Molecular Ecology (2000) 9, 107–118

PRIMER NOTES

Microsatellite primers for the wild brown capuchin monkey *Cebus apella*

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Keywords: *Cebus*, microsatellites, monkeys, paternity, primers

Received 26 April 1999; revision accepted 19 June 1999

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Molecular ecological studies are not common in primatological research. Part of the problem with monkeys from Central and South America (New World) is the difficulty of obtaining DNA from the animals, given their arboreal life. This problem can now be overcome with the new methods developed to extract DNA from fecal material, food wedges and urine. The second problem is the lack of microsatellite primers that can be used to amplify the small amounts of DNA found in the fecal samples. Here, I report the characterization of five microsatellite primers isolated from *Cebus apella*, and two primers isolated from *Lagothrix lagotricha*. The use of these primers in other neotropical primate species is also reported.

Five micrograms of genomic DNA from *Cebus apella* and *Lagothrix lagotricha* were digested with *Sau*3AI and fragments in the range of 400–1000 bp were selected for cloning into pBluescript-SK cut with *Bam*HI. Eight-hundred colonies of each species were screened with $(GT)_{10}$ and $(CT)_{10}$ DIG-labelled probes and positive clones were detected using a DIG detection kit (Boehringer Mannheim). Positive clones were sequenced on an Applied Biosystem ABI 373A automated sequencer, and analysed using Perkin Elmer DNA Sequencing Software (version 2.1.1). Out of the five clones sequenced from the *Lagothrix* library, four contained microsatellites, and out of the 13 clones sequenced from the *Cebus* library, nine contained microsatellites. Primers were designed using GeneRun software. Seven of the 14 pairs of primers designed are reported in Table 1. PCR conditions were optimized on *Cebus* DNA extracted from blood using standard phenol–chloroform extractions. DNA amplifications were performed in a total volume of 25 μL (10 mM Tris-HCl (pH 8.9), 50 mM KCl, 20 μM of each dNTP, 0.5 U of native *Taq* polymerase (Perkin-Elmer/Cetus) and 4 pmol of each of the appropriate primer). One primer of each pair was labelled with a fluorescent dye (either TET or HEX). The optimum $MgCl_2$ and BSA concentrations as well as optimum annealing temperatures for the different primers are shown in Table 1. The thermal cycling (on a PTC-100™ Programmable thermal controller, MJ Research Inc.) was performed at the following conditions: 2 min at 92 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at the selected annealing temperature and 30 s at 72 °C and an extra extension time of 10 min at 72 °C. PCR products were visualized on 3% agarose gels stained with ethidium bromide. One microlitre of each PCR product was mixed with 2 μL of formamide, and 0.5 μL of the size standard (GeneScan TAMRA-500, Applied Biosystems) and the TAMRA buffer, denatured at 95 °C for 2 min, and loaded on 5% 'Sequagel' (National Diagnostics) with 1× TBE buffer, in the Applied Biosystems 373A automated sequencer. Allele sizes were established using the GeneScan software. The primers, both those derived from the *Cebus* library and those from the *Lagothrix* library, were tested in three populations of *Cebus apella*: the colony at the animal centre of the NIH; a group from Parque Natural Nacional Tinigua, Macarena Colombia; and a group from Parque Nacional de Iguazú in Argentina. DNA from the last two populations were extracted from fecal samples using the Constable *et al.* (1995) protocol, while the NIH samples were extracted from blood (and some individuals from both blood and faeces as a control for the fecal amplifications) following standard protocols. In addition, the primers were tested in DNA samples extracted from tissue of other neotropical primer species using the same PCR conditions as before but with an annealing temperature of 45 °C. The results are shown in Table 2.

Table 1 Summary of the different *Cebus apella* polymorphism data for the different microsatellites. GenBank Accession nos were AF109994–AF104000. PEPCx indicates a marker derived from *C. apella* while PEPLx denotes a marker derived from *Lagothrix lagotricha*

Locus ID	Primer sequences (5 – 3)	Repeat structure	*n*	No. of alleles	Size range (bp)	Annealing temp. (°C)	$MgCl_2$(mM)
PEPC3	CATGGACTGCAATTCAAGCC ACTTCCAGCCTCCAAAACTATG	$(GT)_{13}$	30	5	292–325	55 °C	2.5
PEPC8	TTCAGGATGCATCAAATGATT TAGCAGTCTATTTAGGTGTTAAT	$(CA)_{16}$	74	11	128–168	50 °C	2.5
PEPC40	GACAGAGCAAGACTCCATCTC GATCAGTAAACACATGTGCAT	$(CA)_{18}(CT)_{14}(CA)_9$	30	4	280–290	55 °C	1.5
PEPC59	CAGTGGCAACTCTGTAAGGA GTGGAGTCAACATGCAGAGG	$(GT)_{18}$	71	7	302–321	55 °C	1.5
PEPL4	TGGAAGTTACGCTCAGCCTTC CTGGAGCATCTTGTAGTGCCAG	$(TG)_{12}$	65	9	292–308	55 °C	1.5

N is the number of individuals tested.

Table 2 Cross-species amplifications data for *Cebus apella* and *Lagothrix lagotricha* microsatellites. Neotropical primates. Successful amplification is indicated by the observed genotype (in bp) of the single sample per species that was analysed

Locus	PEPC3	PEPC8	PEPC40	PEPC59	PEPL4
Alouatta seniculus	265	171/189	+	256	–
Aotus trivirgatus	268	150	+	232/256	–
Ateles sp.	251/262	176/189	+	320	+
Brachiteles arachnoides	263/281	180	+	253/257	+
Callicebus molloch	258/275	176	+	+	–
Callimico goeldii	266		+	+	–
Callithrix jacchus	259	172/174	+	221/232	–
Chiropotes satanas	244	208	+	+	+
Lagothrix lagotricha	211/222	277	+	288/290	296/298
Leontopithecus rosalia	264/304	209	+	226/236	–
Pithecia pithecia	266/307		+	233	–
Saguinus geoffrogyi	264/327	146	+	281	–
Saimiri sciureus	327		+	277	–

– indicates no amplification.

The primers reported here give evidence of the high polymorphism found in microsatellites compared to other molecular markers. Given the high polymorphism in these primers they offer a powerful tool for studies on pedigree and population structure of neotropical primers.

Acknowledgements

This work has been supproted by FONDO FEN-Colombia; COLCIENCIAS, Colombia, Banco de la República de Colombia and Sigma Delta Epsilon Graduate Women in Sciences. I would like to thank Dr Charles Janson, Mario Di Bitetti, Dr Gayle Burns and Dr Ines Horovitz for providing the samples and Dr Daniel Dykhuizen, Dr Rafael Zardoya and Dr Sabine Loew for technical assistance.

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Polymorphic microsatellite DNA markers for the marine gastropod *Littorina subrotundata*

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Keywords: gene flow, *Littorina*, microsatellite, population structure

Received 15 May 1999; revision accepted 24 July 1999

Coastal habitats of the northern temperate zone are home to over 19 species of *Littorina* (Reid 1996). Due to its widespread abundance and phenotypic diversity, members of this genus have been the model system of choice for numerous ecological and evolutionary studies (Reid *et al.* 1996 and references therein). Although mitochondrial DNA has been useful in the study of population structure in *Littorina*, it is clear from recent data (Kyle & Boulding 1998) that faster-evolving markers are needed for fine-scale metapopulation analysis. To this end, we have isolated 12 polymorphic microsatellite loci for *Littorina subrotundata* (Carpenter 1864), a direct-developing species from the North Pacific. We report preliminary data evaluating their potential utility as high-resolution genetic markers for this model taxon. The general methods for the isolation of simple sequence loci outlined in Rassmann *et al.* (1991) were followed, with the following alterations.

Total genomic DNA from the visceral mass of *L. subrotundata* was isolated via organic extraction with methylene chloride/isoamyl alcohol and subsequent ethanol precipitation (Claxton *et al.* 1997). DNA from five individuals was combined, digested with the restriction enzymes *Hae*III and *Rsa*I and size selected using 1% agarose gel electrophoresis. DNA fragments of 300–1000 bp in length were ligated into the *Eco*RV site of the pZErO plasmid vector (Invitrogen) which was then transformed into Top F *Escherichia coli* cells (Invitrogen) via electroporation using standard methods (*E. coli* Pulser, Bio-Rad).

Approximately 32 000 colonies were screened with [γ^{33}P]-ATP-labelled AAT_{10}, $AAAT_6$ (hybridization $T = 52$ °C), AAG_8, AAC_9 and $GATA_7$ (hybridization $T = 55$ °C) oligonucleotide probes according to the methods of Shaw (1997; P. W. Shaw, personal communication). DNA sequences of inserts from clones positive in two successive hybridizations were obtained using the manufacturer's suggested cycle sequencing protocol for the ABI model 377 Version 2.1.1. using M13 sequencing primers and labelled ddNTPs.

Of the 50 sequences obtained, 48 contained unique microsatellites composed of at least one trinucleotide repeat array. PCR (polymerase chain reaction) primers for 10 of these loci were designed using Primer V0.5 (Lincoln & Daly 1991) and Gene Runner V3.0 (Hastings software) programs and are reported in Table 1. Two dinucleotide loci, VAM-F1 and

Table 1 Characterization of *Littorina subrotundata* microsatellites

Locus*	Repeat motif	Primer sequence (5 –3)	T_a (°C) used in PCR	Product length (bp)†	Number of alleles‡	Product size range (bp)	H_O	H_E§
Lsub4	$(ATA)_{28}$	CCCCATTTGATAACAGGAACC GCTTTGAACTTCGGATAAGGC	62	153	18 (27)	143–210	0.96	0.92
Lsub6	$(TCAA)_6CA(AAT)_{27}$	GCCTTTAACAACAGGTCTTGG CTCTGGCACCTATAAACACGC	60	230	17 (27)	203–251	0.48	0.92
Lsub8	$(ATA)_{29}$	CCAGTGACCAGATCATAGCG GGAATTGTAAAGTGCTTGGAGC	62	234	16 (27)	186–258	0.78	0.90
Lsub9	$(ATA)_{25}$	TGTGACTGTGCTACAACTGCC TGACAAATTGTGATATGTTGATGC	62	231	15 (24)	195–237	1.00	0.92
Lsub13	$(TTA)_7C(TAT)_{30}$	TCCCAGTGCCGTGTATCC ACAGGAAGCGACTATGGTGC	64	240	19 (27)	189–240	0.89	0.93
Lsub14	$(ATT)_{19}$	GGCTTGGTCAGTGGATTATTG CGCTTTGAACTTTTGATAAGGC	64	133	14 (26)	97–172	0.73	0.85
Lsub16	$(CTT)_{11}$	TTTGCTGCTCTGAAAATTCC GGAGTCTAAGCCCATGAACG	60	140	8 (23)	137–200	0.30	0.76
Lsub32	$(CAA)_{13}TTG(AAC)_5$ $TG(AAC)_{10}$	ATCACATCGCACACGCTTAC ACGGTGTGTCATCATCAACG	62	229	10 (27)	205–247	0.81	0.84
Lsub62	$(AAC)_{14}$	CGCTTTCCCGTTATACCAAC CACCGTAAAACCTTGTGAGC	64	240	12 (27)	231–246	0.93	0.84
Lsub63	$(CAA)_5AA(CAA)_2CA$ $(CAA)_7CAT(CAA)_{13}$	GCCAATTGTATAACGGACTGTG TAAGTGCGGGTATGGTCAAC	58	142	16 (27)	130–184	0.78	0.91
VAM-F1	$(TC)_7N_{10}(TC)_{27}$	TTGGTAGTTAGGGCGTAGAAAG ACCTGCAACCAGAATACACAG	60	212	16 (25)	196–262	0.80	0.88
VAM-B3	$(CT)_{30}(CA)_7$	CTTGTTATCTCTCGTATCCGGG AGAACCAGCAAGCGTAAAGC	62	173	22 (28)	143–213	0.75	0.93

*Genbank Accession numbers: AF167443–AF167454.

†Based on the sequence of the cloned fragment.

‡Number in parentheses indicates the number of individuals sampled at that locus.

§Using the formula $H_E = 1 - \Sigma P_i^2$ where P_i is the frequency of the ith allele.

VAM-B3 (Table 1), had been isolated at an earlier date, employing an enrichment method (Naish & Skibinski 1998).

Allelic variation was assayed in 27 individuals collected from Prasiola Point, Vancouver Island, British Columbia, Canada (48° 50 N, 125° 08 W). PCR amplifications were carried out in a final volume of 20 μL containing 10 ng of genomic DNA, 0.4 μM of each primer, 50 μM of each dNTP, 1.5 mM of $MgCl_2$, 0.5–1.0 units of *Taq* polymerase and 1× PCR buffer containing 20 mM Tris-HCl (pH 8.4) and 50 mM KCl (Gibco-BRL). Cycling parameters were: 5 min at 95 °C followed by 28 cycles of 30 s at 95 °C and 40 s + 1 s per cycle at the optimal annealing temperature (Table 1) using a PTC-100 thermocycler (MJ Research). PCR products were electrophoresed in 5% nondenaturing polyacrylamide gels and visualized by silver staining (Naish & Skibinski 1998). Sizes of PCR products were determined by comparison to a 3-bp allelic size standard made from the PCR products of different individual snails and calibrated with the original clone.

For VAM-F1 and VAM-B3 (Table 1), the PCR conditions were modified as follows: a final volume of 5 μL was used, the dNTP concentration was 40 μm, the forward primer was end-labelled with [γ^{33}P]-ATP and used at a concentration of 0.02 μM and the reaction included 0.1% Tween-20. Cycling parameters were: 5 min at 95 °C followed by 30–40 cycles of 30 s at 95 °C, 30 s at the optimal annealing temperature (Table 1) and 30 s at 72 °C. PCR products were resolved on 6% polyacrylamide gels under denaturing conditions and visualized by autoradiography. Allele sizes were determined by comparison to an M13mp18 sequencing size standard (Amersham).

Table 1 outlines the allelic variation, product size range and heterozygosity observed at each of the 12 loci. All loci were moderately to highly polymorphic with a population of 23–27 individuals showing between 8 and 22 alleles per locus and an observed heterozygosity in the range of 0.30–1.00. These preliminary results indicate the potential utility of microsatellite DNA markers in future studies of intraspecific variation, local gene flow and population substructure in *Littorina subrotundata*.

Acknowledgements

The authors warmly acknowledge A. Hollis for sequencing the microsatellites. This study was supported by an NSERC research grant to EGB.

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New microsatellite markers for assessment of paternity in the squid *Loligo forbesi* (Mollusca: Cephalopoda)

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Keywords: Cephalopoda, *Loligo*, microsatellite DNA, population genetics, squid

Received 26 July 1999; revision accepted 7 August 1999

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The availability of microsatellite DNA markers for the veined squid, *Loligo forbesi*, has allowed detailed analysis of population structuring and preliminary investigation into mating strategy and male–male competition in this northeastern Atlantic species (Shaw & Boyle 1997; Shaw 1997; Shaw *et al.* 1999). Mating involves the transfer of sperm packets (spermatophores) from male to female, after which eggs are laid in encapsulated strings (Boletzky 1997). Competition for mates between males has been observed in loliginid squid, where dominant males guard females against other males and smaller, 'sneaker' males (Hanlon 1996). Microsatellite markers have been used to demonstrate that multiple mating in *Loligo forbesi* can result in multiple paternity of the offspring of a single female, i.e. within an egg string (Shaw & Boyle 1997). To facilitate these studies, additional microsatellite loci have been isolated from *L. forbesi*.

A size-selected partial genomic library (Rassmann *et al.* 1991) was constructed using genomic DNA from ethanol-preserved mantle of *Loligo forbesi* extracted using a proteinase K, phenol–chloroform procedure (Jones *et al.* 1997). DNA from eight individuals was pooled to a total of 10 μg and digested with *Alu*I and *Rsa*I (Promega). DNA fragments of 300–600 bp were selected by electrophoresis and ligated into the PCRscript vector (Stratagene) following the manufacturer's instructions for cloning blunt-ended polymerase chain reaction (PCR) products. The ligation was used to transform strain XL-1 blue competent cells (Stratagene). Colonies positive for recombinant plasmids

Table 1 Microsatellite loci in *Loligo forbesi*. Core repeat motif, primer sequences (F, forward; R, reverse; RB, 'PIG-tailed'), optimal Mg^{2+} concentration and annealing temperature (T), and expected product size from cloned alleles are given for six loci. Number of alleles, observed (H_O) and expected (H_E) heterozygosity detected within a sample of 46 individual *L. forbesi* are given. Number of alleles found at each locus, and at seven of the previously reported *L. forbesi* loci (Shaw 1997; Shaw *et al.* 1999) in 12 individuals of *L. vulgaris* are given where clean products were obtained. Nucleotide sequences for cloned fragments have GenBank/EMBL accession numbers AF167997 (Lfor11) to AF168002 (Lfor16), respectively

Locus	Repeat motif	Primers	Mg^{2+} (mM)	T (°C)	Product size (bp)	No. of alleles	H_E (%)	H_O (%)	No. of alleles (*L. vulgaris*)
Lfor11	$CTT_{13}ATT_{13}$	F: GAGGAAATGCTTCATAAC	2.0	55	219	23	93.4	95.6	—
		R: CTGAGGAGACTTGCTGAC							
Lfor12	TTC_{29}	F: TGCCCAAAATACTAAACCAC	1.5	57	247	31	96.6	87.0	—
		R: CGACTCCCCTGGCTGACCAA							
Lfor13	CT_{18}	F: TCATGGATTGTTAATTTTAAGG	2.0	55	234	12	89.5	82.6	monomorphic
		R: TCAAGGCAGTTACATACAGG							
		RB: GTTTCTTCAAGGCAGTTACATACAGG							
Lfor14	CT_{38}	F: CTGCCATACCACCAACATTC	1.5	55	241	21	91.2	91.3	3
		R: AGCGAAAACCTACAACAATC							
		RB: GTTTCTTAGCGAAAACCTACAACAATC							
Lfor15	TA_{14}	F: ACATTCTCTACTACTTGGAC	2.0	55	275	21	86.5	82.6	—
		R: ATTTTTCAACAGTTTCTTTTAG							
		RB: GTTTCTTATTTTTCAACAGTTTCTTTTAG							
Lfor16	$CT_{14}AT_1CT_6$	F: TCCAAATAAGTCTAAAAAGCC	2.0	57	157	11	88.0	80.4	5
		R: GGCACAGATGACACGAGAG							
		RB: GTTTCTTGGCACAGATGACACGAGAG							
Lfor1*									13
Lfor2									—
Lfor3		Shaw (1997)							14
Lfor4		Shaw *et al.* (1999)							—
Lfor5									14
Lfor6									10
Lfor7									—

were streaked onto gridded plates (2802 colonies in total). Colony lifts were probed with a number of repeat motif oligonucleotides (AAG, AAC, GAAA, CAAA) and dinucleotide polymers (AG/CT, AC/GT) (Shaw 1997; Piertney *et al.* 1998). Colonies positive for repeat motifs were used as template for PCR using primers corresponding to the T3 and T7 promoter sites on the PCRscript vector. Sequencing of the PCR products was performed by cycle sequencing using the ABI Prism Big-Dye terminator kit (PE Applied Biosystems) which were run on an ABI Prism 377 DNA sequencer, according to the manufacturer's instructions. Primers were designed from the flanking sequences surrounding a range of microsatellite repeats (Table 1). Forward primers of optimized primer sets were labelled with fluorescent dyes compatible with the PE Applied Biosystems Genescan system and alleles were scored from 4% acrylamide denaturing gels run for 1 h at 750 V on an ABI Prism 377 DNA sequencer.

PCR reactions were performed using the following cycling conditions: 2 min at 94 °C, 30 cycles of 1 min at 92 °C, 1 min at the annealing temperature (Table 1) and 10 s at 72 °C, under block control on a Hybaid 'PCR express' thermal cycler. A second program resulted in higher yields for some loci: 2 min at 92 °C, 10 cycles of 92 °C for 30 s, 30 s at 55 °C reducing by 0.5 °C per cycle, then 25 cycles of 92 °C for 30 s and 55 °C for 30 s, and a final extension for 5 min at 72 °C. Reaction mixes (10 μL) contained 20 ng of DNA, each deoxynucleotide triphosphate at 200 μM, each primer at 500 nM, 0.02 units of *Taq* polymerase (Bioline), 1× PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 0.01% Tween-20) and $MgCl_2$ at concentrations given in Table 1. 'PIG-tailed' (Brownstein *et al.* 1996) reverse primers (RB) were also tested, and worked under lower stringency than the conventional (R) primers. The product sizes obtained with each of the RB primers were fully consistent with those obtained using the R primer, suggesting that there was no allele size variation caused by unpredictable terminal 'A' addition.

The results of the PCR optimization and screening for variation within a sample of 46 individuals are presented in Table 1. Each primer set, and primers from seven loci reported by Shaw (1997) were also tested on DNA extracted from 12 individuals of the closely related *Loligo vulgaris*. Seven of the 13 primer sets were optimized with these samples, six of which were variable (Table 1). These preliminary data suggest that the loci should also be useful for the study of population genetics in *L. vulgaris*.

Acknowledgements

This work was conducted under NERC DEMA programme grant GST/02/1723.

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Isolation of microsatellite loci for paternity testing in *Phillyrea angustifolia* L. (Oleaceae)

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Keywords: microsatellite markers, Oleaceae, *Phillyrea angustifolia*

Received 4 July 1999; revision accepted 31 July 1999

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Male and hermaphrodite individuals co-exist within the same population of *Phillyrea angustifolia* and hermaphrodites produce viable pollen (Lepart & Dommée 1992). However, effective male function of hermaphrodites in populations of *P. angustifolia* is still debated and a direct estimation of male reproductive success via paternity analysis is necessary. Microsatellite loci have been proved to be useful for paternity testing in trees (Chase *et al.* 1996). Here we report 10 primer sets designed to amplify $(GA)_n$ and $(ATT)_n$ microsatellites in *P. angustifolia*.

Nuclear DNA was extracted (Pillen *et al.* 1992) from one individual, digested with the restriction enzyme *Asc*I, and size-fractionated on agarose gel according to Mörchen *et al.* (1996). DNA fragments ranging from 0.3 to 1.5 kb were ligated into *Eco*RI-cut dephosphorylated pUC19 cloning vector. After transformation into DH5α competent cells (Clontech Laboratories), 13 500 recombinant clones were transferred onto nylon membranes with a Biomek 1000 robot (Beckman) and hybridized with 13 ^{32}P-labelled oligonucleotide probes as described by Epplen (1992).

We obtained a total of 317 positive clones: 90 were revealed by the $(TC)_8$ probe, 81 by $(AAC)_5$, 49 by $(TAA)_6TA$, 16 by $(CA)_8$, 14 by $(GATA)_4$, 13 by $(AT)_{12}$, 12 by $(ACG)_5$, 10 by $(GGAT)_4$, 9 by $(GAA)_5$, 8 by $(CAC)_5$, 8 by $(CT)_4(CA)_5$, 4 by $(GCC)_5$, and 3 by $(TAC)_5$. We selected 74 clones and sequenced them on a Li-Cor automated DNA sequencer 4000L, using the Sequitherm Long-Read Cycle Sequencing kit (Epicentre Technology). Eighteen clones contained repeats of at least nine di- or six trinucleotides. Primer pairs were designed using PRIMER3 (Rozen & Skaletsky 1998) with the following parameters: melting temperatures (50–60 °C), GC content (25–60%), and no. 3 GC clamps.

Total DNA was extracted from frozen leaves using a SDS–PVP method (Saumitou-Laprade *et al.* 1999). Polymerase chain reactions (PCR) were carried out in a total volume of 15 μL containing 10 ng of template DNA, 3.5 mM $MgCl_2$, 200 μg/mL of BSA, 100 μM dNTP, 0.2 μM each primer (one of which was fluorescence labelled either with IRD-700 or IRD-800), 20 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.4 units of Ampli-Taq DNA polymerase (Perkin-Elmer). PCR was carried out on a Perkin-Elmer Gene-Amp system 9600 (95 °C, 5 min, followed by 94 °C, 45 s, annealing temperature (Table 1), 45 s, 72 °C, 30 s, for 30 cycles, final extension 72 °C, 2 min. Amplification products, loaded on 7% denaturing polyacrylamide gels, were analysed on a Li-Cor automated DNA sequencer 4200. For each locus, the allele size was determined relative to the sequenced positive clone using a pUC19 sequence as one nucleotide size standard.

Amplification and polymorphism were tested on a sample of 16 individuals from Languedoc-Roussillon (southern France). Of the 18 primer sets tested, 10 revealed a polymorphic locus (Table 1). These 10 loci were analysed on 33–43 individuals collected in two small populations in Les Salins de Giraud (Camargue, southern France). Number of alleles and allele size range were scored, and values of H_E and H_O were determined using FSTAT software version 2.3 (Goudet 1995). Of the 10 loci presented, seven exhibited more than five alleles, and five loci showed significant differences between expected and observed heterozygosity. These differences were always due to an excess of homozygotes. In addition, we also investigated whether the primers amplify in four other Oleaceae species: *Olea europea*, *Ligustrum vulgare*, *Jasminum fruticans*, and *Fraxinus excelsior*. With the exception of one locus, amplification was successful at least in one of the tested species (Table 2).

Table 1 Primers and characteristics of the polymorphic microsatellite loci in *Phillyrea angustifolia*. Allele size range in base pairs (bp) and number of alleles, gene diversity (H_E), observed frequency of heterozygotes (H_O), and test for Hardy–Weinberg deviation (*P*-values) within the two Salins populations

Accession nos	Locus	Repeat sequence	Primer sequence (5′ to 3′)	Annealing temp. (°C)	Size range (bp)	No. of different alleles	H_E	H_O	*P*-values	No. of individuals tested
AF145338	PA(GA)$_1$	(AG)$_{32}$	F: TTTTGAGGATCATTAGCAGCC R: AAATTGGAAATGGATTCCTCT	56	83–117	12	0.809	0.725	0.061	40
AF145339	PA(GA)$_2$	(GT)$_5$(GA)$_{17}$	F: AAGGAAGATGGGTCTTTGGG R: ATTCAGCTCCTCCACCTTCA	60	111–115	3	0.497	0.558	0.906	43
AF145340	PA(GA)$_4$	(AG)$_{25}$	F: CGACGGTCGTAGAATTGGA R: CAATCTACCCACGCCCTAAC	65	58–114	17	0.92	0.636	0.001	33
AF145341	PA(GA)$_5$	(GA)$_{12}$	F: TCTTTGCTTCGTTGCTTTTG R: TCTTGCTCCCTCGACATTTT	56	114–156	12	0.88	0.860	0.281	43
AF 145342	PA(GA)$_6$	(GT)$_{14}$(GA)$_{13}$	F: AGAGAGAGTTGGGAAAGGGG R: AGATTCAGATGCCGAGGATG	56	103–137	14	0.881	0.814	0.13	43
AF145343	PA(GA)$_8$	(AG)$_{30}$	F: TTGTAGTTGGCCATCATATACATTC R: TCACATTGAGATGATAATACGAAAGTT	55	129–178	15	0.904	0.559	0.001	34
AF145344	PA(GA)$_9$	(GA)$_{11}$	F: CAACACTCAACAGCCACCAC R: GGACCGTCATTATGTGAGGC	61–56*	144–166	10	0.831	0.595	0.001	42
AF145345	PA(GA)$_{11}$	(GA)$_{13}$	F: TTCCAAAACTCGTTCTCTGATTC R: GGTTACAGCAGTAGTGAGGAGC	56	106–117	5	0.466	0.561	0.991	41
AF145346	PA(ATT)$_1$	(TTA)$_{11}$	F: TTCACCCCGTTCAGTTTTTC R: AGAAGCCGAGTAATGAAATTGC	55	71–147	20	0.928	0.690	0.001	42
AF145347	PA(ATT)$_2$	(TAA)$_6$	F: CACCTCCCGGTTAACAAGAA R: TGACGCGGTTATTTTGTGAA	60	115–136	3	0.193	0.116	0.025	43

* Touch down.

Table 2 Cross-species amplification with the 10 pairs of *Phillyrea angustifolia* primers. An individual of each species was tested

Species	PA(GA)$_1$	PA(GA)$_2$	PA(GA)$_4$	PA(GA)$_5$	PA(GA)$_6$	PA(GA)$_8$	PA(GA)$_9$	PA(GA)$_{11}$	PA(ATT)$_1$	PA(ATT)$_2$
Olea europea	1	1	m	1	m	0	m	1	m	1
Ligustrum vulgare	m	1	0	1	m	0	2	1	0	0
Jasminum fruticans	0	0	1	2	m	0	0	2	2	0
Fraxinus excelsior	1	2	1	2	1	0	1	2	1	1

0, 1, 2: no, one, or two PCR products detected; m, multiple banding pattern.

Acknowledgements

This research was supported by a Procope research contract no. 96038. We thank Jacques Lepart for providing the plant material, Monika Mörchen for her help in establishing the genomic bank and Carole Raby for her help in optimization of the PCR conditions.

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Variable microsatellite loci for the leafcutter ant *Acromyrmex echinatior* and their applicability to related species

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Keywords: *Acromyrmex echinatior*, allele frequency differences, leafcutter ants, microsatellite primers

Received 18 June 1999; revision accepted 21 August 1999

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Ants of the tribe Attini are limited to the Neotropics. The 12 known genera comprise approximately 200 species which are all special among the Formicidae because of their ectosymbiotic interaction with a mutualistic fungus (Cherrett *et al.* 1989). This 'unholy alliance' is a profound evolutionary and ecological success story. Leafcutter ants are dominant herbivores in many neotropical ecosystems and genetic studies of their phylogeny and social organization have recently advanced considerably (e.g. Schultz 1999). The genera *Acromyrmex* and *Atta* cover a diverse array of social organization, with some species having multiple queens in at least some of their colonies (Bekkevold *et al.* 1999), whereas other species exhibit the highest level of queen mating so far known in ants (Fjerdingstad *et al.* 1998; Boomsma *et al.* 1999). The recently estimated degree of multiple mating in *Acromyrmex* (Boomsma *et al.* 1999) was determined with genetic markers of low variability (2–3 allozymes and one moderately variable microsatellite marker). This produced accurate estimates of relatedness but could not avoid relatively high nondetection errors of multiple paternity (Boomsma & Ratnieks 1996). To increase the resolution of such studies in the future and to be able to test hypotheses for the evolution of multiple queen-mating more accurately, we describe here five polymorphic microsatellite markers for *Acromyrmex echinatior* and report on their applicability and polymorphism in two other sympatric *Acromyrmex* species, *A. octospinosus* and *A. insinuator*. *Acromyrmex insinuator* is a recently described social parasite of *A. echinatior*, a host species that was previously known only as a form of *A. octospinosus* (Schultz *et al.* 1998).

DNA was extracted by high-salt purification from *A. echinatior* workers preserved in ethanol. The genomic library was constructed by ligating size-selected fragments (200–500 bp) from a *Sau*3A1 digest into the plasmid vector pBluescript II SK+ which was cut with *Bam*Hl. Vectors were transformed into competent *Escherichia coli* cells and recombinants were identified by blue–white selection. A total of 2000 recombinant clones was transferred onto nylon membranes which were hybridized with a mixture of three DIG-labelled oligonucleotide probes (CT_{10}, GT_{10} and ATT_{10}). This screening yielded 56 clones with a positive signal. Inserts of the putative microsatellite-containing clones were amplified with T7 and reverse primers. The amplification products were run on a 1%

Microsatellite	Repeat in clone	Primers (5 –3)	T_a (°C)
Ech1390	$(GA)_{13}$	F: CTCCACATTGACACACTACTCG	53
		R: GACGAAAAGAGAGGTAATCG	
Ech4126	$(AC)_{11}(AT)_5$	F: GTTCATTCACGAGACCTGTAA	59.5
		R: GAACGTCAGTTTTCACGACA	
Ech3385	$(CA)_6TAGCA_3CATA(CA)_8$	F: TGAAAGTAGAAACACGGATGA	53
		R: GCCCTAGAACAAATTACATCG	
Ech4225	$(CA)_{17}$	F: CTTTTCTCCATTTCTTCGTG	52
		R: AGAGGGATAAAGTGGAAAATAA	
Ech3197	$(AC)_5A_3(AC)_8$	F: AAGATGATTTAGCTAAGGGAAG	52
		R: CGCCAGATTATTACAAAGTGA	

Table 1 Microsatellite loci isolated from *Acromyrmex echinatior* and annealing temperatures (T_a). Cloned sequences have been registered with the GenBank database under the Accession nos AF160985–AF160989

Table 2 Polymorphism of five microsatellite loci in three *Acromyrmex* species. Number of alleles (N_A), allele size range (bp) and expected and observed heterozygosity (H_E and H_O, based on diploid individuals) are given. Sample sizes: *Acromyrmex echinatior* ($n = 32/30/16$, queens + workers/males/colonies), *A. octospinosus* ($n = 30/8/9$) and *A. insinuator* ($n = 8/4/5$). H_E and H_O could not be calculated for Ech4225 in *A. insinuator* because amplification failed in most of the diploid individuals tested

	Acromyrmex echinatior				*A. octospinosus*				*A. insinuator*			
Locus	N_A	Size range	H_E	H_O	N_A	Size range	H_E	H_O	N_A	Size range	H_E	H_O
Ech1390	3	107–111	0.516	0.384	15	107–149	0.851	0.880	3	107–131	0.227	0.500
Ech4126	8	151–189	0.708	0.642	14	151–195	0.841	0.785	3	157–169	0.408	0.0
Ech3385	7	139–177	0.729	0.588	13	143–199	0.831	0.637	4	159–211	0.582	0.333
Ech4225	9	281–327	0.785	0.333	20	285–331	0.909	0.428	4	301–331	—	—
Ech3197	1	123	0.0	0.0	3	121–125	0.609	0.127	2	123–129	0.197	0.0

agarose gel, cut out and purified with a QIAquick gel extraction kit (Qiagen). Sequencing of this DNA was performed on an automated sequencer (ALFexpress™, Pharmacia-Amersham) with Cy5-labelled T3 and/or KS primers. Fifteen clones were found to contain microsatellite sequences and for five of them primers were designed using the Software Oligo 4.1 (Table 1). Routine amplification of all *Acromyrmex* samples was performed with the following conditions for 6 µL reactions: 0.1 U *Taq* polymerase (Pharmacia-Amersham), 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.0) 0.2 mM dNTPs, 2 pmol of each primer and 1 µL of genomic DNA, run for 3 min at 94 °C, 30 s at 93 °C, 30 s at the annealing temperature (Table 1), 40 s at 72 °C, then 39 cycles of 30 s at 93 °C, 7 min at 72 °C and finally cooling down to 10 °C. Allele sizes were scored by comparison with internal size markers using the software Allele Links (Pharmacia-Amersham).

The utility of the newly obtained microsatellite markers was tested by genotyping queens, males and adult workers from ant colonies of three *Acromyrmex* species commonly found around Gamboa, Panama and collected there in 1996 and 1998 (Table 2). Four out of the five loci were polymorphic in all species tested. Mean expected heterozygosities ranged from 0.2 to 0.9 (Table 2). In contrast to the expectation that they should be most variable in the species from which they had been isolated (e.g. Ellegren *et al.* 1997), all markers tested here were most variable in *Acromyrmex octospinosus*. A possible explanation might be that *A. echinatior* is less common and more patchy in its distribution than *A. octospinosus*, so that genetic drift may have eroded variation. The observed even lower genetic variation in the social parasite *A. insinuator* would be consistent with this hypothesis. Given their high variability across species, the five microsatellite markers described here may prove to be variable in other *Acromyrmex* species as well.

Acknowledgements

We thank Palle Villesen, Jane Frydenberg and Camilla Håkanson for help and advice in the laboratory, the Smithsonian Tropical Research Institute for providing working facilities in Panama and Panama's INRENARE for permission to sample and export ants. Funding was obtained through the research network 'Social Evolution' financed by the European Commision via the Training and Mobility of Researchers (TMR) programme.

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Isolation and characterization of microsatellite loci in the Pacific giant salamander *Dicamptodon tenebrosus*

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Keywords: Caudata, *Dicamptodon tenebrosus*, microsatellite loci, Pacific giant salamander, primers

Received 27 June 1999; revision accepted 13 August 1999

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The Pacific giant salamander (*Dicamptodon tenebrosus*) is a fossorial species that inhabits forested areas ranging from northwestern California to southwestern British Columbia. It is considered an obligate old growth species and breeds in small, fast-flowing mountain streams (Nussbaum *et al.* 1983). In British Columbia, *D. tenebrosus* is restricted to the Chilliwack River and part of the Fraser River drainage basins, which are largely disturbed and fragmented by agriculture and silviculture. The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) recognizes *D. tenebrosus* as vulnerable to deforestation and habitat fragmentation, which are among the leading factors linked to global amphibian decline (Duellman & Trueb 1986; Blaustein & Wake 1995).

A genetic study was initiated in 1998 in order to assess the impacts of current forest practices on the population structure and movement patterns of *D. tenebrosus* in British Columbia. Microsatellite markers were chosen for this study on the basis of their sensitivity to fine population structure and minute tissue requirements (Bruford *et al.* 1996). We report the development and characterization of six polymorphic microsatellite loci in *D. tenebrosus*.

Genomic DNA was extracted from ethanol-preserved tail fin tissue from three larval *D. tenebrosus* using standard phenol–chloroform extraction. Approximately 150 μg of DNA were digested with *Alu*I, *Hae*III, *Hin*cII and *Rsa*I, and fractionated on a 1% low-melting-point agarose gel. Fragments ranging in size from 200 to 700 bp were recovered from the gel with β-agarase I (New England Biolabs). Putative microsatellite-bearing fragments were cloned and isolated as in Glenn (1995). Approximately 5000 clones from two partial genomic libraries were screened with six [γ^{32}P]-dATP labelled oligonucleotides; $(GT)_{15}$ $(GTGA)_8$ $(GACA)_8$ $(AAG)_{10}$ $(TAA)_{10}$ and $(TAG)_{10}$. Twenty-five distinct microsatellite loci were isolated and sequenced using an ABI 377 Prism automated sequencer. Primers for PCR amplification were designed using the programs OSP (Hillier & Green 1991) and Primer3 (Rozen & Skaletsky 1998). PCR conditions were initially optimized on 1% agarose gels using $MgCl_2$ and annealing temperature (T_a) gradients. Final PCR assays were performed on a PTC-100™ Programmable Thermal Controller (MJ Research), with 100 ng of template DNA and 0.5 U *Taq*

Table 1 Primer sequences, repeat array of cloned allele, number of detected alleles (*A*), size range of PCR products in base pairs, annealing temperature (T_a), and expected (H_E) and observed (H_O) heterozygosity. Polymorphism data are based on screening of 72 individuals

Locus	Primer sequences (5 –3)	Repeat array	*A*	Product size range	T_a (°C)	H_E	H_O	Accession no.
*Dte*4	F: TGCTTCTGCCACCATAGCC R: AGAGCCAGCCTTTGTTGCG	$GT(GC)_3(GT)_3(GCGT)_2GTG$ $G(GCGT)GC(GCGT)_2(GT)_8$ $GC(GCGT)_2GTGC$	17	163–229	62	0.496	0.571	AF149305
*Dte*5	F: GGAGGAGTTTTTGAAGTTG R: ATTCTCCAAACATTCTCCC	$(AG)_3CG(AG)_4AA(AG)_{17}$	4	186–216	55	0.220	0.075	AF150725
*Dte*6	F: GGTAGTCATGGTGATGCTG R: CACTCCCCTATTCTCCCTAC	$CACTA(CA)_{18}$	10	134–172	55	0.151	0.033	AF150726
*Dte*8	F: CTGCATACATTGCATCTCCG R: CCGCAAGGTCATCTTCACTAAC	$(CT)_{16}$	7	176–192	55	0.223	0.198	AF150727
*Dte*11	F: ACACATGGTTGCTCACTC R: TAGTGTGTGGCATTAAGGG	$(GT)_3(CA)_6CG(CA)_4CG(CA)_5(CT)_3$	12	88–116	62	0.379	0.228	AF150728
*Dte*14	F: AGGAGTGAGACAGGGTGAGC R: CACCTCTCCTCCTCTTCCAG	$(GA)_{17}$	10	118–142	62	0.375	0.500	AF154879

Table 2 Number of alleles successfully amplified in 18 closely and distantly related species of salamanders. *N* refers to the number of individuals assayed per species

Family and species	*N*	Dte4	Dte5	Dte6	Dte8	Dte11	Dte14
Ambystomatidae							
Ambystoma californiense	3	2	1	—	3	—	—
A. cingulatum	3	2	1	—	2	—	—
A. gracile	2	2	1	—	2	—	—
A. laterale	2	1	1	—	3	—	—
A. mexicanum	2	1	1	—	2	—	—
A. texanum	3	2	1	—	1	—	—
A. tigrinum	3	2	1	—	1	—	—
Cryptobranchidae							
Andrias davidianus	3	2	1	—	2	—	—
Dicamptodontidae							
Dicamptodon atterimus	3	1	1	1	1	1	1
D. copei	6	1	3	—	1	2	1
D. ensatus	6	1	1	—	4	—	—
Plethodontidae							
Aneides ferreus	3	1	1	—	3	—	—
Desmognathus monticola	3	3	1	—	1	—	—
Eurycea bislineata	3	2	1	—	2	—	—
Plethodon vehiculum	3	3	1	—	2	—	—
Speleomantes flavus	3	3	1	—	3	—	—
Salamandridae							
Paramesotriton sp.	3	2	1	—	1	—	—
Triturus carnifex	2	1	1	—	2	—	—

DNA polymerase (Gibco/BRL) in 10 μL of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 800 μM dNTPs, 0.5 pmol of [γ^{32}P]-dATP labelled forward primer, 2.5 pmol unlabelled forward primer, and 6 pmol of reverse primer. The PCR profiles for each locus consisted of one cycle of 2 min denaturation at 95 °C, 1 min annealing at T_a + 2 °C (see Table 1 for T_a), and 1 min extension at 72 °C, five cycles of 1 min at 94 °C, 1 min at T_a + 1 °C, and 1 min at 72 °C, and 25 cycles of 45 s at 92 °C, 30 s at T_a, and 30 s at 72 °C. The last cycle was followed by a final 10 min extension at 72 °C. PCR products were separated on 6% denaturing polyacrylamide gels and autoradiographed. Alleles were scored using a standard M13 sequence.

A total of 72 *D. tenebrosus* samples (1–14 larvae from 11 Washington and Oregon populations) were assayed to assess levels of polymorphism at each locus. Unambiguous PCR products of the expected size were successfully amplified at 11 loci. Of these, six were polymorphic with 4–17 alleles (Table 1). Populations with > 10 samples were tested for deviations from Hardy–Weinberg expectations at each locus using the software TFPGA 1.3 (Miller 1997). *P*-values ranged from 0.089 to 1.0 across all loci and populations. Expected (H_E) and observed (H_O) heterozygosity calculated across all samples ranged from 0.151 to 0.660 and 0.033 to 0.449, respectively. Homologous products were successfully amplified in 18 closely and distantly related species under the same PCR conditions (Table 2). All species except *D. copei*, *D. ensatus*, and *D. atterimus* shared the same most common allele as *D. tenebrosus* at locus *Dte*4, *Dte*5 and *Dte*8.

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) currently regulates several endangered salamander species including *Andrias davidianus* and *Ambystoma mexicanum*. The six microsatellite markers reported here may be useful tools for assessing the population structure of *D. tenebrosus* and related salamander species of conservation concern.

Acknowledgements

We thank Robert Murphy, Ross McCaulloch (Royal Ontario Museum), Jacquelyn Brinkman, David Green (Redpath Museum), Tom Titus (University of Oregon), and John Richardson (University of British Columbia) for kindly providing salamander tissue samples. We also thank members of the 'Taylor laboratory' for all their pearls of wisdom. This research was supported by grants from FRBC, HCTF, and WWF, an NSERC grant to EBT, and the Department of Zoology, UBC.

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