)	T_a	Size range (bp)	No. of alleles	H_O
FEMSATL1	AF004829	$(Ttaaaa)_{2...}(Gt)_{19}$	F: AGCAGCATTTATGAATGTTC R: ATCAACTGAAGATGACGACG	60	170–212	10	0.73
FEMSATL2	AF023473	$(Ct)_{28}$	F: TCTTTATCATCAAAAAATAA R: TACAAGGTGATATCACTTCT	50	174–224	5	0.375
FEMSATL4	AF006069	$(Ca)_2(Ag)_{24}$	F: TTCATGCTTCTCCGTGTCTC R: GCTGTTTCAGGCGTAATGTG	52	164–228	9	0.75
FEMSATL5	AF028803	$(Ga)_{41}$	F: GGATTGAGATTCAATTTGCA R: TCCGAGTGATGCCTACTCTA	54	107–183	12	0.75
FEMSATL8	AF020394	$(Gt)_{23}$	F: TGTAGCTCAGGATTGGCAAT R: AGCGTTGTCCTTAACTTTT	52	138–188	13	0.69
FEMSATL10	AF020396	$(Ct)_{17}(Ca)_5ct(Ca)_7(Ta)_2tg(Ta)_2$	F: TTGAGCAACATGTAATTATG R: AAATATCCGGTGCTTGTGTA	51	174–252	11	0.81
FEMSATL11	AF029882	$(Ga)_{20}(Ta)_4$	F: GATAGCACTATGAACACAGC R: TAGTTCTACTACTTCAAGAA	52	180–226	11	0.19
FEMSATL12	AF020397	$(Ga)_6ca(Ga)_8$	F: TTTTTGGAACCCTTGATTTT R: GATGGACGGGCATTCTTAAT	52	180–262	9	0. 44
FEMSATL16	AF029880	$(Ca)_3(Cg)(Ca)_{10}(Ta)_2(Ca)_3$	F: TTTAACAGTTAACTCCCTTC R: CAACATACAGCTACTAATCA	52	180–200	4	0.5
FEMSATL19	AF020400	$(Ca)_6cggc(Ca)_{13}$	F: CTGTTCAATCAAAGATCTCA R: TGCTCGCATATGTGCAGATA	52	174–214	12	0.88

after silver staining according to a modified protocol (Streiff & Lefort 1997).

The sequences of the cloned alleles of each characterized microsatellite locus are given in Table 1. All the 10 loci analysed were polymorphic and the number of alleles observed varied from 4 to 13 alleles in 16 trees analysed. Heterozygosity varied from 0.19 to 0.88. For the locus FEMSATL5, many alleles were found to be shorter than expected. Three alleles from three homozygous trees were sequenced in comparison to the cloned allele. Sequencing confirmed that a major deletion event had occurred at this locus in all three alleles (deletion of 29, 33 and 35 repeat units). In addition, two of these alleles displayed an additional insertion of 7 bp at the end of the 5′ flanking region and also an important deletion of 15 bp in the 3′ flanking region of the alleles.

The usefulness of eight loci for DNA profiling has been tested in 14 other species of the genus *Fraxinus* and 11 other genera of the *Oleaceae* family. When amplification was positive, it always yielded a PCR product of expected size and no other product was observed (Table 2). One locus (FEMSATL5) appears to be specific to *F. excelsior* while two other loci (FEMSATL1 and FEMSALT2) are specific to the genus *Fraxinus*. FEMSATL12 only amplified in *F. angustifolia* which is close to *F. excelsior*. We observed a decline in amplification success with an increased genetic distance at the family level.

This work has proven very successful and allowed the characterization of 10 new microsatellite markers from a very efficient enrichment method. These markers proved to be suitable for population studies in different species of the *Oleaceae* family and the *Fraxinus* genus.

Acknowledgements

Dr Francois Lefort was supported by European Union FAIR contract No. CT 965004. We thank the ‘Arboretum National des Barres’, ENGREF, France and the CRBF, Université Laval, Sainte-Foy, Québec, Canada for providing samples of *Fraxinus* and other *Oleaceae* species.

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Table 2 Amplification results of eight microsatellite loci of *Fraxinus excelsior* in 14 other *Fraxinus* species and 11 other *Oleaceae* species. (Assays resulting in a positive amplification of PCR products of expected size are indicated by +, those showing no amplification are indicated by –.)

Species	FEMSATL1	FEMSATL2	FEMSATL4	FEMSATL5	FEMSATL11	FEMSATL12	FEMSATL16	FEMSATL19	No. of amplified loci
Fraxinus sp.									
Fraxinus americana L.	+	–	+	–	+	–	+	+	5
Fraxinus angustifolia Vahl.	+	+	+	–	+	+	+	+	7
Fraxinus chinensis var. *rhynchophylla* (Hance) Hemsl.	+	–	+	–	+	–	–	–	3
Fraxinus cuspidata Torr.	+	+	+	–	+	–	+	+	6
Fraxinus longicuspis Siebold & Zucc.	+	+	+	–	+	–	–	+	5
Fraxinus mandshurica Rupr.	+	+	+	–	+	–	+	+	6
Fraxinus nigra Marshall	+	+	+	–	+	–	+	+	6
Fraxinus ornus L.	+	–	+	–	+	–	+	+	5
Fraxinus pallisiae Wilmott	+	–	+	–	+	–	+	+	5
Fraxinus pennsylvanica Marshall	+	–	+	–	+	–	+	+	5
Fraxinus quadrangulata Michx.	+	–	–	–	+	–	–	+	3
Fraxinus syriaca Boiss.	–	–	–	–	+	–	–	–	1
Fraxinus tomentosa Michx. f.	+	+	+	–	+	–	+	+	6
Fraxinus velutina Torr.	+	+	+	–	+	–	+	+	6
No. of species with a product of expected size	13	7	12	0	14	1	10	12	
Oleaceae									
Abeliophyllum distichum Nakai	–	–	–	–	–	–	–	–	0
Chionanthus virginens L.	–	–	–	–	–	–	–	–	0
Fontanesia fortineï Labill.	–	–	–	–	–	–	–	+	1
Foresteria neomexicana Poir.	–	–	–	–	+	–	–	+	2
Forsythia x intermedia Zabel.	–	–	–	–	–	–	–	–	0
Jasminum arborescens Roxlo.	–	–	–	–	–	–	–	–	0
Ligustrum ovalifolium Hassk.	–	–	+	–	+	–	–	+	3
Olea europea L.	–	–	+	–	+	–	–	+	3
Osmanthus heterophylles Green.	–	–	–	–	+	+	+	+	4
Phillyrea angustifolia L.	–	–	+	–	+	+	–	+	4
Syringa vulgaris L.	–	–	–	–	+	–	–	+	2
No. of species with a product of expected size	0	0	3	0	6	2	1	7	

Isolation and characterization of microsatellites in iris

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Keywords: *Iris*, Louisiana irises, hybridization, microsatellites, paternity

Received 13 February 1999; revision accepted 4 March 1999

The Louisiana iris species complex consists of three widespread species (*Iris fulva* Ker-Gawler, *I. hexagona* Walter and *I. brevicaulis* Raf.; Viosca 1935) and a rare diploid hybrid species (*I. nelsonii* Rand.). While these species have relatively wide ranges (with the exception of *I. nelsonii*) and distinct ecological preferences, they occur sympatrically in southern Louisiana where interspecific matings have led to the production of hybrid populations (e.g. Viosca 1935; Arnold *et al*. 1990). Although the ecology, taxonomy and evolution of the Louisiana irises have been studied for over 50 years, direct estimates of gene flow, mating patterns and hybrid fitness in natural populations are lacking. To this end, we developed a suite of five microsatellite loci from two species of Louisiana iris, *I. brevicaulis* and *I. fulva*, that will allow us to take a paternity-based approach to the study of these phenomena in natural *Iris* populations.

Cloning and screening procedures followed the methods of Aldrich *et al*. (1998). Total genomic DNA was isolated from fresh leaf tissue of a single *I. brevicaulis* and a single *I. fulva* individual using the DNeasy Plant Mini Kit (QIAGEN). The DNA was digested with *Mbo*I and size selected by excising 300–700 bp fragments from a 2% agarose gel (QIAquick Gel Extraction Kit, QIAGEN). The size-selected DNA fragments were then ligated into the *Bam*HI site of a lambda vector (ZAP Express, Stratagene) and packaged (ZAP Express Gigapack III Gold packaging extract, Stratagene). The packaged libraries were used to infect XL1-Blue *Escherichia coli* cells and plated in top agar. Plaques were lifted with nylon membranes (Hybond N+, Amersham) and screened with a [γ^{32}P]-dATP end-labelled $(GA)_{10}$ probe. Seventeen plates carrying ≈1000 plaques/plate were screened for each species. Candidate plaques were picked and rescreened at a lower density to minimize false positives. A total of 27 *I. brevicaulis* and 26 *I. fulva* clones were amplified using the M13 forward and reverse primers, purified with the High Pure PCR Product Purification Kit (Boehringer Mannheim) and sequenced on an automated sequencer using the ABI Dye Terminator Prism Kit (ABI/Perkin-Elmer). Primers were designed for eight *I. brevicaulis* and seven *I. fulva* loci. Of these, three of the *I. brevicaulis* and two of the *I. fulva* primer pairs produced amplification products that were both interpretable and polymorphic in at least one of the species (Table 1).

Polymerase chain reactions were performed in 20 μL volumes containing 50 ng of genomic DNA, buffer [50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol and 1% Triton X-100), 2.0 mM $MgCl_2$, 8 pmol of each primer, 125 μM of each dNTP and 0.8 units of *Taq* DNA polymerase (Promega)]. Amplification conditions consisted of an initial 2 min denaturation at 94 °C, followed by 30 cycles of 94 °C for 1 min, 56–62 °C for 1 min and 72 °C for 1 min. The 30 cycles were followed by 5 min at 72 °C. Reactions were carried out in a Perkin-Elmer Cetus 9810 Thermal Cycler. Polymorphisms were detected by end-labelling one primer with [γ^{32}P]-dATP using T4 polynucleotide kinase (1 μCi per 5 pmol of primer), resolving fragments on 6% polyacrylamide gels and visualizing them by autoradiography.

The five primer pairs produced interpretable, polymorphic amplification products in *I. brevicaulis*. In contrast, IB141 consistently failed to amplify in *I. fulva*, even after multiple attempts to redesign the primers, and IF061 amplified as many as four alleles in each *I. fulva* individual, suggesting that this locus has been duplicated in this species. In order to assess allelic variability at all five loci, we screened 41 *I. brevicaulis* and 19 *I. fulva* individuals collected from natural populations of these two species. The number of alleles ranged from

Table 1 Characteristics of five microsatellite loci cloned from two species of Louisiana iris, including locus name, GenBank Accession no., primer sequences, repeat motif, annealing temperature, size of the sequenced allele and total number of alleles from the two species combined. The number of alleles, observed heterozygosity and expected heterozygosity are reported for each locus in each species separately

Locus (Accession)	Sequence (5′–3′)	Repeat	T_a (°C)	Size (bp)	Alleles (total)	*I. brevicaulis*			*I. fulva*		
						Alleles	H_O	H_E	Alleles	H_O	H_E
IB025 (AF124505)	GATCTCACATCGTTTGGTC CGATAACCCAACTTCACTAC	$(GA)_{27}$	56	104	25	17	0.71	0.91	13	0.94	0.90
IB141 (AF124506)	CTGAACCACCGGTCACAAG GAAGCCATGTTGAAGTTGTCC	$(GA)_{15}$	56	156	12	12	0.90	0.83	—	—	—
IB145 (AF124507)	TGTTGCGGGATTAAGGAGAC CAACGAGAAGAATTATCCGAAAG	$(GA)_{15}$	56	164	22	19	0.83	0.92	10	0.76	0.83
IF061 (AF124508)	TTGGGACAACCATTTGAGGA CGCCGACAAGACCCTGAC	$(GA)_{24}$	62	235	16	16	0.92	0.90	—	—	—
IF073 (AF124509)	TGGCTCTACCTTCACCACAAC CCGAACCCAGAATGGAAGTG	$(GA)_{19}$	58	195	14	8	0.60	0.83	9	0.95	0.85

8 to 17 in *I. brevicaulis* and from 9 to 13 in *I. fulva* (Table 1). Overall, there was a total of 12–25 alleles per locus in the two species combined. In all cases, expected heterozygosities were quite high, ranging from 0.83 to 0.92 in *I. brevicaulis* and from 0.83 to 0.90 in *I. fulva*. Only two of the five loci (IB141 and IF061) conformed to Hardy–Weinberg expectations in *I. brevicaulis* when tested with the probability test of GENEPOP (Raymond & Rousset 1995), whereas all three loci assayed in *I. fulva* were consistent with Hardy–Weinberg expectations. The significant heterozygote deficits ($P < 0.05$) at IB025, IB145 and IF073 in *I. brevicaulis* could be due to the presence of null alleles. Alternatively, these deviations could be an artefact of our sampling strategy. That is, we could have created an apparent heterozygote deficit (i.e. a Wahlund effect) by sampling a substructured *I. brevicaulis* population across which allele frequencies at these three loci were strongly differentiated. The relatively high level of variation described above, combined with the fact that three of our five loci are informative in both *I. brevicaulis* and *I. fulva*, suggests that these markers are well suited for paternity studies in hybrid *Iris* populations.

Acknowledgements

We thank P. Aldrich, D. Erickson, K. Hufford and G. Kochert for their assistance. This work was supported by NSF Grant DEB-9703853 (MLA) and NSF/USDA/DOE Training Grant BIR-9220329 (JMB).

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