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PRIMER NOTES

Microsatellite loci to determine population structure of the Patagonian toothfish *Dissostichus eleginoides*

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The Patagonian toothfish (*Dissostichus eleginoides*), found in sub-Antarctic waters, is currently being fished off the east and west coasts of South America and on sea-mounts and submarine ridges of the Indian Ocean. The species is dispersed throughout the Southern Ocean, crossing a number of political and management zones. With little known of its migrations or life cycle, the extent to which the populations are separated is poorly understood (CCAMLR 1995). However, a knowledge of population distribution is vital for the effective management and sustainability of this fishery.

Microsatellites are polymorphic markers, useful for population genetics studies, including analysis of fishery stock structures (O'Connell & Wright 1997). We report here the isolation and characterization of five *D. eleginoides* microsatellite loci. To evaluate their suitability for population assessment, each microsatellite locus was analysed in samples from one of the main Australian fishing grounds, Macquarie Island.

A genomic DNA library was constructed from DNA extracted from muscle tissue of a single individual (Sambrook *et al*. 1989). DNA was digested with *Sau*3A enzyme and the 500–750 bp fraction ligated into the dephosphorylated *Bam*HI site of the vector, pGEM™28–3Zf(+) (Promega) (Reilly *et al*. 1999). Ligated plasmids were transformed into XL-1 Blue supercompetent cells (Stratagene). Cells were plated onto selective media and replicated onto uncharged nylon membrane filters (Boehringer Mannheim).

The library, consisting of approximately 25 000 recombinant clones, was screened for microsatellite repeats with a $(CA)_9$ probe, and later with a combined $(AAT)_9$ and $(AAC)_8$ probe. Probes were 3′-end-labelled with digoxygenin molecules, and standard hybridization and wash conditions were used (Boehringer Mannheim 1995). The nucleotide sequence of positive candidate clones was determined with ABI Prism™ Dye Terminator Cycle Sequencing (Perkin-Elmer) using double-stranded plasmid DNA, prepared by the alkaline lysis method (Sambrook *et al*. 1989). Sequencing products were analysed on an ABI377 Prism DNA autosequencer. PCR primers were designed for unique flanking regions of the microsatellite repeats. Oligonucleotides were synthesized by Bresatec, and one primer from each pair was labelled with a fluorescent tag.

For microsatellite analysis, total genomic DNA was extracted from 50 mg of alcohol-preserved muscle samples (Sambrook *et al*. 1989). PCR amplifications were performed in a Perkin-Elmer thermocycler 9600 as 25 μL reactions containing 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 0.2 mg/mL gelatin; 1.5 mM or 2.5 mM $MgCl_2$ (Table 1); 0.4 μM of each primer; 200 μM dNTPs; 0.5 units *Taq* F1 DNA polymerase (Fisher Biotech); and ≈ 20 ng genomic DNA template. Denaturation for 3 min at 95 °C was followed by 35 cycles made up of 30 s at 96 °C, 30 s at the annealing temperature (Table 1) and 1 min at 72 °C. The final step was a prolonged extension of 20 min at 72 °C. The amplified products were diluted, mixed with formamide loading-dye containing Tamra size standards (ABI), heat-denatured and then loaded on a 4% denaturing acrylamide gel. The samples were run on an ABI377 Prism DNA autosequencer and analysed with accompanying software (Genotyper® 2.0).

Between 15 and 17 fish were examined from each of two sites about 60 km apart off Macquarie Island. Of 11 loci tested, five were consistently scorable with suitable levels of polymorphism for a population-structure study. Genotypes for each locus were determined and allele frequencies estimated. Each of the five loci was highly polymorphic,

Table 1 Characteristics of the five Patagonian toothfish microsatellite loci developed

Locus	Primer (5′–3′)	$[MgCl_2]$ (mM)	Repeat motif	Alleles (*n*)	Anneal temp. (°C)	GenBank Accession no.
cmr*De*4	GCCTTCCCAAACCTGAGC ACCCCCTCATCCCAACAC	2.5	$(CAA)_8$	12	52	AF105071
cmr*De*13	GAGAGAAGACAGGATAAACAC TGGCTAAAGCCTTTTTAAC	2.5	$(CAA)_7$	7	48	AF105072
cmr*De*9	TGAGGAGCATCCTAATAC AACCAATAGAATCCAGAG	2.5	$(CA)_{32}$	24	48	AF105073
cmr*De*30	CACTGACCTTTAACCTGCG CCAGCCAAAAAACCTCAC	1.5	$(CA)_{14}$	9	50	AF105074
cmr*De*2	GAGACCTCTGACAGGGTAG TGACAGATGTTTTCTGATTAAG	1.5	$(CA)_{29}$	16	50	AF105075

Table 2 Data analysis of the five microsatellite loci at two sites off Macquarie Island. The probability of intersample homogeneity (*P*) is shown along with calculated Hardy–Weinberg observed (H_O) and expected (H_E) heterozygosity values and their associated *P*-value, HW(*P*)

Locus	Site	Sample size	Alleles (*n*)	Allele size range (bp)	Homogeneity* (*P*)	H_O	H_E	†HW (*P*)
cmr*De*4	1	17	11	266–299	0.244	0.882	0.849	0.999
	2	15	9	260–287		0.933	0.851	0.999
cmr*De*13	1	17	6	171–189	0.025	0.588	0.557	0.118
	2	17	6	174–189		0.588	0.590	0.570
cmr*De*9	1	17	13	212–270	0.135	1.000	0.905	0.995
	2	15	18	216–284		1.000	0.904	0.996
cmr*De*30	1	16	7	161–181	0.046	0.813	0.660	0.515
	2	17	7	165–181		0.588	0.651	0.038
cmr*De*2	1	17	14	119–147	0.637	1.000	0.898	0.998
	2	17	14	121–149		0.824	0.917	0.996

*Calculated according to Roff & Bentzen (1989).
†Calculated according to Zaykin & Pudovkin (1993).

displaying between seven and 24 alleles. A Monte-Carlo χ^2 statistical test (Roff & Bentzen 1989) was used to determine the homogeneity of allele frequencies at the two sites (Table 2). The allele frequencies were different ($P < 0.05$) at two loci (cmr*De*13 and cmr*De*30), suggesting that the two samples may not be homogenous. Combining probabilities from all loci (Sokal & Rohlf 1981) gave a *P*-value of 0.019, also indicating that the two samples may be different. A Monte-Carlo χ^2 test (Zaykin & Pudovkin 1993) was used to determine whether the observed genotype frequencies in each sample accorded with Hardy–Weinberg equilibrium. With the exception of locus cmr*De*30 at site 2 ($P = 0.038$), no samples deviated significantly from Hardy–Weinberg expectations. Larger sample sizes are required for a more accurate assessment of population structure.

The markers we developed show considerable promise as a means to discriminate stock structures of Patagonian toothfish. They would be useful not only for the management of the Australian toothfish fisheries at Macquarie and Heard Islands, but also to help regulate other international sub-Antarctic fisheries of this species.

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Interspecific variation in microsatellites isolated from tuco-tucos (Rodentia: Ctenomyidae)

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Table 1 Characteristics of microsatellite loci isolated from *Ctenomys haigi*. T_a is the annealing temperature used in PCR reactions. Repeat motifs were sequenced from cloned PCR products, as described in the text. For each locus, the repeat motif for *C. haigi* is listed first, followed by the repeat motif for *C. sociabilis*. Data on allelic variability and expected and observed heterozygosities are based on a sample of 15 adults per species. GenBank Accession nos are shown

Locus	Primer sequences (5′–3′)	T_a (°C)	Repeat motif	No. of alleles detected	Product sizes (no. of bp)*	H_E	H_O	Genbank Accession no.
Hai1	TAGAAGTGGAAGGCATAGCTC	50	$(CA)_{21}$	8	119–143	0.84	0.79	AF093145
	AACTAGCCTGTATTACCATTC		$(CA)_3CG(CA)_6$	1	119	0.00	0.00	
Hai2	TCTGAGCTGTGTAGTGAGAGC	52	$(CA)_{15}$	6	180–192	0.76	0.67	AF093146
	GTAGTTTACAGGGTTTCTCCC		$(CA)_{13}$	1	172	0.00	0.00	
Hai3	CAATTGCCCATGCTTCTCTAT	60	$(CA)_{26}$	8	158–174	0.70	0.80	AF093147
	TGATCAGAGCTCGTTCTACCA		$(CA)_{26}$	1	174	0.00	0.00	
Hai4	CACCCTGTAGACCCTTCACTA	58	$(CA)_{18}$	9	158–184	0.77	0.81	AF093148
	CATCATCACTCTGGCTGAATC		$(CA)_{25}$	1	180	0.00	0.00	
Hai5	AGTTCACCATCACCCAGCAG	56	$(CA)_{17}$	3	199–213	0.44	0.46	AF093149
	ACCTATCGTGCCTCCAAGTGA		$(CA)_{10}$	1	195	0.00	0.00	
Hai6	CTTGTAGTGTAAATCCATGCTCAT	54	$(CA)_{21}$	4	128–146	0.50	0.53	AF093150
	CTCAGTGGAGTAAGTTGCCTG		$(CA)_8$	2	112–114	0.07	0.07	
Hai7	TTCTCATTCCACTGACTTCATTC	62	$(CA)_{23}$	7	187–203	0.71	0.73	AF093151
	TCACAATGCTGCTGCTGA		$(CA)_{25}$	4	199–225	0.63	0.57	
Hai8	GAAAAGAGTGAGAGTGCCAACT	56	$(CA)_{22}$	10	125–153	0.79	0.88	AF093152
	CTAAGTGTTGATGGAGCAAGG		$(CA)_{17}$	1	135	0.00	0.00	
Hai9	TCCAAAGGAATTGCAGACAAG	56	$(CA)_{11}$	3	213–217	0.50	0.67	AF093153
	TGAGGCTAAACGATGGGGTAT		$(CA)_{14}$	1	221	0.00	0.00	
Hai10	AGAGTGACCACGAACATGGA	58	$(CA)_9AT(CA)_8$	3	305–313†	0.13§	0.20	AF093154
	AGTTAGCTGAGAAACATGGGTG		$(CA)_{21}$	3	313–321	0.34	0.20	
Hai11	AAAAGGAAAGAGAAGCTAGA	60	$(CA)_{26}$	9	172–194	0.84	0.87	AF093155
	TGAAGATGGTAGTGTTACTGTC		$(CA)_{20}$	2	180–182	0.13	0.13	
Hai12	TACATACCCAGTCCTGATGTGG	56	$(ATT)_{15}$	6	123–138	0.76	0.75	AF093156
	ACGCCTGTAATCCCAGAGCT		$(ATT)_{12}$	1	132	0.00	0.00	
Hai13	GCAGGCTAAGACAGCCATTAA	54	$(TA)_9(CA)_5TA(CA)_8$	6	139–153‡	0.51§	0.58	AF093157
	GACAGCTGGCCAAATTTTTC		$(CA)_5$	1	95	0.00	0.00	

*Determined using M13 mp18 sequence as a size standard.
†Total length variation over 3 alleles = 8 bp; portion of repeat motif that varies not determined.
‡Total length variation over 6 alleles = 14 bp; portion of repeat motif that varies not determined.
§Heterozygosity estimated assuming no size homoplasy of alleles.

Microsatellites are widely used in studies of population genetic structure due to their often high levels of intraspecific variability. Although the utility of these markers is increased if primers designed for one species are used to amplify loci from numerous taxa, concerns have arisen regarding interspecific differences in repeat motifs that may influence levels of variability (Estoup *et al*. 1995; van Treuren *et al*. 1997). In particular, interruption of simple dinucleotide repeats has been linked to decreased allelic diversity (e.g. Pépin *et al*. 1995), suggesting that microsatellite structure should be considered when choosing loci for interspecific comparisons of genetic variation.

In the current study, microsatellites were developed to compare population genetic structure in two species of ctenomyid rodents: the Patagonian tuco-tuco (*Ctenomys haigi*) and the colonial tuco-tuco (*C. sociabilis*). Microsatellite loci were isolated from a genomic library for *C. haigi*; the decision to use *C. haigi* as the source for microsatellites was based on the quality of genomic DNA extracted from three individuals of each species. *Sau*3AI-digested DNA from a single *C. haigi* was ligated into pBK-CMV phagemid vector (ZAP Express Vector Kit; Stratagene). Ligation products were packaged (Gigapack III Gold Packaging Kit; Stratagene), after which the packaged phage was incorporated into XL1-Blue MRF′ cells (Stratagene) that had been heat shocked at 37 °C for 15 min. The resulting library was screened using a $(CA)_{15}$ probe that had been end-labelled with [γ^{32}P]-ATP (Richardson 1965). Clones that scored positive during two successive rounds of screening were sequenced in both directions using M13 Reverse and T7 Promoter primers (United States Biochemical).

PCR primers were designed for 20 repeat-containing sequences using Primer 0.5 (Whitehead Institute, MIT). Thirteen of these primer pairs produced well-resolved PCR products for both *C. sociabilis* and *C. haigi* (Table 1). Amplification of microsatellites was carried out in a 15-μL volume containing

30 ng of DNA, 0.2 μM each primer, 0.2 mM dNTP, 1× *Taq* buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl), and 0.75 units of *Taq* polymerase (Boehringer Mannheim). Amplification conditions were 94 °C for 5 min, followed by 34 cycles of 94 °C for 30 s, T_a for 30 s, and 72 °C for 45 s, with a final extension of 72 °C for 5 min. Locus-specific annealing temperatures are given in Table 1. To assess allelic variation at each locus, DNA samples from 15 adults per species were amplified as above, but with one primer per locus end-labelled with [γ^{32}P]-ATP. PCR products were electrophoresed on 6% polyacrylamide sequencing gels and then visualized via autoradiography. Radioactively labelled M13 mp18 sequence was used as a size standard on all gels.

To compare repeat motifs in *C. haigi* and *C. sociabilis*, we cloned (TA Cloning Kit; Invitrogen) and then sequenced (T7 promotor primer) PCR products from one individual per species per locus. Repeat motifs at 10 (76.9%) of the loci surveyed did not differ between the study species (Table 1). In contrast, interspecific differences in repeat motif were detected for the three remaining loci (Hai 1, 10, 13). At each of these loci, one species exhibited a simple repeat motif while the other exhibited a compound motif composed of two or more distinct repeat segments. Two of these compound repeats were present in *C. haigi*, indicating that imperfect repeat motifs occurred in the source, as well as the non-source, species for microsatellites.

Although the effects of imperfect repeats on allelic diversity in tuco-tucos have yet to be determined, interrupted repeat motifs are thought to be associated with decreased levels of microsatellite variability in other taxa (Garza *et al*. 1995; Pépin *et al*. 1995; Jarne & Lagoda 1996). If perfect and imperfect repeats differentially affect allelic diversity then interspecific variation in repeat motifs may confound cross-taxa comparisons of genetic variability. Nearly 25% of the loci surveyed in this study exhibited interspecific differences in repeat motif, underscoring the need to examine microsatellite structure when comparing patterns of genetic variability among species.

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Microsatellites in grayling (*Thymallus thymallus*): comparison of two geographically remote populations from the Danubian and Adriatic river basin in Slovenia

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There are two geographically separate populations of grayling (*Thymallus thymallus*) in Slovenia inhabiting the Danubian drainage (Danubian type) and the Soča river system (Adriatic type). The two populations have their own phenotypic characteristics which make them morphologically different (Voljč & Ocvirk 1982). No detailed analysis has been performed to evaluate these differences at the molecular level, except for one microsatellite locus (Snoj *et al*. 1999). Intensive stocking of the Soča drainage with Danubian grayling during the last few years could cause an uncontrolled mixing of both populations and potentially threaten the existence of Adriatic grayling in Slovenia. To help differentiate these populations for conservation purposes and to assess the diversity between populations, we compared both types of grayling for genetic polymorphisms.

To identify genetic polymorphisms between the Adriatic and Danubian grayling, we examined several microsatellite loci. DNA was extracted from erythrocytes (Medrano *et al*. 1990) and a genomic library was constructed as described by Rassmann *et al*. (1991). Briefly, size-selected (200–800 bp) *Sau*3AI genomic restriction fragments were ligated into the *Bam*HI-restricted pBluescript vector (Stratagene) and propagated into Epicurian Coli Cells (Stratagene). Recombinant colonies were probed with $(CA)_n$ and $(AG)_n$ polymers. Screening of the library was performed with the Chemilluminescence

Table 1 Core sequences of microsatellite loci BFRO005, BFRO006, BFRO007, BFRO008 and BFRO009, primer sequences and GenBank Accession no.

Locus	Repeat	Primers*	GenBank Accession no.
BFRO005	$(CA)_{17}$	CGCATCTGTATGAAAAACCT	AF115407
		TGGTTTGGTAGGAGTTTCGT	
BFRO006	$(CA)_{15}$	GCCTGGTTTTACCCTTTAGA	AF115408
		AGGCATTTTACACTGGCATT	
BFRO007	$(AG)_{13}$	AGACCCCCAAAAACTATGCT	AF115409
		TAAGGTCCCCAACACTACGA	
BFRO008	$(CA)_{26}CG(CA)_{24}$	TCTCCCCACTGTAAGTACGC	AF128890
		GCATTGATTGTCCTACATTA	
BFRO009	$(CA)_8T(AC)_4T(AC)_8G(CA)_6$	AAATTGTCCCCGTTGGCAGA	AF128891
		ACATACACCGCAACACCCAG	

*The sequences of the forward (top) and reverse primers are given in the 5′ to 3′ orientation.

Quick-Light™ Genome Mapping Probe Kit (FMC, Bioproducts) according to the manufacturer's instructions. Plasmids from positive clones were cycle-sequenced using universal T3 and T7 primers and the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) following the manufacturer's protocol. The extension products were analysed using the ABI Prism 310 automated sequencer. Sequence data for the microsatellites studied have been deposited at the GenBank Data Library (Table 1).

Microsatellite polymorphisms were analysed for five loci (BFRO005, BFRO006, BRO007, BFRO008 and BFRO009) using primers designed from unique sequences flanking a microsatellite repeat. These loci were amplified using the polymerase chain reaction (PCR), with the profile of initial denaturation of 94 °C for 3 min and 30 cycles of 94 °C for 45 s, 55 °C for 15 s and 72 °C for 5 s, in the MJ Research PTC-100 Thermal cycler. All PCRs (10 μL) contained 50 ng of template DNA, 0.5 μM of each primer, 0.2 mm dNTP, 1.5 mm MgCl2, 20 mm Tris-HCl, 50 mm KCl and 0.5 U of Taq polymerase (PE Applied Biosystems). Aliquots of fluorescently labelled amplified DNA were mixed with formamide and GENESCAN-350 (TAMRA) Size Standard (PE Applied Biosystems) and genotyped on the ABI Prism 310 Genetic Analyser using the GeneScan™ Analysis Software 2.1.

Identified alleles at five microsatellite loci and their frequencies of the Adriatic type (34 animals) and three geographically remote populations of the Danubian type (49 animals) are given in Table 2. The loci BFRO005 and BFRO008 had eight and seven alleles and were moderately polymorphous with polymorphism information content (PIC) values (Botstein *et al*. 1980) of 0.486 and 0.675, respectively. The locus BFRO006 was characterized by three alleles (PIC = 0.371) and the loci BFRO007 and BFRO009 by two alleles exhibiting only a low level of polymorphism (PIC values of 0.189 and 0.211, respectively). Nevertheless, all five loci indicate genetic distinctiveness between the Adriatic and Danubian type of grayling, with nine alleles unique to the Adriatic type (see χ^2 and heterozygosity values in Table 2). However, only five alleles (111 at locus BFRO005, 140 at BFRO006, 176 at BFRO007, 233 at BFRO008 and 241 at BFRO009) were present in the Adriatic population

Table 2 Allele frequencies, observed and expected heterozygosity (H_O and H_E) at five microsatellite loci in the Adriatic and Danubian type of grayling in Slovenia

Locus	Allele (bp)	Adriatic	Danubian
BFRO005*	111	0.39	0.00
	119	0.03	0.04
	121	0.00	0.01
	123	0.06	0.00
	128	0.02	0.02
	130	0.42	0.83
	132	0.06	0.10
	134	0.02	0.00
H_O		0.67	0.30
H_E		0.55	0.30
BFRO006*	134	0.10	0.05
	138	0.43	0.95
	140	0.47	0.00
H_O		0.60	0.09
H_E		0.47	0.10
BFRO007*	176	0.29	0.00
	182	0.71	1.00
H_O		0.42	/
H_E		0.47	/
BFRO008*	223	0.06	0.00
	225	0.03	0.11
	229	0.32	0.57
	231	0.03	0.13
	233	0.49	0.00
	235	0.04	0.19
	237	0.03	0.00
H_O		0.66	0.62
H_E		0.62	0.48
BFRO009*	239	0.66	1.00
	241	0.34	0.00
H_O		0.45	/
H_E		0.38	/

*Allele frequencies for all five loci differ significantly (χ^2-test, $P < 0.001$). Calculation was performed using GENEPOP Software 3.1c (Raymond & Rousset 1995).

at sufficiently high frequencies to be useful as a diagnostic tool for the differentiation between the Adriatic and Danubian type of grayling.

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Polymorphic microsatellite markers in *Ictalurus punctatus* and related catfish species

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Although several types of efficient molecular marker systems such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) have been used in catfish (Liu *et al*. 1998a, b), microsatellite markers offer several advantages. They are abundant, codominant, small in size, and highly polymorphic among highly related individuals, which provides tools for ecological studies of closely related populations (Hughes & Queller 1993). Such population genetic analysis, when conducted in proper families established for genetic linkage and quantitative trait loci (QTL) analysis, would make it possible to localize genes and construct genetic linkage and QTL maps.

Little is known about population structures and genetic resource variations of catfishes at the molecular level. This was at least in part due to a lack of highly polymorphic markers before microsatellite markers were reported (Waldbieser & Bosworth 1997). Many ecological issues involving genetics need to be addressed for catfishes. For instance, channel catfish is the most important cultured fish in the United States, but its genetic resource variations are not known.

We previously reported high levels of evolutionary conservation of microsatellite flanking sequences among several catfish species (Liu *et al*. 1999). We present here development of 30 microsatellite markers, their PCR primers, amplification conditions, and informativeness in the Auburn resource families.

Channel catfish (*Ictalurus punctatus*) small-insert libraries were constructed as previously described (Liu *et al*. 1999). Double-stranded DNA was sequenced and PCR primers designed using OLIGO (National Biosciences, Inc., Plymouth, MN, USA). Among the 30 microsatellite loci, 21 loci harbour simple microsatellite repeats and nine harbour composite microsatellites.

Sixty-five clones were sequenced, of which 47 clones produced enough sequences appropriate for designing PCR primers. All designed primer pairs were first tested with one fish for the amplifiability of the microsatellite loci. Thirty pairs of primers successfully amplified their microsatellite loci using genomic DNA of channel catfish (Table 1). Approximately 200 ng of genomic DNA was amplified in PCR reactions of 50 μL containing 50 mM KCl, 10 mM Tris (pH 9.0 at 25 °C), 0.1% Triton X-100, 0.25 mM each of deoxynucleotide triphosphate (dNTPs), 1.5 mM $MgCl_2$, 20 μM each primer, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI, USA; or Life Technologies, Bethesda, MD, USA). The general temperature profiles used in initial amplification trials were 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min, for 35–40 cycles. An initial denaturing period of 1 min at 94 °C was used. Following amplification, samples of 3 μL were mixed with 1 μL of loading dye and electrophoresed on 10% acrylamide gels or sequencing gels. To optimize PCR conditions for efficient amplification, every pair of primers was initially tested at 55 °C. For those primers that did not efficiently amplify the expected products at 55 °C, they were tested at 45 °C, 50 °C, or 60 °C. The highest temperature that efficiently amplified the expected products is listed in Table 1. Under these conditions, the PCR amplification results were consistently obtained.

Loci with shorter repeat units appeared to have lower levels of polymorphism (Table 1). This is consistent with the slippage theory for microsatellite polymorphism (Levinson & Gutman 1987; Tautz 1989). Therefore, microsatellite clones containing low repeat numbers should be avoided in future efforts. The high informativeness of these microsatellite markers in the resource families should facilitate genetic linkage and QTL mapping in catfish. Microsatellites were shown to be highly conserved among several ictalurid catfish (Liu *et al*. 1999). That means that the markers developed from channel catfish will probably work as well in closely related ictalurid catfishes. These polymorphic microsatellite markers thus provide valuable tools for various ecological studies (Morin *et al*. 1994; Small *et al*. 1998) such as genetic resource analysis, relative contribution, stocking success evaluation, population

Table 1 Characterization of channel catfish (*Ictalurus punctatus*) microsatellite heterozygosity and usefulness in Auburn resource families. Numbers of alleles, observed (H_O) and expected (H_E) heterozygosities were derived from testing 40 individuals from two populations of channel catfish

Locus names	Accession nos	Repeat units	Primer sequences	Annealing temp. (°C)	PCR product (bp)	Usefulness in resource families				No. of alleles	H_E	H_O
						1	2	3	4			
Ip059	AF114754	$(CA)_8$	TGTTGTATGCTTCCTGATTACAG TTTATAAAGGGATCAAAGGTGC	55	257	–	–	+	+	3	0.47	0.43
Ip077	AF114755	$(GT)_{15}$	GAAACACAATGTACAGTAAGCTG GCTGCTTCTTATGGAATCTC	55	114	+	+	+	+	4	0.71	0.58
Ip080	AF114756	$(TG)_{10}$	CTCTTAAGGCACATATCTATGTC ACTCCACATACCGGTGTCCTTG	50	125	–	–	–	–	2	0.20	0.08
Ip248	AF115390	$(CA)_{20}$	CCTCTTGCATCTTTACATTACAT TTCTCACTCTCAGACAACCAC	55	98	–	–	–	–	3	0.48	0.10
Ip265	AF114782	$(CA)_{32}$	AGAGGTTGAAATAAAACACAGCC AAGACCCCACTTCCATCATC	55	220	+	+	+	+	5	0.78	0.60
Ip266	AF114781	$(GT)_{13}T_4$ $(GT)_4$	TGCATTCACTTTGTCTGTCGAT AACACACTACAGAGTCCCATGCT	55	210	+	+	+	+	4	0.68	0.63
Ip268	AF114780	$(GT)_{13}$	ACCGTTATAATACAACAACATAT ACAGCTGTTAATGCTAAATGC	55	290	+	+	+	+	4	0.67	0.65
Ip308	AF114779	$(GT)_{11}$	TCAGCTTACTGTACAGACACCTG AACCACTTTAGCTGGAAGACC	55	120	–	–	–	–	2	0.07	0.05
Ip314	AF114778	$(CA)_{12}$	AAACAAGTTCAACTGGTACCTGA GCTTGGACCCCACAGATTAC	55	101	+	+	+	+	4	0.73	0.78
Ip317	AF114777	$(TG)_3TC$ $(TG)_{24}$	GATGAGAATTGAGCTGTATCGG ATTCTGGAGTGTACAGAAACG	50	121	+	+	+	+	5	0.74	0.55
Ip326	AF114776	$(CA)_{16}$	AACTCATGCATTATACAGAGC TGTGGACCTCACCTTGCCTGG	50	144	+	+	+	+	4	0.73	0.58
Ip329	AF114775	$(GT)_{13}$ $(GA)_{13}$	TGTCACTGAGCCATTTAACCC GTGGCAGGTCACTGTGGGCAG	50	144	+	+	+	+	5	0.74	0.75
Ip349	AF114774	$(GT)_{16}$ $(AG)_{20}$ $Nn(CAT)_7$	CAGCAGCCTTCCTCATGGTGCC TCACCACATTAACATCTCATCC	50	290	+	+	+	+	6	0.78	0.83
Ip350	AF114773	$(TC)_{22}AG$ $(TG)_4(TC)_5TT(TC)_9$	GCCTCATGAAAAATCTCAGACC GTAGGCAAGGTGCTCAGATCTC	50	174	+	+	+	+	4	0.56	0.48
Ip379	AF114772	$(CA)_{13}$	CCGAGAGATACGGGGTACAC GGAGCTTGCAGGACAGAAC	55	169	+	+	+	+	4	0.52	0.20
Ip384	AF114771	$(AAAT)_{12}$	CATCTGTAACACCTGCCAGCC TAGGCATGTCCAAGAAGTAAGGG	50	189	+	–	+	+	3	0.54	0.50
Ip420	AF114770	$(GA)_9$ $(CA)_{13}$	CAGCATTCACGATAAAAAGTGGG CCTAATGAGGCTCAGCTTTGATG	50	103	+	+	+	+	3	0.60	0.68
Ip427	AF114768	$(CA)_{24}$	CATTTTGCTAGGTGCGCGCACG GGTGCCTTTATATGTATATAC	50	91	+	+	+	+	5	0.74	0.78
Ip429	AF114767	$(CA)_{16}$	CATCTTACATCTTTACATTAC CCACCAAGCCCATCCCTCCAAC	50	100	–	–	–	–	3	0.18	0.05
Ip477	AF114766	$(CA)_{25}$	GCCAATAATTACACCAGAATG GGAACCAGCTGTTAAAACTG	55	114	+	+	+	+	4	0.62	0.55
Ip504	AF114765	$(TC)_{20}$	CCTGGAGAAGGTCTGTATCTCT GGACAAACATCTACAATTAGGCT	55	111	+	+	+	+	4	0.60	0.65
Ip546	AF114764	$(TG)_{19}$ $(GA)_{15}$	GAAGGAAAAGAATGTAGACAATG CTCTATTTCTTTCCTTTTCACTG	55	121	+	+	+	+	5	0.68	0.70
Ip547	AF114763	$(GA)_{14}$	GTCAGCATGGAAGAGGGATG GATGTTAACTTGCTCTTATCC	55	150	+	+	+	+	3	0.47	0.38
Ip565	AF114762	$(CA)_7$ $(CT)_{23}$	ATAAAGTAGTGCATCCAGGTCGC CAGGACGATGTGAACAGGAGAG	55	164	+	+	+	+	4	0.70	0.85
Ip591	AF114761	$(GT)_7$ $(GA)_{20}$	CTGCTTTAGGTCCACCCACTGC AGGCACTTGACATTTAGCCTGC	55	133	+	+	–	–	6	0.56	0.33
Ip603	AF114760	$(GA)_{28}$	CCCCTGGGAAGCATTTCCTACTG ACTCCCAGTTCAATGAGAATGC	50	156	+	+	+	+	5	0.66	0.73
Ip605	AF114759	$(GA)_{17}$	GGGATGAGTAGAAAGAAAGAGGG TTACAGTGTGGCTTTGAAGCTAC	55	103	+		+	+	3	0.44	0.23
Ip607	AF114758	$(GA)_{24}$	TCAGGCACAAATCTTGTGATGG TTGTAGTTCTGCCTCTAACCGC	50	174	+	+	+	+	7	0.74	0.83
IpTr09	AF114769	$(CA)_{15}$	GCTGACCGGTTCGTGTATATCT TCTCAAAACAGTAATCTCCACCG	55	350	+	+	+	+	4	0.51	0.53
IPTr39	AF114757	$(CT)_{11}$	GGTCTGGGTCAGTGCGTAGTGA GCTGACCGGCAGAGACGAGAG	55	140	+	+	+	+	3	0.54	0.50

structures, strain and paternity identification, and molecular systematics.

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Isolation and characterization of long compound microsatellite repeat loci in the land snail, *Cepaea nemoralis* L. (Mollusca, Gastropoda, Pulmonata)

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The study of land snails has been fundamental to understanding the origins and maintenance of genetic diversity. The realization that the frequencies of *Cepaea* shell colour and banding morphs are affected by selection against predation by thrushes (Cain & Sheppard 1950) was followed by the discovery of 'area effects', patterns of gene frequencies in which particular shell-colour or banding morphs predominate over areas much larger than the Mendelian population (Cain & Currey 1963). Studies of allozymes helped to estimate the relative contributions of selection, gene flow and history in bringing about differences between neighbouring populations in area effects (Johnson 1976; Ochman *et al*. 1983). I have now developed microsatellite primers for *Cepea nemoralis*, with the continuing aim of studying the relationships between populations of land snails.

A genomic library of *C. nemoralis* DNA was constructed following the method of Armour *et al*. (1994), enriching for two trinucleotide (GAT, GCT) and three tetranucleotide (CCAT, GACA, GATA) repeats. Approximately 30% of 500 clones gave a hybridization signal, and after discarding clones in which there was no obvious repeat, or the repeat was directly adjacent to the cloning site, 18 pairs of primers were designed. Five pairs gave reproducible and easily interpretable PCR products. The observed and expected heterozygosities found at these five loci in a population from the Marlborough Downs, UK is shown in Table 1. Amplification was tested in species related

Table 1 Characteristics of five microsatellite loci cloned from *Cepaea nemoralis*. F, forward primer; R, reverse primer. For details of the repeat type, see Table 2 below. The number of alleles and heterozygosities were those found in a sample population ($n = 30$); the allele size range was that found after widespread British sampling. The clone sequences have GenBank Accession nos AF139019 to AF139023

Locus	Primer sequences (5′–3′)	Annealing temp. (°C)	No. of cycles	No. of alleles	Allele size range (bp)	Observed heterozygosity	Expected heterozygosity
*Cne*1	F: TGCGGAAACGATGACAAAG R: GCCATCTGCTCCCTTCTTG	55	29	7	199–751	0.67	0.71
*Cne*6	F: AAGCCGGGCCGGGTTCTCT R: GCCATATTATTTGACACTAGTTGAC	50	30	7	288–552	0.79	0.80
*Cne*10	F: CGCCCACGCTTATCGGTATGACACG R: GGCAGGTAAACTGTCTCTGTTGCTG	65	26	4	129–450	0.41	0.56
*Cne*11	F: CAATACTGGCCGACTTGTGACAAAC R: GTATGTCTTCCAGTGGTTTTATCTCAC	55	34	13	317–392	0.97	0.90
*Cne*15	F: TCAGCTTCTGCTATTGCTTCTACTG R: AGGTGTGACATCAGTTCCCCTAACC	55	29	2	143–176	0.41	0.33

Table 2 Exact compound repeat sequences of each microsatellite clone. PCR products were cloned and sequenced for 1–2 alleles of *Cne*1, *Cne*6, and *Cne*11. Their sequences are aligned under the sequence of the clone

Locus		Repeat sequence
*Cne*1	Clone:	$(ACT)_{16}$ $(GCT)_8$ $(ACT)_{10}$ $(GCT)_{14}$ $(ACT)_3$ $(GCT)_6$ $(ACT)_6$ $(GCT)_{46}$ $(ACT)_9$
	PCR product:	$(ACT)_{19}$ $(GCT)_8$ $(ACT)_9$ $(GCT)_{15}$ $(ACT)_3$ $(GCT)_6$ $(ACT)_6$ $(GCT)_{57}$ $(ACT)_{10}$
*Cne*6	Clone:	$(TCTA)_{14}$ $(TCTG)_{30}$ $(CCTG)_2$ $(TCTG)_{24}$ $(CCTG)_2$ $(TCTG)_5$ $(CCTG)_2$ $(TCTG)_{16}$ $(CCTG)_2$ $(TCTG)_6$
	PCR product:	$(TCTA)_{19}$ $(TCTG)_{21}$ $(CCTG)_1$ $(TCTG)_5$
*Cne*10	Clone:	$(GAT)_8$ (GCT) $(GAT)_{12}$
*Cne*11	Clone:	$(AAG)_4$ $(AAC)_3$ $(AGC)_{26}$ $(AAC)_5$ (AGT) (GAC) $(AAC)_3$ $(AGC)_2$ AGT $(GAC)_{10}$
	PCR product:	$(AAG)_4$ $(AAC)_3$ $(AGC)_9$ $(AAC)_9$ (AGT) (GAC) $(AAC)_4$ $(AGC)_2$ AGT $(GAC)_{16}$
	PCR product:	$(AAG)_4$ $(AAC)_3$ $(AGC)_{20}$ $(AAC)_7$ (AGT) (GAC) $(AAC)_4$ $(AGC)_2$ AGT $(GAC)_{14}$
*Cne*15	Clone:	$(CTG)_{14}$ $(CCG)_2$ $(CGT)_3$

to *C. nemoralis*, but failed in all except *C. hortensis*, in which most loci appeared to be monomorphic. All repeats were compound and some alleles were very long (Table 2).

For the routine screening of microsatellites, DNA was extracted from *C. nemoralis* using Nucleon Phytopure kits (Nucleon Biosciences). For PCR, 5 pmol of forward primer was labelled at the 5′ terminus with [γ^{32}P]-ATP using T4 polynucleotide kinase (Sambrook *et al*. 1989). Five pmol of the complementary reverse primer was provided with 0.1 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 U Thermoprime^{PLUS} polymerase (Advanced Biotechnologies) and 1 μL of 10× PCR buffer (750 mM Tris-HCl pH 8.8, 200 mM ammonium sulphate, 0.1% v/v Tween 20). All PCR reactions/amplifications were carried out in a 10 μL final volume using approximately 50 ng of template DNA. The standard PCR conditions were 96 °C for 1 min, followed by *n* cycles of 94 °C for 20 s, *t* °C for 20 s, and 72 °C for 1 min. The annealing temperatures (*t*) and numbers of cycles (*n*) used are shown in Table 1. A relatively long extension time ensured the amplification of very long alleles (up to 750 bp). The amplification of the loci *Cne*1 and *Cne*10 was improved by the use of a high-dNTP PCR buffer (50 mM Tris-HCl pH 8.8, 12 mM ammonium sulphate, 5 mM $MgCl_2$, 7.4 mM 2-mercaptoethanol, 1.1 mM each dNTP and 125 μg/mL bovine serum albumen, final concentration).

Many of the microsatellite primers amplified alleles that differed in size corresponding to more than 150 repeat units. To confirm that these alleles differed only in their number of repeats, rather than by insertions in the flanking sequences, a number of PCR products for *Cne*1, *Cne*6 and *Cne*11 were cloned using the pGEM®-T vector systems (Promega) and sequenced. All sequenced alleles differed only in their number of repeats (Table 2).

Physical linkage of the microsatellite loci to one another and to the major colour and banding loci was also tested, using approximately 100 offspring from a mating of two virgin snails (yellow 00300 × pink 00000; see Cain & Sheppard 1950 for details of nomenclature). Most comparisons were not significant, except for evidence of linkage between: *Cne*11 and *Cne*10 ($\chi^2 = 114.3$, 1 d.f., $P < 0.001$, recombinants = 8/58) and *Cne*11 to the 'midbanded' locus ($\chi^2 = 8.8$, 1 d.f., $P < 0.01$, recombinants = 7/31). Linkage of *Cne*10 to the midband locus was not significant ($\chi^2 = 3.0$, 1 d.f., not significant, recombinants = 18/48). Crosses involving the least polymorphic locus, *Cne*15, were uninformative so that linkage could not be checked. No mutations of the microsatellite loci were observed in the scoring of offspring.

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Polymorphic microsatellite markers for Atlantic halibut, *Hippoglossus hippoglossus*

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Table 1 Estimated variability at five microsatellite loci for Atlantic halibut from the Bay of Fundy, Canada

Locus	Primer sequence (5′–3′)	Accession number	Annealing temp. (°C)	Size range (bp)	*N*	*A*	H_O	H_E	*P*
*Hhi*A44	CAACTGTGGGTATGTGCCTG GTGTCAGCACTGTGCTTAAACC	AF133243	55	136–234	110	18	0.87	0.86	0.924
*Hhi*C17	TTAGGTCTGATCACCGCTATG GTTTACAAAGGTTTCTGATGGC	AF133244	55	114–168	110	22	0.89	0.95	0.196
*Hhi*D34	GCCTGGTCTCATTGTGTTCC AGGTTAAATGATTTCCTGAAGCTG	AF133245	55	184–226	110	9	0.80	0.73	0.563
*Hhi*I29	GCTTCGGTTACACCTTTGC AGGACAGTGAGGATGTCCG	AF133246	55	98–134	110	14	0.85	0.86	0.577
*Hhi*J42	CACAAACTCAAGATGTTGCG AAGCTCACTGGAAAATAATACCC	AF133247	55	112–144	110	13	0.67	0.79	0.025

N, total number of alleles sampled; *A*, number of different alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity; *P*, exact probability for departure from Hardy–Weinberg equilibrium (null hypothesis rejected at $P < 0.05$).

Atlantic halibut (*Hippoglossus hippoglossus*), the largest of the flatfish, is distributed throughout cold, boreal and subarctic waters, on both sides of the north Atlantic (Scott & Scott 1988). A growing body of allozyme data has suggested that there may be more than one panmictic population of halibut from Greenland eastward. (Foss *et al*. 1998). To the west of Greenland, no genetic analysis of Atlantic halibut has been undertaken, although two stocks, the Gulf of St Lawrence and Scotian Shelf, are currently recognized on the bases of differences in growth rate, age composition and movement (Trumble *et al*. 1993). To further our understanding of the genetic structure of Atlantic halibut in the western Atlantic, we have developed microsatellite markers for use in population studies.

An amplicon was generated by digesting halibut genomic DNA with *Bgl*II, ligating primer-adapters to both ends of the restriction fragments, then amplifying the ligation product using the polymerase chain reaction (PCR). The adaptors, primers and procedures used to generate the amplicon are described by Lisitsyn *et al*. (1993). We found that by using a restriction enzyme with a 6-bp recognition sequence, longer flanking regions remained on either side of the repeat. This simplified subsequent design of primers for amplification of the microsatellite using PCR. The production of an amplicon facilitated cloning.

The amplicon was subjected to electrophoresis through a 2% NuSeive®GTG® agarose gel (FMC Bioproducts) and the 400–650 bp fragments recovered using the Wizard™ PCR DNA purification system (Promega) according to the manufacturer's instructions. The recovered fragments were ligated into the pCR® 2.1 vector (Invitrogen) and the ligation product used to transform TOP10F′ One Shot™ competent cells (Invitrogen) according to the manufacturer's instructions.

A library of 1500 colonies was screened with a (GT) ×11 oligonucleotide probe which was 5′-end-labelled with [γ^{32}P]-ATP (Taylor *et al*. 1994). One hundred positive clones were identified of which 50 were subjected to cycle sequencing reactions using fluorescent labelled ddNTP. The nucleotide sequence of reaction products was determined using an automated sequencer (Applied Biosystems). Primers were designed using C-Primer (Bristol & Andersen 1995).

Polymerase chain reactions were performed in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer) using the following temperature profile: initial denaturation for 5 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. An aliquot of each product was mixed with an equal volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF), denatured at 85 °C for 5 min, then subjected to electrophoresis through an 8% highly denaturing polyacrylamide/formamide gel (Litt *et al*. 1993). Gels were dried without fixing, then exposed to X-ray film overnight with intensifying screens at –70 °C.

Five primer pairs were used to amplify microsatellite loci in a sample of 55 Atlantic halibut from the Bay of Fundy, Canada. Estimates of heterozygosity and tests for Hardy–Weinberg equilibrium were carried out in GENEPOP 3.1c using a Markov chain method with 4000 dememorization steps to estimate an exact *P*-value (Raymond & Rousset 1995).

All five primer pairs generated highly polymorphic PCR products, which detected a high proportion of heterozygous individuals in the sample of halibut from the Bay of Fundy (Table 1). At locus *Hhi*J42, homozygous genotypes were observed in significant excess. This could be the result of a null allele present in the population at this locus. At all other loci, alleles were distributed according to Hardy–Weinberg expectation.

Primers were also tested for cross-species amplification in three other species of flatfish; winter flounder (*Pleuronectes americanus*) ($N = 1$), yellowtail flounder (*Pleuronectes ferrugineus*) ($N = 2$) and Canadian plaice (*Hippoglossoides platessoides*) ($N = 1$). All primers successfully amplified microsatellites in all species except for *Hhi*C17, which did not amplify in yellowtail flounder. Variant alleles were observed for winter flounder at *Hhi*A44, *Hhi*D34 and *Hhi*J42, for yellowtail flounder at

*Hhi*A44 and *Hhi*J42 and for Canadian plaice at *Hhi*A44, *Hhi*C17 and *Hhi*J42. This suggests that these loci may be informative for other species of flatfish in addition to halibut.

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Characterization and isolation of microsatellite loci from the endangered North Atlantic right whale

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As a consequence of historic whaling pressures, the western North Atlantic right whale (*Eubalaena glacialis*) is one of the most endangered of all large cetaceans (Knowlton *et al*. 1994). Although photoidentification techniques for individual identification of whales and more than 18 years of monitoring have provided extensive behavioural and life-history data, individual-specific genetic profiles would augment the study by allowing whales for which only low-quality photographs exist to be identified (see Palsbøll *et al*. 1997).

Microsatellite loci are known to have high levels of genetic variability in many taxa due to the high rates of mutation (Dietrich *et al*. 1992; Bruford & Wayne 1993). Moreover, as microsatellites are noncoding, allele frequencies will be subject to random genetic drift, making these markers useful for studying levels of genetic diversity and population structuring (Nauta & Weissing 1996).

Skin samples were collected from *E. glacialis* in the waters off eastern Canada between 1988 and 1997. Samples were stored in DMSO (20% DMSO, 0.25 M sodium-EDTA, saturated with NaCl, pH 7.5; Amos & Hoelzel 1991) and DNA was extracted using phenol–chloroform extractions (Brown *et al*. 1991; Schaeff *et al*. 1993). Samples were also available for several South Atlantic right whales (*E. australis*) from near Peninsula Valdes, Argentina (Schaeff *et al*. 1993).

Restriction digests of 1–3 mg of genomic DNA were conducted in 25 μL volumes using three blunt-end cutting restriction enzymes (*Rsa*I, *Eco*RV, and *Alu*I; Gibco-BRL). DNA fragments of < 450 bp were size separated on a 2% agarose gel. Cloning was carried-out as described in Rassman *et al*. (1991). Insert sequences were determined using the PRISM™ Ready Reaction Dye Deoxy Termination kit (Applied Biosystems Inc.), a Perkin-Elmer 9600 Thermal Cycler, and an Automated DNA Sequencing System (373A, Applied Biosystems). Primers were developed for 14 of 34 microsatellite sequences identified (Primer version 5; Lincoln *et al*. 1991).

Amplifications using the polymerase chain reaction (PCR) used 20–50 ng of DNA, 0.2 μM of each primer, 20 nm of γ^{33}P end-labelled primer, 2.0–2.5 mM $MgCl_2$, 0.70 U of *Taq*, 1× reaction buffer, and 0.20 mM dNTPs (Gibco-BRL). PCR products were size-separated on a 6% denaturing, polyacrylamide gel and visualized by autoradiography. Alleles were sized relative to the clones. Loci were tested for deviations from Hardy–Weinberg equilibrium (HWE) when > 58 chromosomes had been screened (Exact test; Raymond & Rousset 1995).

Loci were considered monomorphic when the most common allele had a frequency of > 95% (N > 58 chromosomes). Only two of 13 and two of 10 polymorphic loci deviated significantly from HWE in *E. australis* and *E. glacialis* (rw26 & rw18 and rw31 & rw34, respectively). Allelic diversity was as great or greater in *E. australis* than in *E. glacialis* at all but one locus (rw34).

The potential use of these microsatellites (Table 1) in the study of toothed whales was assessed using seven randomly selected primer pairs on six beluga whale samples (*Delphineptera leucas*). All seven primer pairs produced PCR products and, despite the small sample size, four of the seven loci were variable (rw2–17, rw2–19, rw21, and rw26).

For the right whales, the microsatellite loci will allow individual-specific profiles to be developed. In addition, for *E. glacialis*, the exclusionary power (PE) for paternity analysis is over 98% when the identity of one parent is known. In contrast, high allelic diversity in *E. australis* provides a PE ≅ 99%

Table 1 Microsatellite loci identified from the genome of the North Atlantic right whale

Locus	Repeat sequence	GenBank Accession no.	Primer sequence (5′ > 3′)	PCR product (bp)	T_a (°C)	No. of alleles		H_O		*Nc*	
						nA	sA	nA	sA	nA	sA
rw18	$(TG)TA(TG)_{19}$	AF156294	F: AGAGGGAAGCAAACTGGA R: GAAGGNTGCCAGACACCC§	195	52	5	16	0.5	0.9*	378	58
rw26	$(TG)_{16}(TA)_2$	AF156295	F: GTCCATCCATATTACTGC R: CAGTTATACCTCAATGAAGC	165	56	2	11	0.4	0.9†	386	54
rw31	$(TG)_{20}$	AF156296	F: TATTCATGGAGTGCTTTGG R: CCTAGAGTCCAGTGTGGTA	130	54	5	7	0.5*	0.8	354	50
rw25	$(TG)_{18}$	AF156556	F: CTTAACATGGAAGGCTCCC R: GCCAAGCATTGGGACTTTTG	140	54	1	4	—	—	0.8	60 20
rw2–17	$(GT)_5A(GT)_{13}$	AF156297	F: ATCTGGCATTTGTTTTAAAATAATCC R: CCAGAAAGAATAATGTAATAAACCC	166	52	1	3	—	—	0.4	60 58
rw2–19	$(AC)_{12}$	AF156298	F: AGTTCCATAGGGCTGCTCAC R: TTCCATTTTTGGGTTCAATC	96	52	1	5	—	0.7	60	22
rw4–10	$(GT)_{17}$	AF156555	F: ATGGCATTACTTCATTCTTT R: GCCAAACTTACCAAATTGTG	177	54	2	9	0.3	0.8	50	20
rw34	$(CA)_{25}$	AF156299	F: AGCCCCATAACGGCGCATA R: GGGAGCCAGAACCTGATAC	122	57	11	2	0.7†	—	368	18
rw48	$(TG)_{23}$	AF156300	F: CCAATGACTTTTCCCTGTA R: GATACCGCAGTGTGTCCTG	112	57	6	6	0.4	—	370	20
rw2–12	$(TG)_4A(TG)_2ACGCACAC(GT)_7T(TG)_5$	AF156301	F: TGACACTTTTCCGCTTTAGG R: AAAAGCTTCCATCCTAACCA	86	52	1	2	—	—	60	22
sam25	$(TG)_{16}(TA)_2$	AF156302	F: CTGCAAATGGCATTACTTC R: CCAAACTTACCAAATTGTG	182	53	2	7	—	—	20	18
rw4–5	$(TG)_{15}TATGTAT(GA)_{10}AT(GT)_2$	AF156303	F: AGGTCTTTCATTGCTGCC R: ACGGAAATCAGAAAGCCTTA	115	55	2	6	—	—	18	20
rw4–17	$(TG)_{18}A(T)_8$	AF156304	F: TATCCTGCAACCTTGCTGA R: TCACAGATGACATGACCTTG	104¶	55	4	9	0.7	—	362	22

H_O, observed heterozygosity; Nc, no. of chromosomes screened; nA, North Atlantic right whale; Sa, South Atlantic right whale; T_a, annealing temperature.
PCR cycles consisted of 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 52–57 °C, 45 s at 72 °C and a final 10-min step at 72 °C.
*Heterozygote deficiency ($P < 0.01$).
†Heterozygote excess ($P < 0.05$).
§Sixth position was represented by both G and C.
¶In addition to the ~104 bp target product, rw4–17 had a 'fixed' band of 80 bp in both species. No size overlap was observed between alleles from the target region and the 'fixed' band for either species.

when neither parent is known. These microsatellite-based profiles will augment the *E. glacialis* catalogue by identifying new whales and confirming the identity of whales when photographs are of low quality.

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Microsatellites from the Amazonian tree *Dinizia excelsa* (Fabaceae)

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A central problem in conservation biology is to determine whether spatially isolated rainforest trees experience reproductive isolation. To address this question, we developed microsatellite primers to infer paternity in remnant populations of the Amazonian tree *Dinizia excelsa* (Fabaceae).

Dinizia is one of the most phylogenetically isolated Legume genera, marking the divergence between the Mimosoid and Caesalpinioid subfamilies (Herendeen & Dilcher 1990). *D. excelsa* is endemic to *terra firme* rainforests of the central Amazon, is insect pollinated and bears indehiscent pods that are wind dispersed. Members of this species include some of the largest trees in Amazonia, attaining 60 m in height and 2.5 m in diameter (Ducke 1922). The prized hardwood accounts for ≈ 50% of regional timber sales (Barbosa 1990). Because of their value for timber and shade, large individuals are often left standing in pastures. Shade trees, along with individuals in continuous and fragmented forest, provided an experimental system to examine the breeding structure of remnant populations (Dick 1999).

Microsatellites were isolated with standard methods to construct small insert genomic libraries (Rassmann *et al*. 1991). Genomic DNA was digested with *Sau*3A and 300–900 bp fragments were purified from 1.7% agarose (Gene Clean II; Bio101). DNA fragments were ligated to pBS SK+ plasmid (Stratagene) and transformed into XLR-1 Blue cells (Stratagene). Transformed cells were plated and colonies were lifted with Hybond-N+ membranes (Amersham). We probed the colony lifts with 13 oligonucleotides [$(AAAG)_7$ $(ACG)_{10}$ $(CA)_{15}$ $(CCG)_{10}$ $(CTC)_7$ $(CAC)_7$ $(TC)_{10}$$(CAT)_7$ $(TAG)_7$ $(AAG)_7$ $(GGAT)_4$ $(AAT)_7$ $(GATA)_4$] labelled with Digoxigenin-11-dATP for nonradioactive detection (Boehringer Mannheim). We screened 2500 recombinant colonies. Fifty-eight positive clones were sequenced, yielding 29 microsatellite loci. Primer pairs for 16 loci were designed with AMPLIFY version 1.2 (Engels 1993).

To test the loci for polymorphism, template DNA was extracted from 121 individuals from six populations using DNeasy spin columns (Qiagen). Alleles were initially labelled by adding dye-labelled nucleotides (FdUTPs: Applied Biosystems Inc.; ABI) to the polymerase chain reaction (PCR) in a FdUTP:dNTP ratio of 1:1000. Dye-labelled primers were later developed for five loci used in the paternity study. Alleles were sized on an automated DNA sequencer (ABI 377) using GENESCAN version 2.1 (ABI) and Rox 400 size standard (ABI). The PCR cocktail (10 μL total) contained 250 μM of each dNTP, 25 mM $MgCl_2$, 1.25 units of *Taq* polymerase (Qiagen), and 0.5 μM of each primer. PCRs were performed on an MJ Research PTC-200 thermal cycler using the following thermal protocol: 5 min at 94 °C; 25 cycles of 45 s at 94 °C, 1 min at the primer-specific annealing temperature, and 30 s at 72 °C; ending with 15 min at 72 °C.

Seven loci amplified reliably and were polymorphic (Table 1). Five of these loci (DE27, DE37, DE44, DE48, DE54) were used

Table 1 Microsatellite loci of *Dinizia excelsa*, with number of individuals sampled (*n*), number of alleles observed (*k*), observed heterozygosity (H_O), expected heterozygosity (H_E) and expected exclusion probabilities (P_E) calculated by CERVUS (Marshall *et al*. 1998). All sequences have been deposited in GenBank (AF143976, AF143979, AF143980, AF143982, AF143986, AF143987, AF143988)

Locus	Repeat array	Primer sequences (5′–3′)	Annealing temp (°C)	Clone size (bp)	*n*	*k*	H_O	H_E	P_E
DE27	$(AAG)_8$	GCATTTAAAAATTTAAATGTAGGG GTGCAGTTTTGGATTCTTTGC	60	118	121	5	0.54	0.49	0.23
DE37	$(AC)_{20}$	TAGAATGTGCGCGCACGTC GTGTATAACTGGTGTCACCC	60	128	115	11	0.72	0.73	0.51
DE44	$(GT)_{13}$	ACGCTTAAAGGCTATTGAACC CAAATTTAAAAATAGATTAATTGAAAC	60	144	119	9	0.66	0.64	0.40
DE48	$(GA)_{27}$	AGAAGAATTAGGGAGGGACG GAATAAAAGCATGCTTTATTTTTC	60	143	106	31	0.80	0.94*	0.87
DE54	$(CT)_{39}$	GTGCAATGGGACAAAGCTTC TCCCATTGCTCAAAGACTCG	60	175	84	21	0.62	0.93*	0.85
DE60	$(AAT)_7$	CAACGCAAATAAGGCCTAACC CATATATACCTGGGCTTACAG	62	238	23	2	0.35	0.29	—
DE64	$(AAT)_7$	ATTCCACTGAGGCAAATCCC CCTCCGGCATTAACTCAGG	60	134	36	2	0.03	0.03	—

*Significant excess of homozygotes ($P < 0.05$) in some of the sample populations.

for paternity analyses. Six loci that did not amplify reliably but may be useful with different primers are (repeat array followed by GenBank Accession no.): $(GA)_{13}$ (AF143977); $(TG)_{13}$ (AF143978); $(CT)_{19}$ (AF143981); $(TG)_{11}$ (AF143983); $(GA)_{24}$ (AF143984); and $(CT)_{23}(AT)_{12}$ (AF143985).

The five loci used for paternity yielded 77 alleles with a mean of 15 alleles per locus. Observed and expected heterozygosities ranged from 0.49 to 0.94. Hardy–Weinberg equilibrium was tested with Fisher's exact test of GENEPOP version 3.1d (Raymond & Rousset 1995). A significant excess of homozygotes was observed in three populations for DE48 and in five populations for DE54 (Table 1), presumably the result of null alleles. The expected exclusion probabilities for single loci ranged from 0.23 to 0.87 with a multilocus expectation of > 0.995 (Marshall *et al*. 1998). Together, these loci provided enough variation to resolve paternity for a large portion of the *D. excelsa* seed population.

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Amplifying dolphin mitochondrial DNA from faecal plumes

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Molecular studies are increasingly used to support cetacean conservation and management (Hoelzel 1994). Biopsy-darting

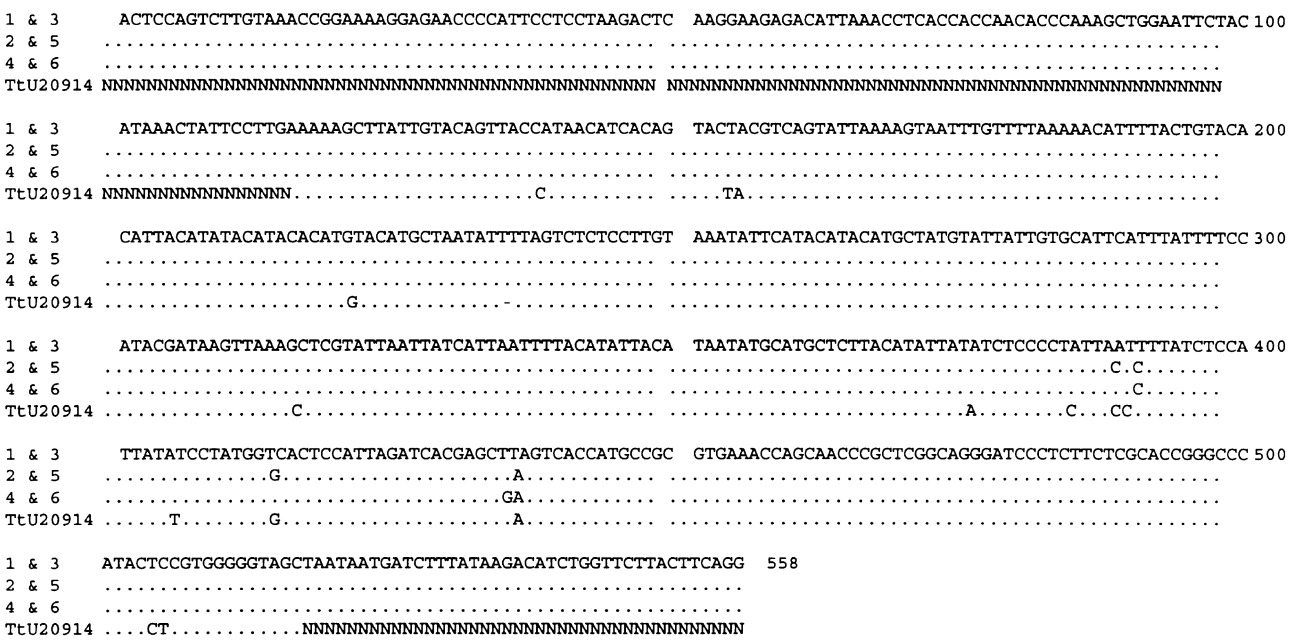

Fig. 1 Aligned control region sequences of DNA extracted from five *Tursiops truncatus* faecal samples (1 through 5; GenBank Accession no. AF155160–AF155162), one *T. truncatus* skin sample (6), and a 400-bp *T. truncatus* sequence from GenBank.

(e.g. Lambertsen 1987) and collecting sloughed skin (e.g. Amos *et al*. 1992) have provided DNA from several species, but these methods are often impractical. An alternative is to extract DNA from faeces, as previously applied to terrestrial (e.g. Gerloff *et al*. 1995) and semiaquatic (Reed *et al*. 1997) mammals, and to floating dugong faeces (Tikel *et al*. 1996). Until now, however, the diffuse form of cetacean 'faecal plumes' has prevented their collection and genetic analysis. Here, we demonstrate the feasibility of collecting faeces, extracting total DNA, and reliably amplifying mitochondrial DNA (mtDNA) markers from free-ranging dolphins.

Five faecal samples were collected from bottlenose dolphins (*Tursiops truncatus*) in the Bahamas (26°0′N 77°25′W). Upon observing defaecation, snorkelers collected sinking faeces in 100 mL plastic vials. Samples were immediately preserved in 20% DMSO/5 M NaCl (Amos & Hoelzel 1991), and frozen at –20 °C within 10 h.

Faeces were thawed immediately preceding extraction (total storage time 157–227 days). Cross-contamination was avoided by using disposable plasticware, and by cleaning metal instruments with ethanol, and flaming. One millilitre of faeces was suspended in 2 mL of 2× CTAB buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB) (Milligan 1992), and mixed by rotation. Debris was cleared by centrifugation, and 2 mL of lysate transferred to a 2-mL microcentrifuge tube. Following high-speed centrifugation, 1.5 mL of cleared suspension was extracted twice with 0.5 mL of chloroform, and DNA precipitated by adding 0.67 mL of isopropanol.

DNA pellets were dissolved in 1.8 mL of guanidine thiocyanate (GITC) buffer (5 M GITC, 0.1 M Tris-HCl, pH 6.4) at ambient temperature overnight. Three grammes of diatomaceous earth particles (Sigma D-5384) were washed, three times, in 50 mL of molecular-grade water, and centrifuged. Pelleted diatoms were then suspended in 12 mL of water and vortexed to create a slurry. One hundred microlitres of diatomaceous slurry was added to the microfuge tube to recover the DNA. Diatoms were pelleted, resuspended in 600 µL of GITC buffer, transferred to the insert of a 10-µm mesh VectaSpin microtube (Whatman, no. 6838 0002) and centrifuged to remove buffer. The diatoms, and DNA, were washed twice with 700 µL of ethanol, then incubated at 55 °C for 30 min to evaporate residual traces of ethanol. DNA was eluted in 130 µL of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), incubated at 55 °C for 30 min, centrifuged to pellet diatom traces, and 100 µL was transferred to a new 0.2-mL tube.

The origin and quality of extracted DNA was determined by polymerase chain reaction (PCR) amplification of 558 bp of the 5′ section of the mtDNA control region, using primers L15926* and H16498 (Eggert *et al*. 1998). PCR reactions were carried out in a 50-µL volume containing 10 µL of DNA. Extraction blanks, positive and negative controls were included in all PCR reactions to detect contamination. Products were detected and quantified on ethidium-stained 1% agarose gels. The desired fragment was successfully amplified from the DNA of all five faecal samples.

To confirm that amplified mtDNA fragments originated from *T. truncatus*, both strands of purified (QIAquick PCR purification kit [Qiagen]) PCR products were sequenced using BigDye sequencing kits (PE Biosystems), and detected on an ABI 377 automated DNA sequencer. Each faecal sample was amplified and sequenced twice. These sequences were aligned to a published *T. truncatus* mtDNA sequence (GenBank), and to the sequence from a *T. truncatus* skin sample obtained in the Bahamas (Fig. 1). All five faecal sequences showed a high degree of similarity to the published sequence (15

substitution sites; 14 transitions), and to the sequence from dolphin skin (five substitution sites; five transitions). This indicates that the amplified mtDNA sequences originated from dolphin DNA, and not from contaminating sources of faecal (e.g. prey) DNA.

Low quantities of target DNA can cause complications when using faecal-derived DNA in nuclear genotyping (Taberlet & Waits 1998). However, our mtDNA analyses produced reliable results from the sequence of a single fragment, suggesting that faeces may represent a useful supplement to conventional sources of DNA for future studies of cetacean population genetics.

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