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PRIMER NOTES

Isolation and characterization of polymorphic microsatellite markers in *Abudefduf luridus* (Pisces: Pomacentridae)

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The bluefin damselfish, *Abudefduf luridus* is commonly found in rocky shallow waters of the Macaronesian Islands. During the breeding season males prepare and defend nests in an open rock within their territories. Females deposit egg masses in single layers (clutches) and return to their territories after spawning. Males care for the eggs until the hatching of planktonic larvae. Behaviour observations have shown that deserted nests are generally reoccupied by other males who take care of present eggs and begin attracting females, similar to the redlip blenny (Santos 1995). Competition for territories is high and is a central factor of mating and reproductive success of the males.

A. luridus is, therefore, a promising case study species for the genetic analysis of reproductive strategies and male mating success. For this purpose, we present in this paper the development of microsatellite markers for further population analysis.

Genomic DNA from *A. luridus* muscle tissue was isolated using a phenol–chloroform extraction protocol (Sambrook *et al*. 1989). Approximately 10 µg of DNA was digested with *Sau*3A (Gibco) and size selected (300–800 bp) by 1.5% LMP (low melting point) agarose gel electrophoresis. Microsatellite enriched partial genomic library was performed according to the procedure of Kijas *et al.* (1994) with modifications. Size-selected fragments were ligated to adaptors (AdapF: 5′CTCTTGCTTACGCGTGGACTC-3′ and AdapR: 5′GATC-GAGTCGACGCGTAAGCAAGAGCACA-3′) and hybridized with 5′biotinylated, 3′aminated $(CT)_{15}$ oligonucleotides bound to streptavidin-coated magnetic spheres (magnesphere, Promega, Madison, WI). The hybridization was followed by several washing steps in 2× SSPE (0.3 M NaCl, 20 mM $NaH_2PO_4H_2O$, 2 mM EDTA, pH 7.4) and the enriched DNA was then eluted in water. DNA fragments were polymerase chain reaction (PCR) amplified, using AdapF as a primer, and ligated into *Bam*HI digested and dephosphorylated pUC19 vector (Promega). The ligation was performed overnight cycling between 10 °C 30 s and 30 °C 30 s, in 10 µL using, 50 ng vector, 50 ng purified PCR product, 30 mM Tris-HCl pH 7.8; 10 mM $MgCl_2$; 10 mM DTT; 1 mM ATP and 10 U of T_4 ligase (Promega, madison, WI). One tenth of the ligation was transformed into 50 µL *Escherichia coli* DH5α supercompetent cells (Gibco BRL) following Sambrook *et al.* (1989). A total of 400 colonies were screened according to the method described in Waldbieser (1995), based on PCR amplification with pUC19 forward or reverse universal primers and a $(CT)_{10}$ primer. Forty-six clones with a clear amplification were digested with *Pvu*II restriction enzyme (Gibco) to determine the insert size. Thirty positive clones were selected and sequenced using the dideoxy chain termination method (Sanger *et al*. 1977). The sequencing reactions were performed manually (T7 sequencing Kit, Pharmacia), using α35S dATP and forward/reverse M13 universal primers. Sequencing products were then separated by electrophoresis on standard acrylamide denaturing gels. Among them, seven sequences allowed the definition of primers (Table 1). Clear amplification was obtained with the following conditions: 20 µL total volume containing 20–50 ng of genomic DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5–1.5 mM $MgCl_2$ (Table 1), 60 µM of dCTP, dGTP, and dTTP, 12 µM dATP, 0.16 µL α35S dATP (12.5 mCi/mL, 1250 Ci/mmol), 2 µM each primer, 1 U *Taq* DNA polymerase (Gibco Brl Life Technologies, Gaithersburg, MD). The PCR was performed in a Robocycler (Stratagene Cloning Systems, La Jolla, CA) and consisted of an initial denaturation of 4 min at 94 °C followed by 30 cycles for 60 s at 94 °C, 60 s at annealing temperature (Table 1), and 60 s at 72 °C, with a final elongation of 10 min at 72 °C. Microsatellite polymorphism was analysed in 25 adult individuals from three populations from the islands of Açores and Madeira. Parameters and tests were computed using the GENPOP software version 3.1b (Raymond & Rousset 1995). The seven loci show a relatively high polymorphism, with 2–13 alleles per locus. Both expected and observed heterozygosity were very similar over all populations (Table 1) for each locus, except for locus 5. Hardy–Weinberg equilibrium could not be rejected in each population, except for the same locus. A genotypic disequilibrium was found between locus 3 and 7 in population 2 ($P < 0.05$) and over all populations ($P < 0.05$). No genotypic nor genic differentiation was found between populations. This could be due to the small sample sizes ($n = 11$, 9 and 5).

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Table 1 Characteristics of seven microsatellite loci in *Abudefduf luridus*. Primer sequences, repeat motifs, annealing temperatures (T_a), PCR final concentrations of $MgCl_2$, number of alleles (for $n = 25$ individuals), observed (H_O) and expected (H_E) heterozygosities are shown

Locus	Primer sequences 5′–3′	Repeat motif	T_a °C	$MgCl_2$(mM)	Sizs (bp)	No. of alleles	H_E	H_O	Accession no.
2AL2	F: CAAGTCAGACTCTGAACTCG R: TCAATGACTGAACCAGACAC	$(CT)_{29}$	52	1	243	13	0.90	0.92	AF208493
2AL5	F: TTCTGTACCATCGGTTGCGT R: TGTTCTACTTCGGGTTCCTC	$(AG)_4C(GA)_{10}GGA(GGTA)_3$	50	1	190	5	0.59	0.64	AF209036
2AL10	F: TAGTCTACCCCAGGGGAGAA R: CCATAATCCACACTCCTGTC	$(GA)_{19}$	52	1	151	9	0.84	0.88	AF209037
2AL13	F: GTCCTCTGAGCCACAGTGTA R: TGTCGTGAACTCCGCTGATG	$(CT)_{10}…(CT)_8$	52	1	235	9	0.87	0.88	AF209038
2AL15	F: GACGCTGCTTCCCTGTTGGC R: CCTCTGCGTGGTCATTTCTC	$(AC)_3…(AAG)_8G_5A(AG)_4$	50	0.5	127	8	0.76	0.56	AF209039
2AL24	F: ATGACACGCATGGCTAACCT R: CATAGACGCACATTGACTGG	$(GGGA)_2…(AGG)_6$	52	1	168	2	0.46	0.44	AF209040
AL34	F: ATCCTGCCCGGCCTTCAGTA R: TGTTCAGAGTGTTCTGCCCT	$(GT)_{17}$	51	1.5	131	3	0.34	0.32	AF209041

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Microsatellite markers for the European Roe deer (*Capreolus capreolus*)

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With a population size of approximately six million animals, the European roe deer is the most numerous wild cervid species in Europe. Its territory ranges from Italy in the south to Portugal in the west, Norway in the north and the Ural mountains in the east. It has been postulated that due to habitat fragmentation a new ‘roe deer field ecotype’ has emerged (Pielowski & Bresinski 1982). Microsatellite markers could be used to investigate whether or not the classification of a ‘field ecotype’ in roe deer is genetically justified. For cervids, microsatellites have been characterized among others in red deer (Roed 1998), reindeer (Wilson *et al*. 1997) and the Indian swamp deer (Ali *et al*. 1998).

Roe deer genomic DNA was extracted from 200 μL EDTA-blood samples. Microsatellites were identified and subsequently amplified using the PCR-based isolation of microsatellite arrays (Lunt *et al*. 1999). PCR amplicons were sequenced on an Automated Sequencer (ABI 310C) which was also used for fragment length analyses (primers were 5′-labelled with the fluorescence dye 6-FAM). PCR reactions (25 μL) were set up as follows: 50 ng DNA, 10 mM Tris, pH 8.8, 0.5 U *Taq*-DNA polymerase (Perkin Elmer), 3 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 1 μg BSA, 200 nM of each primer. After an initial denaturation of 3 min at 94 °C, PCRs were run for 35 cycles on a GeneAmp 2400 (Perkin Elmer). Cycle conditions were: 30 s 94 °C, 30 s annealing temperature (Table 1), 30 s 72 °C. Final extension was for 30 min at 72 °C. Roe deer primer sequences, annealing conditions as well as heterozygosities and polymorphic contents ($n = 20$) are also listed in Table 1.

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Table 1 Loci, primer sequences and additional characteristics of seven roe deer microsatellites. Expected (H_E) and observed (H_O) heterozygosities as well as polymorphic information contents (PIC) for a group of 20 roe deer have been calculated using CERVUS® version 1.0 (Marshall *et al.* 1998)

Locus	Primer sequences	T_a [°C]	Repeat motif	No. of alleles	Allele size range	H_E	H_O	PIC	Accession number
*Roe*01	F: 5′-AAATTTGGCTCTGCAATCGG–3′ R: 5′-ACACAAAAGCCACCCAATAC–3′	53	$(CA)_4TA(CA)_7$	7	112–132	0.658	0.714	0.593	AF164070
*Roe*03	F: 5′-TAACTCCTCACTGGGGCGCG–3′ R: 5′-TCCCAAATCCATAGGCCCAG–3′	54	$(CG)_2(CA)_4N_6(CA)_4$ $(CG)_3(CA)_{11}$	6	116–138	0.794	0.615	0.728	AF166357
*Roe*05	F: 5′-TAATATGAATCACTTGGATG–3′ R: 5′-TCCACCTCCCAGCCTGCATC–3′	54	$(GT)_2(AT)_2CT(GT)_{20}$ $N_5(AGG)_2G_5$	6	160–172	0.826	1.000	0.753	AF166355
*Roe*06	F: 5′-GTTCCTAGCCCAGTGCTC–3′ R: 5′-TGCAGACCTGGCAGAC–3′	49	$(CA)_5(CATG)_2(CA)_2$ $GA(CA)_{13}$	7	89–109	0.738	0.600	0.688	AF164067
*Roe*08	F: 5′-AAGCCGCGCTTGAAGGAG–3′ R: 5′-ATCAAGCTCCCCTCTTCG–3′	49	$(CA)_{10}CG(CA)_6$	7	69–89	0.729	0.800	0.673	AF166356
*Roe*09	F: 5′-TTGGCGTCATTCCAACAGAG–3′ R: 5′-TCACAGCAGAATGTCATCTG–3′	51	$(CA)_2CGTA(CA)_4$ $TA(CA)_8$	3	175–179	0.624	0.500	0.533	AF164069
*Roe*10	F: 5′-GAAGACCCTGTGCGATC–3′ R: 5′-TGAGGTTGCTCCCAAGTGTC–3′	45	$(AC)_8CAGACA(A)_7$ $T(A)_3(AC)_8$	2	190–192	0.495	0.714	0.354	AF166358

T_a, annealing temperature; f, forward; r, reverse.

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Isolation and characterization of microsatellite loci in the yellow perch (*Perca flavescens*), and cross-species amplification within the family Percidae

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Fishes belonging to the Percidae family are economicaly important, with a particular emphasis on the following commercial species, *Perca flavescens*, *Stizostedion vitreum* and *S. canadense* in North America, and *P. fluviatilis and S. lucioperca* in Europe. New genetic tools are needed to improve the management and the selective breeding programmes. Previous studies used allozyme and mitochondrial DNA (mtDNA) as genetic markers to characterize population genetic structure in yellow perch (Billington 1993; Todd & Hatcher 1993) and walleye (Stepien & Faber 1998). Although very useful for large scale population structure and phylogeography, these markers may be of limited interest for fine scale studies and parental analyses. Microsatellites loci have proven themselves to be very useful markers in such situations (O'Reilley & Wright 1995; De Garcia Leon *et al*. 1998). Although Borrer *et al*. (1999) and Wirth *et al*. (1999) have reported walleye microsatellite primers which amplified reliable products when used with yellow perch DNA, no microsatellite markers cloned from *P. flavescens* have yet been published. We report in this paper the isolation and characterization of 10 microsatellite loci cloned from *P. flavescens*, and examine cross-species amplification of these loci in European perch (*P. fluviatilis*), sauger (*S. canadense*), zander (*S. lucioperca*) and walleye (*S. vitreum*).

Genomic DNA was extracted from a yellow perch liver using a standard proteinase K phenol–chloroform technique (Sambrook *et al*. 1989) and pooled in a partial *Sau*3A1 digest. Fragments ranging from 400 to 900 bp were purified from a 1.7% agarose gel using DEAE paper (Sambrook *et al*. 1989). These fragments were then ligated into the *Bam*HI site of phosphatase treated pUC18 ('Ready to go' kit, Pharmacia). Competent Dh5α *Escherichia coli* cells (Gibco BRL) were transformed with the ligation products and grown on agar plates. Colonies were blotted on Hybond N+ nylon membranes (Amersham) which were hybridized with synthetic $(TC)_{10}$

Table 1 *Perca flavescens* microsatellite primer sequences, amplification conditions, number of alleles detected (A), observed (H_O) and expected (H_E) heterozygosity in a sample of the Saint-François lake, Québec ($N = 38$). Characteristics of the predominant continuous repeat region in each cloned allele are also given. Accession nos (GenBank) refer to clone sequences from which the primers were designed

Locus	Primer sequence	T (°C)	Size range (bp)	Repeat type	A	H_O	H_E	Accession no.
Pfla LI	F: AAGCAGCCTGATTATATATC R: CAGACAATTAAACATGCAAC	50	108–134	$(GA)_{27}$	8	0.73	0.76	AF211826
Pfla L2	F: GTAAAGGAGAAAGCCTTAAC R: TAGCATGACTGGCAAATG	53	208–232	$(CA)_{23}$	6	0.63	0.67	AF211827
Pfla L3	F: GCCGAATGTGATTGAATG R: CGCTAAAGCCAACTTAATG	53	130–186	$(TG)_{18}$	6	0.58	0.75	AF211828
Pfla L4	F: AAAGGGAAAAGGCTACGGTG R: ATCAGCAGTGCTTATGTTTG	53	98–152	$(TC)_{37}$	18	0.67	0.82	AF211829
Pfla L5	F: TGAGAGCCCATGAATTAC R: GCAAACACAGCCAATTTAG	53	134–156	$(GT)_{27}$	4	0.52	0.65	AF211830
Pfla L6	F: GCATACATATAAGTAGAGCC R: CAGGGTCTTCACTATACTGG	52	148–170	$(TG)_{18}$	5	0.52	0.71	AF211831
Pfla L7	F: TGTTCTGGTGAGCATTTG R: ACTGGGTTCCTTCATTAC	53	152–172	$(GT)_{29}$	10	0.38	0.82	AF211832
Pfla L8	F: GCCTTATTGTGTGACTTATCG R: GGATCTTTCACTTTTTCTTTCAG	53	176–200	$(TG)_{39}$	5	0.40	0.39	AF211833
Pfla L9	F: GTTAGTGTGAAAGAAGCATCTGC R: TGGGAAATGTGGTCAGCGGC	53	214–300	$(TG)_{24}$	11	0.67	0.71	AF211834
Pfla L10	F: TCCACCCTTTGATAAGGGAC R: ACAAATCTCCTGTCAAACGC	53	218–222	$(TG)_{14}$	2	0.17	0.25	AF211835

$(TG)_{10}$ $(CAC)_5CA$, $CT(CCT)_5$, $CT(ATCT)_6$, and $(TGTA)_6TG$ probes labelled with the DIG oligonucleotide kit (Boehringer, Mannheim). The DIG nucleic acid detection kit (Boehringer, Mannheim) was used for detection. Among the 2200 clones of the partial library screened, 144 (6%) were identified as positive. DNA from 17 positives was extracted using the QIAprep plasmid DNA prep kit (Quiagen) and the nucleotide sequence determined by cycle sequencing using AmpliTaq®DNA polymerase FS (Perkin Elmer) and an ABI 377 automated sequencer. All these clones contained microsatellites and primers were designed using the program OSP (Hillier & Green 1991). Ten primer sets amplified polymorphic and scorable perfect repeats products in *P. flavescens*. Characteristics of these pairs are described in Table 1.

To examine microsatellite variability, genomic DNA was extracted from fin tissues using a standard phenol–chloroform procedure. PCR was performed in a 11.5-μL volume with 10–50 ng of DNA, 300 pmol of each primer, 75 μM each nucleotide, 1.2 mM $MgCl_2$, 10 mM Tris-HCl pH 9, 50 mM KCl, 0.25 units of *Taq* polymerase (Perkin Elmer) and 1.5 μCi [^{35}S]-dATP. PCR reactions were carried out in a Perkin Elmer 480 DNA thermal cycler as follows: an initial denaturation of 3 min at 96 °C, followed by 30 cycles at 96 °C for 30 s, 30 s at the locus specific annealing temperature (see Table 1) and 1 min at 72 °C, 5 min of final elongation at 72 °C. PCR products were separated by electrophoresis in 6% denaturating polyacrylamide gels and bands were visualized using autoradiography.

We examined variability at each locus using a sample of 48 yellow perch from the lake Saint-François, Québec, Canada (Table 1). The polymorphic loci had 2–18 alleles per locus and expected heterozygosities ranged from 0.17 to 0.73.

The results of cross-amplification are shown in Table 2. As expected, more successful amplification were obtained in *P. fluviatilis*, with eight amplified loci and heterozygosities ranging from 0 to 0.71 (mean 0.49). In *Stizostedion* species, nearly 50 per cent of the loci amplified and heterozygosity ranged from 0 to 0.7 (mean 0.22) for walleye, from 0.30 to 1.0 (mean 0.54) for sauger and from 0 to 0.63 (mean 0.35) for zander. The genetic variability assessed by microsatellite typing significantly outnumbered the genetic variability inferred from mtDNA (Billington 1993) and these new loci are, therefore, useful tools for selective breeding programmes and fine scale population structure. These results confirm the usefulness of *P. flavescens* microsatellite markers for the genetic characterization of closely related species of ecological and economical interest.

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Table 2 Number of alleles and observed heterozygosity values from cross-species amplification within the family Percidae using *Perca flavescens* microsatellite primers. Amplification was attempted on *N* individuals. Number in parentheses are the number of individuals that amplified for each species/locus combination

		Locus									
Species	*N*	*Pfla* L1	*Pfla* L2	*Pfla* L3	*Pfla* L4	*Pfla* L5	*Pfla* L6	*Pfla* L7	*Pfla* L8	*Pfla* L9	*Pfla* L10
Stizostedion canadense	10	7(10) $H = 0.70$	12(10) $H = 1.00$	4(10) $H = 0.30$	—	—	—	—	9(10) $H = 0.70$	—	—
Stizostedion vitreum	10	3(10) $H = 0.20$	2(8) $H = 0.10$	2(10) $H = 0.10$	—	—	—	—	8(10) $H = 0.70$	1(9) $H = 0$	—
Stizostedion lucioperca	8	— $H = 0.38$	3(6) $H = 0$	2(8)	—	—	—	—	4(8) $H = 0.63$	2(7) $H = 0.38$	—
Perca fluviatilis	14	5(14) $H = 0.57$	6(14) $H = 0.36$	—	7(14) $H = 0.71$	6(14) $H = 0.64$	6(14) $H = 0.50$	—	1(13) $H = 0$	8(13) $H = 0.50$	7(14) $H = 0.64$

—, indicates no amplification.

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Isolation of microsatellite markers in the digenetic trematode *Schistosoma mansoni* from Guadeloupe island

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Schistosomes are dioecious blood helminth parasites of human beings and rodents in tropical countries. Development of methods allowing more precise characterization of intraspecific genetic variation and population structure will greatly improve our understanding of schistosome epidemiology and transmission patterns.

In Guadeloupe (French West Indies), numerous foci have been surveyed during several years for the ecology and dynamics of *Schistosoma mansoni* populations among its murine definitive host (*Rattus rattus*) and intermediate mollusc host (*Biomphalaria glabrata*) (Théron & Pointier 1995). Distribution of genetic diversity within and among adult and larval schistosome populations was previously studied using isozymes (Rollinson *et al*. 1986) and RAPD markers (Barral *et al*. 1996; Sire *et al*. 1999). However isozymes loci were not sufficiently polymorphic, and RAPD markers are dominant markers which prevent access to the heterozygosity. The aim of this work was to detect high polymorphic markers such as microsatellites to analyse the fine local structure of infra-populations of schistosomes within and between individual hosts.

After complete digestion of *S. mansoni* DNA with *Sau*3AI, we screened a partial genomic library consisting of about 2894 fragments (size 200–700 bp) ligated into a plasmid pBluescript II SK (+) (Stratagene), digested with *Bam*HI. Colony hybridization was performed with synthetic $(CA)_{10}$ and $(GA)_{10}$ oligonucleotide probes using DIG labelling Kit (Boehringer) according to the protocol of Estoup & Martin (1996). A total of 18 positive clones (0.6% of all colonies screened) were sequenced with [γ-^{33}P] ATP end-labelled with T7 DNA polymerase (Pharmacia) and/or with automatic sequencer (Genome Express). Fifteen sequences containing microsatellites were selected and the primers of corresponding flanking regions defined. In addition, approximately 7000 sequences of *S. mansoni* from EMBL and GenBank databases were checked to detect microsatellites. Eighteen short repeat sequences were selected according to the length of flanking sequences to design primers using OSP version 5.0 software.

Polymerase chain reaction (PCR) was performed in a 40-μL reaction volume containing 30 pmol of each primer, 1 mM dNTPs (Boehringer), 4 μL buffer 10× (10 mM Tris-HCl pH 9.0,

Table 1 Primer sequences and characteristics of *Schistosoma mansoni* microsatellite loci, including locus name, GenBank Accession no., primer sequences, specific annealing temperature (T_a), size of PCR products in base pairs (bp), number of alleles, observed heterozygosity (H_O), unbiased expected heterozygosity (H_E), sample size and repeat array

Locus	Accession no.	Primer sequences (5′–3′)	T_a °C	Size bp	No. of alleles	H_O	H_E	Sample size	Repeat array
SMD25	AF202965	F: GATTCCCAAGATTAATGCC R: GCCATTAGATAATGTACGTG	48	292	3	0.10	0.19	10	$(CA)_{10}$
SMD28	AF202966	F: CATCACCATCAATCACTC R: TATTCACAGTAGTAGGCG	48	240	2	0.08	0.08	12	$(CAA)_5$
SMD57	AF202967	F: TCCTTGATTCCACTGTTG R: GCAGTAATCCGAAAGATTAG	50	297	3	0.40	0.41	10	$(TA)_{22}(GA)_9$
SMD89	AF202968	F: AGACTACTTTCATAGCCC R: TTAAACCGAAGCGAGAAG	51	153	3	0.21	0.28	19	$(TC)_8$
SMD94	AF202969	F: TAACACTCACACATACCC R: AACTAATCACCCACTCTAC	51	184	2	0.06	0.06	17	$(TC)_5$
AI068335	AI068335	F: GTTGAGAGAGAAAAAGAAG R: AGATGTTAGAAAGTGGTG	51	269	2	0.08	0.08	12	$(TG)_{10}$
L46951	L46951	F: CAAACATATACATTGAATACAG R: TGAATTGATGAATGATTGAAG	48	172	2	0.55	0.51	11	$(GAA)_7$
SCMSMOXII	M85305	F: TTCTACAATAATACCATCAAC R: TTTTTTCTCACTCATATACAC	48	295	3	0.60	0.45	10	$(CAT)_9CGT(CAT)_6$
R95529	R95529	F: GTGATTGGGGTGATAAAG R: CATGTTTCTTCAGTGTCC	51	243	4	0.44	0.46	16	$(CAT)_{10}$
SMU31768	U31768	F: TACAACTTCCATCACTTC R: CCATAAGAAAGAAACCAC	48	203	2	0.11	0.53	9	$(GAT)_8$
SMIMP25	X77211	F: CACTATACCTACTACTAATC R: TCGATATACATTGGGAAG	49	219	8	0.90	0.83	19	$(TA)_{16}$

50 mM KCl, 0.1% Triton X-100, Promega), 1.5 mM $MgCl_2$ (Promega), 2 U *Taq* polymerase (Promega) and approximately 20 ng DNA template. The PCR programme consisted of initial denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, annealing temperature for 30 s (see Table 1 for details), 72 °C for 30 s, and a final extension at 72 °C for 10 min in an MJ-Research PTC100 thermocycler. PCR products were mixed with one third volumes of formamide loading buffer and denatured at 95 °C for 5 min, prior to electrophoresis in a denaturing (7 M urea) 8% Long Ranger (Tebu) polyacrylamide gel (18 × 20 cm, Pharmacia). The PCR products were revealed by silver nitrate (Sigma) staining.

A total of 33 microsatellites loci were used to detect length polymorphism. DNA samples from adult schistosomes from two wild rats *R. rattus* trapped in the Dans-Fond locality (Guadeloupe) were extracted using a 5% Chelex-100 (Bio-Rad) extraction method. Of the 33 microsatellite sequences examined, di-, tri- and tetranucleotide repeats were found and the frequencies of perfect, imperfect, and compound repeats were 61, 30 and 9%, respectively. Eleven loci were polymorphic with the number of alleles ranging from 2 to 8 (Table 1). The observed (H_O) and expected heterozygosities (H_E) varied considerably between loci (Table 1). These microsatellite loci will be highly useful to analyse the mono- vs. pluri-infection within intermediate hosts, the recruitment pattern by the definitive hosts inferred from the amount of parasite genetic variability within and between individual hosts, and the population structures of schistosome at different spatial scales (regional vs. local) in a metapopulation system.

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Ten microsatellite loci isolated and developed for the blackspot seabream, *Pagellus bogaraveo* (Brünnich 1768)

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Pagellus bogaraveo is a commercially valuable species of fish that is distributed along the European continental shelf, in the Atlantic and throughout the Mediterranean. It is also found on the slopes of the Macaronesian (mid North Atlantic) islands and their associated seamounts. In this area, *P. bogaraveo* has been the subject of a baited hook and line fishery for over 200 years. Recently, increasing demand for this fish, outside of Macaronesia, has led to modern industrial fishing techniques being employed to increase catches, putting populations under increased fishing pressure. *P. bogaraveo* is a protandrous hermaphrodite and changes in male : female : hermaphrodite frequency ratios, and sizes at maturity have been detected (Krug 1998). Such changes in population parameters may be an early sign of stock collapse and, in the light of over exploitation of other seamount fisheries (e.g. orange roughy, *Hoplostethus atlanticus*, Merrett & Haedrich 1997), it is important that the population structure of *P. bogaraveo* in and around the Azores is resolved. This will allow the construction of a scientifically based management strategy for this species.

In addition to the management and conservation issues surrounding this species, it also appears to exhibit a metapopulation structure, in which geographical isolation of demes around different islands and seamounts, may be correlated with genetic isolation. To define genetically based Management Units and to study the metapopulation dynamics of *P. bogaraveo*, microsatellites provide useful, high resolution, genetic markers. The present paper details the isolation and assessment of variation of 10 microsatellite loci for this species.

The enriched library technique used for finding microsatellites is based on that of Kandpal *et al*. (1994). Genomic DNA was extracted from liver samples, from 14 *P. bogaraveo*, using a phenol–chloroform–isoamyl alcohol technique (Sambrook *et al*. 1989). Combined extractions were partially digested using the restriction enzyme *Mbo*I; from this digest 200–600 bp fragments were gel purified. Double stranded *Mbo*I adaptor molecules (Kandpal *et al*. 1994), were ligated to these fragments, which were then amplified by the polymerase chain reaction (PCR) using the single stranded *Mbo*I adaptor oligonucleotides as a primer. Fragments containing $(CA)_X$ repeats were allowed to hybridize to a biotin-labelled $(CA)_{10}$ oligonucleotide, and were subsequently captured using streptavadin coated magnetic beads. The resulting $(CA)_X$ enriched fragments were further amplified, ligated into pGEM-T plasmids (Promega), then used to transform JM 109 strain *Escherichia coli* (Promega). One hundred and fifty-eight colonies were successfully transformed, then were lysed, and their DNA hybridized to a nitro-cellulose membrane. Digoxygenin labelled (Boehringer Mannheim) $(CA)_{10}$ probes were used to assess the success of microsatellite enrichment. The 36 clones which gave the strongest signal were purified using the Wizard Plus SV Miniprep DNA purification system (Promega). These were then cycle-sequenced using Big Dye terminators (PE applied Biosystems) and the sequences detected by electrophoresis on an ABI 377 sequencer. Of the 36 clones sequenced, 20 provided sequences of sufficient quality for primer pairs to be designed. Primers were designed using Primer 3 (Rozen & Skaletsky 1998). Of the 20 microsatellite primer pairs designed, 10 produced amplifiable microsatellite loci. These loci were assessed for variability on an ABI 377 auto-sequencer using the Genescan application (PE Applied Biosystems). Details of the primers used to amplify these loci, specific fluor-labels and the observed and expected heterozygosity for each locus are given in Table 1. DNA from 18 *P. bogaraveo* was extracted using Qiagen QIAquick DNA extraction kits. This was used as template DNA for the screening of microsatellite loci for amplification and variability. PCR was performed using a Hybaid 'PCR Express'. The PCR conditions for these samples were as follows: amplification in a 10-μL volume containing 30 ng of DNA, 0.7 U *Taq* DNA Polymerase (Qiagen), 8 pmol of each primer (one of which was fluor-labelled, see Table 1), 1 × proprietary buffer (containing Tris-HCl, KCl, $MgCl_2$ $(NH_4)_2SO_4$, pH 8.7), 1 × 'Q – Solution' (Qiagen), and 0.2 mM of each dNTP (Perkin Elmer). Amplification conditions were: an initial denaturation step of 96 °C for 5 mins, followed by 3 cycles of 96 °C for 2 mins, annealing temp for 1 min, 72 °C for one min. This was followed by 25 cycles of 95 °C for 30 s, annealing temp for 30 s, 72 °C for 45 s, with a final extension step of 72 °C for 30 mins. Locus specific annealing temperatures are given in Table 1. Five out of the 10 loci tested demonstrated significant deviation from Hardy–Weinberg equilibrium. As this small sample was from one seamount locality it was not possible to distinguish between null alleles, population substructure effects, sampling effects, or other causes of this difference between observed and expected allele frequencies.

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Table 1 Characterization of 10 *Pagellus bogarveo* microsatellite loci. GeneBank Accession nos are AF209084-AF209092. Based on samples taken from 18 *P. bogaraveo* from the Western Azores

Locus	Repeat sequence	Primer Sequences 5'–> 3 includes fluro-label type		T_m/°C	Number of alleles	Size range (bp)	H_O	H_E
PbMS 1	$(CA)_{34}$	F Fam –	TGGACTTGGACCTGCTGACC	62	18	176–268	0.33‡	0.92
		R	TGTCGCAAAGCCTGGAGGAC					
PbMS 2	$(CTT)_6CTC(CTT)_3$	F Fam –	CTCGCGGTAGTTGTTCACAGAGC	62	11	98–152	0.72	0.88
		R	GCAACAATGGAGGGTTCGTCATC					
PbMS 4	$(CA)_4T(CA)_{22}$	F Hex –	CAACCTGAAGTGAACCGCAGTC	62	14	170–224	0.94	0.90
		R	TCCAGCCCTCTATCACATCAGC					
PbMS 6	$(CA)_{43}$	F Ned –	TGGGACACTGCAGCACAGACAAG	62	17	103–169	0.89‡	0.93
		R	GGAGCCACTCCTTCAACTCAATG					
PbMS 15	$(CA)_{24}GAG(TC)_3$	F	CATGCTATAGGCTGCTCCAG	56	16	174–226	1.00	0.92
		R Fam –	GACAGACGAAACTCTGAGA					
PbMS 16	$(CA)_{33}$	F Hex –	AAACAGTCGGGACACAAAGC	56	18	105–225	1.00	0.93
		R	GGACAGGGGCCTAGTTTATC					
PbMS 17	$(GA)_5$	F	AGATAAACTAGGCCCCTGTCC	56	4	162–172	0.50†	0.65
		R Ned –	GTGGCTGATGATAGCGAAGC					
PbMS 18	$(CA)_{10}$	F Hex –	ACCTCTGCGTTAGCAACAGG	56	14	165–221	0.94	0.87
		R	GAGACTGAACACTTCCGTAGG					
PbMS 19	$(CAG)_{10}$	F Ned –	CAGGGGCCGTAAATCAGG	56	4	168–186	0.50†	0.43
		R	GAGTGGGCGAACATCTGC					
PbMS 20*	$(GTGC)_3(GT)_{11}$	F Ned –	GGCTGTGGAGGTGTGTTAGC	56	8	154–184	0.41‡	0.81
		R	ATTGTGTTTGATTTGGTGAAGG					

*sample based on 17 individuals.
†HO is significantly different from HE at P #0.05 level.
‡HO is significantly different from HE at P #0.0005 level.
Significance of deviation from Hardy–Weinberg equilibrium assessed by Guo and Thompson's analogue of Fisher's exact test, calculated using ARLEQUIN version 1.1 (Schneider *et al*. 1997).

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Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae)

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In response to the Pleistocene ice ages, many organisms living in temperate regions experienced recurrent range shifts and population contractions (Hewitt 1996). In Europe, the concomitant recolonizations of northern regions can result in a latitudinal cline in populations' genetic variability (Merilä 1997). The greater mouse-eared bat (*Myotis myotis*), which is an endemic species restricted to the Western Palaearctic region, is prone to have followed such a scenario. Thus, we expect that North European colonies of *M. myotis*, which were settled more recently, are genetically less variable than southern ones. In order to assess levels of nuclear polymorphism among southern and northern populations of bats, we developed and screened a partial genomic library of *M. myotis*.

Total DNA was extracted from the spleen of one female *M. myotis* (Sambrook *et al*. 1985) and digested with *Sau*3AI. Restriction fragments of 400–900 bp were isolated and ligated into pUC19/Bam*HI* circular vector (Appligene

Table 1 Characteristics and PCR conditions for the 13 microsatellite loci developed in the bat *Myotis myotis*. Temp. is the annealing temperature and Primer the final concentration of each primer. Repeat motifs and fragment sizes are those of the cloned allele

Locus Name	GenBank no.	Temp. (°C)	Primer [μM]	$MgCl_2$ [μM]	Primer sequence (5′–3′)	Repeat motif	Fragment size (bp)
A13	AF203638	55	0.45	2.0	AACGTTCATTCTGCCAAAGG TCATGCTGTTCCACTTCTGG	$(TC)_5TT(TC)_{25}$	237
B11	AF203644	55	0.25	1.5	CATTTACAACGTTACTCCAGGC GCAGTTAATCTGCATTTTGCC	$(TG)_6TA(TG)_{15}$	224
B22	AF203645	55	0.35	2.0	CCAGAAACACCAACGTCATG GGAGGGGTTGTACAGAGAAGG	$(TC)_{22}$	230
C113	AF203647	60	0.25	1.5	ACCTCCCTGCCCTGCAC GCAATGCTTCCTCCAAGTCC	$(ACC)_7$	101
D9	AF203649	60	0.25	2.0	TCTTTCCTCCCCTGTGCTC TCTGGACCCAAAATGCAGG	$(CT)_{29}$	148
D15	AF203651	60	0.45	2.0	GCTCTCTGAAGAGGCCCTG ATTCCAAGAGTGACAGCATCC	$(AC)_{17}$	114
E24	AF203657	60	0.25	1.5	GCAGGTTCAATCCCTGACC AAAGCCAGACTCCAAATTCTG	$(TC)_{32}$	236
F19	AF203661	60	0.25	1.5	GCTAGCCATGGAGAAGGAAG CCCAAATCTGTCTTTCAGGC	$(CA)_{19}$	214
G9	AF203666	55	0.25	1.5	AGGGGACATACAAGAATCAACC TAATTTCTCCACTGAACTCCCC	$(TC)_{19}$	153
G25	AF203667	55	0.25	1.5	TCCTTCCCATTTCTGTGAGG CCATTTCATCCATCCAGTCC	$(AGC)_{11}AAT(AGC)_4$	147
G30	AF203669	55	0.25	1.5	TTGCCAAATTCTGGTATCTTCC AGAGCTTAATGGGGAGGCTG	$(AG)_{24}$	132
H19	AF203675	55	0.25	1.5	GGAATCCGAATCCCTGGC GACATCCCCTCACCCCAAC	$(GT)_{18}CT(GT)_2$	100
H29	AF203677	55	0.35	1.5	TCAGGTGAGGATTGAAAACAC GCTTTATTTAGCATTGGAGAGC	$(CA)_{21}$	182

Oncor). Plasmids were then used to transform XL2-Blue MRF′ Ultracompetent cells (Stratagene). Transformants were screened for inserts containing microsatellites with a mix of $(TC)_{10,}$ $(TG)_{10}$ and $(AAT)_{10}$ DIG-end-labelled (Boehringer Mannheim) oligonuleotides. Among the 4000 insert-bearing clones, 300 were identified as positive and isolated. To increase the yield of true positives, these colonies were directly polymerase chain reaction (PCR) amplified using, in turn, one of the three oligonucleotide probes with one of the forward or reverse primers of the pUC19 sequence (Waldbieser 1995). Following this PCR-based screening, 91 clones were sequenced on an ABI 373XL sequencer (Applied Biosystems). Among them, 56 clones (61%) contained the expected repeat motif, 19 (21%) contained other motifs (mostly TG instead of TC repeats), and 16 clones were false positives (18%). These results confirm that the PCR-based screening is efficient to reduce the number of false positives to be sequenced. Finally, among the 75 clones containing potential microsatellites, 41 had flanking regions appropriate for primer design (using PRIMER version 0.5, Whitehead Institute, Cambridge, USA) and are deposited in the GenBank library (accession nos AF203637–AF203677). Thirteen loci were further optimized and gave reliable results (Table 1).

Markers were tested on 20 *M. myotis* from the nursery colony of Canaleja (Guadalajarra, Central Spain) and 20 from Eysins (near the Lake of Geneva, Switzerland) using fluorescent labelling method. Appropriate concentrations of primers and $MgCl_2$ were optimized for each locus (Table 1). PCR reactions were carried out in 7.5 μL reaction volumes, with 2.5 μL of template DNA (25–100 ng), 0.1 mm of each dNTP, 1 × *Taq* buffer and 0.25 U of *Taq* DNA polymerase (Qiagen). One primer of each pair was fluorescent labelled (from Applied Biosystems) with HEX (locus A13, D15, G25), NED (locus B11, B22, G30, H29) or 6-FAM (locus C113, D9, E24, F19, G9, H19). PCR reactions were overlaid with mineral oil and performed with a PTC-100 thermocycler (MJ research) as follows: 3 min at 95 °C followed by 30 cycles of 45 s at 94 °C, 30 s at the annealing temperature (Table 1), 45 s at 72 °C. The cycles ended with one final extension of 10 min at 72 °C. We designed two post-PCR multiplex combinations to simultaneously reveal in a single lane six and seven loci, respectively. Prior to electrophoresis, the PCR products of each individual were mixed in the following proportions. The first multiplex contained 1.25 μL of C113, 3.0 μL of D9 and 2.5 μL of each B11, E24, G25 and H29. For the second set of loci, 3 μL of each B22, G9 and G30 were mixed with 2 μL of each F19 and H19, and 5 μL of each A13 and D15. PCR products were run on an ABI 373XL sequencer and sized with internal lane standard (GenScan 350-Rox; Applied Biosystems) using the program GENESCAN version 3.1 (Applied Biosystems) and by comparison with the comigrating individual that was used to construct the clone library.

Table 2 Number of alleles, observed (H_O) and expected (H_E) heterozygosity for two nursery colonies of 20 individuals each. None of the heterozygote deficiency within colonies (F_{IS}) was significantly different from zero

	Canaleja (Central Spain), n = 20				Eysins (Switzerland), n = 20			
Locus	No. alleles	H_O	H_E	F_{IS}	No. alleles	H_O	H_E	F_{IS}
A13	15	0.950	0.916	−0.037	11	0.750	0.874	0.142
B11	8	0.900	0.857	−0.051	9	0.850	0.822	−0.034
B22*	15	0.850	0.893	0.049	13	0.750	0.878	0.145
C113	2	0.050	0.050	0	2	0.050	0.050	0
D9	15	0.900	0.925	0.027	13	0.950	0.904	−0.051
D15	10	0.900	0.876	−0.027	10	0.800	0.891	0.102
E24	15	0.800	0.925	0.135	10	0.850	0.878	0.031
F19	9	0.800	0.871	0.082	8	0.850	0.828	−0.027
G9	8	0.800	0.750	0	8	0.850	0.805	−0.056
G25	4	0.700	0.583	−0.201	5	0.650	0.693	0.063
G30	15	0.900	0.929	0.031	11	0.950	0.900	−0.056
H19	7	0.750	0.795	0.056	8	0.900	0.842	−0.069
H29	11	0.950	0.889	−0.068	10	0.900	0.867	−0.038
mean	10.3	0.788	0.789	0.006	9.1	0.777	0.787	0.013

*Showed allelic variation of 1 and 2 bp.

All 40 individuals could be genotyped at the 13 microsatellite loci and no significant heterozygote deficiency at any locus or population was found using FSTAT version 2.9 (Goudet 1995; Table 2). This suggests that null alleles must be either absent or very rare in both assayed populations. Polymorphism was high with a total number of 153 alleles detected. Unexpectedly, number of alleles (10.3 and 9.1) and heterozygosity (0.789 and 0.787) were substantial and comparable in the southern and northern colonies (Table 2). The locus C113 is an exception with only two alleles, the longest of which was nearly fixed in both populations (at frequency of 0.975). Furthermore, both colonies were only weakly differentiated from each other (F_{ST} = 0.011 ± 0.004).

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Polymorphic variable number of tandem repeat (VNTR) loci in the ommastrephid squid, *Illex coindetii* and *Todaropsis eblanae*

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The abundance and commercial importance of ommastrephid squid has led to increased interest in their stock dynamics. The ommastrephid species *Illex argentinus* (Castellanos 1960) and *Illex illecebrosus* (Lasueur 1821) are already targeted by fisheries in West Atlantic waters. Both *Illex coindetii* (Verany 1939) and *Todaropsis eblanae* (Ball 1841) occur in large enough aggregations in the Eastern Atlantic to support substantial fisheries. Genetic markers are being used increasingly to discriminate between stocks to support the establishment of

effective management strategies. Previous studies of squid using allozymes have failed to provide a reliable indication of stock structure due to low levels of genetic variability (Carvalho *et al*. 1992; Brierley *et al*. 1995). In this paper, we report four novel polymorphic microsatellite loci and a minisatellite locus developed for *T. eblanae*, and a new microsatellite locus for *I. coindetii*.

Genomic DNA was extracted from armtips of ethanol preserved specimens using a modified CTAB protocol (Winnepenninckx *et al*. 1993). DNA from both species was digested with *Mbo*I restriction enzyme and digested fragments were separated by agarose gel electrophoresis. Fragments in the size range 300–700 bp were isolated and ligated into *Bam*HI digested and dephosphorylated pUC18 plasmids (Boehringer-Mannheim™) which were used to transform super-competent JM109 *Escherichia coli* cells (Promega™) (Rassmann *et al*. 1991). Approximately 700 *I. coindetii*, and 1400 *T. eblanae* positive recombinants were selected by colour screening and positives were transferred to Hybond-N membranes (Amersham™). The membranes were screened for the presence of microsatellite repeat regions by hybridizing with a mixture of oligonucleotides, consisting of di-, tri- and tetranucleotide repeats (i.e. $(GT)_8$, $(CT)_8$, $(GATA)_4$, $(GACA)_4$, $(ATT)_4$ and $(CAA)_4$) end-labelled with [γ-^{32}P]-dATP. Hybridization was carried out overnight at 42 °C (in 6× SSPE, 0.5% dried milk (MARVEL™), 1% SDS, 6% (PEG)). Positive hybridizations were identified from a strong signal on an autoradiograph. Ten positives from the *I. coindetii* library and 14 from the *T. eblanae* library were identified and sequenced using a thermo sequenase cycle sequencing kit (Amersham™). Primers (pUC18 forward and reverse) were labelled with a 5′ IRD800 label (MWG-Biotech™) and sequences were run out on polyacrylamide gels using a Licor automated DNA sequencer. Primers for the polymerase chain reaction (PCR) were designed in the flanking regions of four *I. coindetii* clones and 11 *T. eblanae* clones using OLIGO 5.0 primer design software (Medprobe™).

Initial testing of primer sets revealed that two of the *I. coindetii* primers and seven of the *T. eblanae* primers amplified PCR products of the expected size, however, three of these failed to amplify products consistently when one of the primers was labelled with a 5′ IRD800 label for screening purposes. The remaining loci (one *I. coindetii* and five *T. eblanae*) were screened in individuals of the appropriate species from a single area, to determine the extent of allelic diversity and compliance with Hardy–Weinberg expectations. PCR reactions were carried out in a total volume of 10 μL, including 100 ng DNA, 0.075 M Tris pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 0.25 mM dNTPs, 0.5 U *Taq* DNA Polymerase (Advanced Biotechnologies™), 1.5–2.0 mM $MgCl_2$ (see Table 1) and 1 μM of forward and reverse primers. Amplifications were carried out on a Hybaid™ thermo cycler with 30 cycles of denaturation at 95 °C for 30 s, annealing for 30 s and extension at 72 °C for 30 s, where optimum annealing temperatures for each primer set are detailed in Table 1. Alleles were resolved on 6% polyacrylamide gels using a Licor automated DNA sequencer, and allele sizes were determined by comparison with size ladders in the range 50–350 bp (Licor™)and 100–1500 bp (Microzone™).

Table 1 Estimated variability of five microsatellite loci, and one minisatellite locus for the ommastrephid squid species *Todaropsis eblanae* and *Illex coindetii*, from the west of Ireland. T_a, annealing temperature, N, total number of alleles sampled, N_a, number of alleles observed, H_O and H_E, observed and expected heterozygosity, respectively

Locus	Primer sequence	Accession no.*	T_a	$MgCl_2$ conc	Allele size range	N	N_a	H_O	H_E	Repeat motif
Icon-32	F: 5′-TTGCAAAACGCGATACAGG R: 5′-GTCTATTGTTAAATACCCGCTTC	AJ251487	54 °C	2.0 mM	464–500	82	18	0.93	0.94	$(TG)_{25}$
Tebl-20	F: 5′-AGAGGAACAGCTGACAAGGGTG R: 5′-ATTACCGACGCAGAAGGACG	AJ251488	54 °C	1.5 mM	199–238	98	12	0.86	0.84	$(AGT)_{15}$
Tebl-25	F: 5′-ATGTGAGCGTGAGAAGGAAAGG R: 5′-TAGAAAATGAAAAGGCAAAGGTG	AJ251489	54 °C	2.0 mM	273–343	94	20	0.98	0.92	$(TG)_{22}$
Tebl-32	F: 5′-CGCAAAATAGGCAGTCATAGAGGG R: 5′-ATACGAAGCGAATGGAGGGA	AJ251490	54 °C	1.5 mM	121–145	78	8	0.87	0.86	$(TGC)_8(TGA)_4TGG(TGA)_2$
Tebl-812	F: 5′-CCCGGACTCAGCATGAACACA R: 5′-TTATGATGGCTGTCGGCTCGC	AJ251491	52 °C	2.0 mM	389–445	82	11	0.88	0.87	$(CTAT)_{10}$
Tebl-MINI	F: 5′-TGCAAGTAAAATTTCCGTTAATATCTA R: 5′-TCCACCTTAAAAGAACAATCCGC	AJ251492	56 °C	2.0 mM	242–1351	94	50	0.89	0.98	$(AGTCAGACTTGTTAGTCAGTC)_{19}$

*Accession nos refer to the cloned sequences as deposited in EMBL DNA database.

Samples of both species collected off the west coast of Ireland were screened for allelic variability for each of the six loci. Estimates of heterozygosity, exact tests for compliance of genotype frequencies with Hardy–Weinberg expectations (Guo & Thomson 1992) and linkage analysis (Raymond & Rousset 1995) were carried out in GENEPOP 3.0 (Raymond & Rousset 1995).

All six loci revealed allelic polymorphism with 8–50 alleles per locus. No significant linkage disequilibrium was detected among the five loci isolated from *T. eblanae*. Neither were any significant deviations from Hardy–Weinberg expected proportions detected (null hypothesis rejected at $P < 0.05$). These preliminary results suggest that these loci will be of value for describing population structures in the species from which they were isolated. Assessment of the potential utility of the primers on related species, revealed that *Icon*-32 amplified products of expected size in both *I. argentinus* and *I. illecebrosus*, while *Tebl*-20 yielded products of expected size in all three *Illex* species as well as in *Todarodes sagittatus*.

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Polymorphic microsatellite DNA loci identified in the African elephant (*Loxodonta africana*)

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Historically, elephants (*Loxodonta africana*) were found on nearly all of the African continent. Because of habitat encroachment and the demand for ivory, elephants are now only found in highly fragmented areas south of the Sahara and have experienced a dramatic decline in numbers (Douglas-Hamilton 1987). Currently, the Convention in International Trade in Endangered Species (CITES) and individual African governments are in the process of making policy decisions regarding the sale of ivory and elephant conservation. Yet, difficulties in censusing elephants makes projecting rates of decline or recovery difficult and little information is known about the genetic diversity present in isolated elephant populations.

Polymorphic microsatellite DNA loci are useful for assessing population-level genetic diversity and can be used to identify populations at risk of extinction (Gottelli *et al*. 1994). These genetic loci feature tandem repeats of 1–6 base pairs (bp) reiterated up to 100 times and large numbers of alleles are due to a high mutation rate (Tautz 1989). They are ubiquitous and relatively evenly dispersed in mammalian genomes (Tautz & Renz 1984; Stallings *et al*. 1991). We report in this paper on the isolation and characterization of 12 polymorphic microsatellite loci in the African elephant genome.

A small insert plasmid library was made from genomic DNA isolated from African elephant blood (*L. africana*) collected from a single individual (Sambrook *et al*. 1989). Genomic DNA was partially digested with restriction enzymes *Bfa*I and *Mse*I, then ligated into the *Nde*I site of a pGEM-5z(+/–) plasmid (Promega). Approximately 16 000 recombinant bacterial colonies were screened with a $(CA)_{15}$ oligonucleotide that was 5′ end-labelled with [γ^{32}P]-ATP. One hundred and five colonies were scored as strongly positive after a secondary screening.

Plasmid DNAs were isolated using a mini plasmid DNA isolation kit (Qiagen). Those clones containing inserts of less than one kilobase (Kb) were sequenced in both directions using the Dye Deoxy terminator cycle sequencing kit (Perkin Elmer/Applied Biosystems) and an Applied Biosystems 373 A sequencer. Plasmids containing noninterrupted repeats of greater than 10 units and suitable flanking sequences were selected for further analysis. Primers were designed to generate 70–300 bp products at annealing temperatures of 55–61 °C using PRIMER version 0.5 (Whitehead Institute, Cambridge MA). The primer sequences, number of repeated units in the sequenced clone, optimized annealing temperatures and approximate sizes of the polymerase chain reaction (PCR) products are reported in Table 1.

PCR amplifications were carried out using 50 ng of genomic DNA in a volume of 10 µL containing 10 µM of a forward primer, 5′ end-labelled with [γ^{32}P]-ATP, and unlabeled reverse

Table 1 Twelve microsatellite loci identified in *Loxodonta africana*

Loci	Repeats*	Forward primers (5′–3′)	Reverse primers (5′–3′)	Size* (bp)	T_a (°C)	Accession nos
FH1	$(CA)_{12}$	GATCAGACCATGGCATGAG	ACAGTCTCCCTTGGGAAGAC	81	55	A206275
FH48	$(CA)_{22}$	GAGTCTCCATAATCAAGAGCG	CCTCCCTGGAATCTGTACAG	178	58	A206279
FH60	$(CA)_{13}$	CAAGAAGCTTTGGGATTGGG	CCTGCAGCTCAGAACACCTG	148	61	A206280
FH65	$(CA)_{19}$	GGCTGTAGCATTTTACACTCCC	CATGAATAAACCCAGCCTCTG	241	60	A206281
FH71	$(CA)_{14}$	GGGATTGGCTAAAATAG	CTAAGCACATCAGGGAC	69	58	A206283
FH40	$(CA)_{17}$	GGCTTTCTAGCCACCTCCTTC	GCTCACATTCACTTGCTGACC	243	60	A206278
FH94	$(CA)_{16}$	TTCCTCCCACAGAGCAGC	ATTGGTTAATTTGCCAGTCCC	229	61	A206284
FH39	$(CA)_{18}$	GTATTCCTGGGCATTCCATG	CTTGGAATATGACCCTGTTTG	242	60	A206277
FH103	$(CA)_{13}$	TGTGCTGCCACTTCCTACAC	GATGTTGAGACAGTTCTGTAAG	154	58	A206286
FH102	$(CT)_{11}(CA)_{14}$	CTTCATTACTGACCTAAACGAG	GGACAGGGCTGGAGAAATATG	179	60	A206285
FH67	$(CA)_{15}$	GCTTCTCTAGAAATGTGTATGC	GGCGTATAGGATAGTTCCAC	97	58	A206282
FH19	$(CA)_{15}$	GAAGCTCATGGTCAAGGTCAC	CTGCATACTCATCGAAGTCACC	185	60	A206276

*As determined from sequencing plasmid clones isolated from genomic DNA library. T_a, annealing temperature.

Table 2 Characterization of microsatellite loci isolated from an African elephant genomic DNA library in African and Asian elephants. Observed heterozygosities, H_O, and expected heterozygosities, H_E, were calculated using the program Genes in Populations (University of California, Davis)

	African				Asian			
Loci	Alleles	H_O	H_E	n	Alleles	H_O	H_E	n
FH1	3	0.217	0.258	23	1	0	0	13
FH48	6	0.609	0.654	23	4	0.769	0.675	14
FH60	5	0.565	0.633	23	6	0.857	0.724	14
FH65	4	0.826	0.647	23	3	0.333	0.375	12
FH71	4	0.636	0.536	23	6	0.714	0.679	14
FH40	5	0.652	0.545	23	2	0.071	0.069	14
FH94	4	0.609	0.556	23	6	0.714	0.765	14
FH39	7	0.826	0.742	23	1	0	0	14
FH103	5	0.565	0.581	23	3	0.643	0.615	14
FH102	3	0.435	0.492	23	6	0.929	0.765	14
FH67	6	0.739	0.651	23	2	0.357	0.497	14
FH19	7	0.739	0.743	23	1	0	0	9

n, number of individuals tested.

primer, 0.5 U of *Taq* DNA polymerase (Bioline), 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$ (except for FH102, where 2.0 mM $MgCl_2$ was used). Reactions were denatured 4 min at 94 °C, then cycled 25 times for 30 s each at 94 °C, the appropriate annealing temperature, and 72 °C in a Perkin Elmer 9600 thermocycler. A final extension was done for 3 min at 72 °C. PCR products were separated on 4–6% denaturing polyacrylamide gels and visualized by autoradiography.

The primer pairs were tested using genomic DNA isolated from captive African elephant and Asian elephant (*Elephas maximus*) blood provided by zoos in the USA. As shown in Table 2, all 12 loci are polymorphic in African elephants. The average heterozygosity (H_O) is 0.618 + 0.401 and there are an average of five alleles ± two per loci. Nine of the 12 loci are also polymorphic in Asian elephants (Table 2), with an average H_O of 0.449 + 0.116 and an average of four alleles ± two per loci. There is little correlation between African and Asian elephants in either H_O values or the number of observed alleles.

These polymorphic microsatellite loci and those colleagues recently described will be invaluable tools in the study of genetic diversity present in the remaining populations of African and Asian elephants (Nyakaana & Arctander 1998, 1999).

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Microsatellite variation in the freshwater snail *Biomphalaria pfeifferi*

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The freshwater snail *Biomphalaria pfeifferi*, the intermediate host for *Schistosoma mansoni* in Africa, is a relevant biological model for analysing the influence of evolutionary forces on the distribution of genetic variability. First, this hermaphroditic snail can both self- and cross-fertilize. Second, it occupies fragmented habitats experiencing annual cycles of flood and drought, which impose wide fluctuation of population size. It is, therefore, no wonder that previous genetic studies on *B. pfeifferi* using allozymes have shown no variability within populations (Mimpfouni & Greer 1990). This prompted us to develop microsatellite markers which revealed polymorphism in other highly inbred snail species (Viard *et al*. 1996).

Snail DNA was extracted from foot tissues using the QIAmp Tissue kit (QIAGEN, Inc.). Isolation of microsatellite loci was performed following Estoup *et al*. (1993), and screening of positive clones following Waldbieser (1995). Briefly, genomic DNA was digested using *Sau*3A, size-selected (300–800 bp) and purified (Promega kit). Restriction fragments were ligated into pUC19 (Eurogentec) and transformed into *Escherichia coli* XL1-Blue (Stratagène). Twelve thousand bacterial colonies were screened using $(GT)_{10,}$ $(GC)_{10,}$ $(GACA)_5$ and $(GATA)_6$ oligonucleotides. Positive clones were purified using the QIAGEN miniprep kit. They were sequenced (Genome Express company) on a 373 XL sequencer (Perkin Elmer) with the Big Dye Terminator sequencing kit. Primers were designed. Loci were amplified using the polymerase chain reaction (PCR) technique and a DNA thermal cycler (PTC 100 MJ Research). PCR reactions were performed using 1.2 mM $MgCl_2$, 60 μM dNTP, 800 nM of forward primer, 96 nM of reverse primer radioactively labelled with [γ^{33}P]-dATP and T4 kinase (Eurogentec), 0.25 Unit of *Taq* DNA polymerase (Eurogentec), 75 mM Tris-HCl pH 9, 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20 and 3 ng of snail DNA in a 12.5-μL total volume. Denaturation for 2 min at 95 °C was followed by 30 cycles under the following conditions: 30 s at 94 °C, 30 s at the locus-specific annealing temperature (Table 1) and 45 s at 72 °C. Amplified alleles were separated by electrophoresis in 5% denaturing polyacrylamide gels and visualized by exposure of dried gels to Kodak films for 16–24 h.

Out of 32 positive clones, 24 harboured a dinucleotide repeat (6–20 repeats), seven a tetranucleotide repeat (5–45 repeats)

Table 1 Repeated motif, sequences of forward (L) and reverse (R) primers (from 5′ to 3′) and annealing temperature (T in °C) of nine microsatellite loci in *Biomphalaria pfeifferi*. The γ^{33}P labelled primer is designed with *. The number of individuals studied ($N_{ind.}$), the size range of alleles (in base pairs) and the number of alleles for all populations (N_{aT}), in Madagascar (N_{aM}) and in Ivory Coast (N_{aCI}) are given for each locus over all populations studied. Only 38 individuals are studied with locus Bpf8 as the Nanarena population couldn't be amplified

Locus	Repeated motif	Primer sequence	T	$N_{ind.}$	Size range	$N_{aT;}$ $N_{aM;}$ N_{aCI}	GenBank Accession no.
Bpf 1	$(CT)_{14}$	L: TCCTATCCTTGTAACTTCTCCAC *R: CGAAACCATGCAAATCAG	52	58	203–207	2; 1; 1	AF189698
Bpf 2	$(GA)_3AA(GA)_4AA$ $(GA)_{10}AA(GA)_6$	L: GCAGCTTCATTCAACATTCC *R: AAATTAACATTTCGCTGAAACAG	52	58	139–153	4; 2; 2	AF189699
Bpf 3	$(TG)_6(TA)_9$	L: CAGTATTTCCCGTGACTGCTC *R: CTTTCCCTTGTTCGATACCATAC	52	58	166–211	2; 2; 1	AF189700
Bpf 5	$(GC)_9(AC)_7ATAC(AT)_3$	L: TGTATGCTGACACTTAAAGAAACC *R: GCTACGCCACTGCTTATGAC	52	58	175–187	3; 2; 1	AF189702
Bpf 8	$(TCTA)_{19}$	L: GGTTCCCATAGCATACAGTGC *R: GGCTTACAAAGAAACAGGCATAC	52	38	141–189	4; 3; 1	AF189704
Bpf 9	$(TCTA)_{45}$	*L: GTGAGGGAGAAGTACCTTGACAC R: GATCTAGTTAGAGAAGATAG	48	58	166–246	5; 4; 1	AF189705
Bpf 10	$(GA)_{20}$	L: TGTCCAGCATGTCCAGTTC *R: CAGAGATGATATTGCAGTCAGG	52	58	235–259	6; 5; 1	AF189706
Bpf 11	$(AC)_8(AT)_{19}$	*L: GAAGTGCTTGCCATTCTTC R: GTCAACTATCACGTGACAGG	48	58	104–106	2; 2; 1	AF189707
Bpf 12	$(GA)_9GGCA(GA)_2(CA)_5GA(CA)_5GA$ $(CA)_4(GA)_7CAGA(CA)_{11}(GA)_7$	L: GACACAAAGAAAGAGATAAGCA *R: GTCGACCTCCCACTCTTC	52	58	307–327	4; 3; 1	AF189708

Table 2 Name (year of sampling), localization (latitude and longitude in degrees, minutes and seconds), number of individuals ($N_{ind.}$), mean number of alleles per locus (N_{all}), observed heterozygosity (H_O), Nei's gene diversity (H_E) and $\hat{f}$-values for the 12 populations studied

Population	Localization	$N_{ind.}$	N_{all}	H_O	H_E	$\hat{f}$
Itasy (1999)	19 02 25/46 44 24	20	2.1	0.03	0.22	0.86
Nanarena (1999)	22 29 68/45 46 06	20	1.0	0	0.00	—
Man (1990)	07 26 12/07 55 06	18	1.1	0	0.01	1

—, monomorphic populations at all loci.

and one both a dinucleotide (5 repeats) and a tetranucleotide repeat (3 repeats). Nine microsatellite loci differing markedly in structure and number of repeats were retained for further analyses (Table 1). Screening was performed on individuals originating from two sites in Madagascar (Itasy and Nanarena) separated by about 500 kilometres, and from an African population (Man, Western Ivory Coast). The allele range was discontinuous at all loci (Table 1). Allelic diversity was rather high, with a mean of 3.3 alleles per locus, and correlated with the number of repeats of the largest stretch of pure repeats across loci (Spearman's coefficient of rank correlation, $P < 0.003$). It was possible to test for genotypic disequilibrium in only nine cases using exact tests implemented by GENEPOP version 3.1d (Raymond & Rousset 1995b). Applying Bonferroni sequential corrections, significant results at the 2% level were obtained for the pairs Bpf8/Bpf12 ($P < 0.011$), Bpf9/Bpf10 ($P < 0.018$) and Bpf10/Bpf12 ($P < 0.0086$) in Itasy. Gene diversity values ranged between 0 and 0.22 (Table 2). A striking result is that no heterozygotes were observed, except in Itasy (Table 2). Departures from Hardy–Weinberg equilibrium were tested within each population using exact tests. Significant heterozygote deficiencies were indeed observed, and $\hat{f}$-values, where $\hat{f}$ is the estimator of F_{IS}, ranged between 0.86 and 1. This indicated that the selfing rate ranged between 0.92 and 1 (for details about estimators, see Viard *et al*. 1996). This and the wide fluctuations of population size are likely causes of the limited variability observed (Viard *et al*. 1996). Exact tests performed over all loci using GENEPOP revealed that all pairs of population were significantly different ($P < 0.05$). F_{ST} were high in all pairwise comparisons, ranging between 0.84 and 0.99 and indicating that most genetic variance was distributed among populations. Substantial differentiation was also observed between the Ivory Coast and Malagasy populations, even if some alleles were shared at some microsatellite loci. Assessing more precisely the relationship between Malagasy and African populations on the basis of microsatellite variability would be a worthwhile task for the future, especially as *B. pfeifferi* has probably been recently introduced in Madagascar.

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Rapid isolation and characterization of microsatellites from the genome of Asian arowana (*Scleropages formosus, Osteoglossidae, Pisces*)

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Asian arowana (dragonfish; *Scleropages formosus*), is an ancient osteoglossid fish from south-east Asia (Kottelat *et al*. 1993) with extreme values on the ornamental and food market. Due to the high demand of the ornamental fish trade the species came close to extinction, and was classified by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as an Appendix I protected fish (Joseph *et al*. 1986). The green, white and gold strains of Asian arowana have lower commercial value than the red one (Kottelat *et al*. 1993).

Asian arowana is now bred regularly in Singapore and sold with CITES' permission, however, the genetic structure of the brood stock is unknown due to the lack of genetic markers. Microsatellites are the ideal DNA markers for studying fish populations because they are highly polymorphic and easy to analyse (O'Connell & Wright 1997). In this paper we describe the first microsatellites from the green strain of Asian arowana.

A $(CA)_n$-enriched plasmid library was constructed according Fischer & Bachmann (1998) with some modifications. Approximately 500 ng genomic DNA was digested with *Rsa*I, then an adaptor produced by hybridizing 5'-phosphorylated 25-mer and 21-mer oligonucleotides (and containing an *Mlu*I restriction

Table 1 Characterization of 21 microsatellites on 25 individuals of green Asian arowana

Locus	Pepeat motif	Primer (5′–3′)	T_a (°C)	No. of alleles	Size range (bp)	H_O	H_E	Genbank Accession no.
D01 (–)	$(CA)_{10}$	F GAATGCTTAAAGTGGCAGTGAA R CTGGCCTTACGCCCTGTGTTAC	55	11	174–216	0.61	0.82	AF219951
D04 (F)	$(GT)_{41}$	F GCTTAAACCCATTACAGACAGG R TTTCTTCATGCAAAACCACTTT	55	19	171–229	0.84	0.94	AF219952
D11 (H)	$(GT)_{16}$	F TGGTTTCCACCTACAGTCCAAAGA R GTTACGAGTATCTGGCCCAATGG	55	4	154–166	0.60	0.74	AF219953
D13 (–)	$(GT)_{12}$ $(CT)_{23}$	F AGCTGCTGTGTCTGTGGTGGTCTA R CATGCCCATGGAGAGGGAGAG	55	14	94–136	0.44	0.91	AF219954
D14 (–)	$(CA)_{12}$	F AAGGGAGCAGCAGTTAGGTAGACG R CCGTGGTGAATTAACATTTCCTCT	55	14	196–246	0.28	0.92	AF219955
D15 (F)	$(GT)_{16}$	F GACTGGCGTCCCGTCCTG R TTAGTGTCAATGGGAAAGAGCATA	50	6	224–236	0.28	0.54	AF219956
D16 (F)	$(GT)_{20}$	F CTTGCGCCCTGTGTTGC R AAGGCCTTTTCTGCTGGTAA	55	10	127–163	0.72	0.74	AF219957
D27 (F)	$(CA)_{17}$	F GTGTCAGTATAGTGAATCTGTAG R TGACAATGGCAGCATAATGAGAT	55	11	97–127	0.52	0.84	AF219958
D31 (F)	$(GATA)_{15}$	F GTTTGTCCCTCCATGCACTGAGAG R GTGATTGCCACATGGTTTTGTTGG	50	15	154–223	0.84	0.88	AF219959
D32 (H)	$(CA)_{13}$	F AGCACCCTGTTACTGGAAGAGA R AGTGTGATGCTTTTGCTTTGAGAA	55	9	236–292	0.84	0.80	AF219960
D33 (H)	$(CA)_{12}AA$ $C(CA)_4$	F TATTACCATGCGCCCAGCACAC R TGGGTGAGCCAGAAGCAGGACT	55	5	130–138	0.40	0.32	AF219961
D35 (F)	$(GT)_{17}$	F GTTTCTTCTAGGTGCTCTGGTTTC R CTACTTACACTGGGTCACTCATCC	55	12	130–156	0.32	0.91	AF219962
D37 (F)	$(GT)_{51}$	F GCCTTACGCCCTGTGTTGC R TGGATATCTGTGAGTGGTGGTGAA	55	13	223–294	0.72	0.88	AF219963
D38 (F)	$(GT)_{24}$	F TTGGGGTCATGCCACTGG R CAATAAATACCAAACAGGGAACC	50	22	179–227	0.61	0.95	AF219964
D42 (F)	$(CA)_{19}$	F AGGAACATCACTGACAACACT R TGGACTAACTAGGAGCACAT	50	20	145–201	0.92	0.94	AF219965
D72 (–)	$(CA)_{14}$	F AGCAGGTTAATTTGGAGACT R CGACCCTGTATGGGACAAG	50	9	78–98	0.60	0.89	AF219966
D85 (H)	$(CA)_{10}$	F GTTCCACAGGGGCTGAGAAAAT R GAGGACGGAACAAAAGCATTGG	55	8	140–154	0.60	0.79	AF219967
D88 (H)	$(GT)_{11}$	F TTTCTTTCTGAGACTGAGG R CAACTCTTATCCACCATTT	50	13	128–164	0.64	0.90	AF219968
D92 (H)	$(GT)_{13}$	F AGTCGCACACCACCACCTCAG R TCAGCGATAACCCCACACCT	55	12	146–174	0.72	0.89	AF219969
D94 (H)	$(CA)_{16}$	F CAGCAGCAGTGACACGGGTTCG R TCGCAGGCTGATTAAAGGTGTG	55	8	195–217	0.64	0.77	AF219970
D95 (T)	$(CA)_9$	F CCTGCGGAAGAAGAAAAGACT R CATGGTGTTGGCTGTGAGGAG	55	10	165–205	0.48	0.79	AF219971

(H), F primer labelled with HEX; (F), F primer labelled with 6FAM; (T), F primer labelled with TET; (–), primers unlabelled.
T_a, optimal annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity.

site) was ligated onto the blunt ends (Edwards *et al*. 1996). The ligation product was polymerase chain recation (PCR)-amplified in 25 μL containing 10 mM Tris-HCl (pH 8.8), 150 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of the 21-mer oligonucleotide as primer, 250 ng template and 2.0 U DyNAzyme II DNA-polymerase (Finnzymes). The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 2 min. The PCR products were then hybridized to a biotinylated $(CA)_{10}$ probe in 6× SSC at 55 °C for 20 min. The products from this hybridization were captured by using streptavidin-coated magnetic beads (350 μg MagnaBind™ from Pierce in 10 μL 6× SSC). The unhybridized genomic DNA and $(CA)_{10}$ probe were washed away and the captured fragments were eluted according to Fischer & Bachmann (1998). The eluate (1–2 μL) was then used as template for PCR amplification (see above for conditions). The amplification products were digested with *Mlu*I and ligated into pPCR-Script Cam vector (Stratagene) linearized with *BssH*II. The ligation products were transformed into XL-gold Kan supercompetent cells (Stratagene) and plated upon LB-agar containing 30 mg/L choramphenicol, 60 mg/L IPTG and 40 mg/L X-gal. More than 99% of the 10 500 clones obtained were white. The insert length was determined by colony PCR using M13–20 and M13 reverse

primers. Approximately 85% (244/288) of the tested clones contained inserts between 250 and 1000 bp. They were purified using GeneClean II Kit (Bio 101), then sequenced using M13–20 or M13 reverse primers using the ABIPrism Big Dye terminator cycle sequencing kit (Applied Biosystems). Extension products were separated on an automated ABI 377 sequencer (Applied Biosystems).

Out of 45 sequenced clones 35 (77.8%) contained CA repeats proving the efficiency of our approach. Twenty-eight out of 35 (80.0%) of these CA clones showed sufficient flanking sequences for primer design (PrimerSelect, DNASTAR).

PCR amplification of microsatellites was performed on a PTC-100 thermal cycler (MJ Research) using 30 ng genomic DNA in 25 μL volume. The concentration of components in the PCR reaction were as above, except for 0.2 μM of the primers. PCR conditions were: 94 °C for 2 min followed by 33 cycles at 94 °C for 30 s, annealing temperature (50 or 55 °C) for 30 s and 72 °C for 30 s with a final extension for 5 min at 72 °C. Unlabelled PCR products were separated either on 8% denaturing polyacrylamide or 4% Metaphor Agarose (FMC BioProducts) gels, then visualized by using silver staining or ethidium bromide staining, respectively. For fluorescent detection on the ABI 377 sequencer, one primer per pair was labelled with either 6FAM, TET or HEX dyes (Geneset). The length of the fluorescently labelled PCR products was determined using GENESCAN and GENOTYPER software programs (Applied Biosystems). Allele sizes of unlabelled PCR products were calculated against a 20-bp ladder. Twenty-eight microsatellite primer pairs were tested in 25 green Asian arowana individuals obtained from farms in Singapore and Indonesia (pedigrees unknown). Twenty-one out of 28 microsatellites showed specific products and polymorphism (Table 1). The average number of alleles was 11.7, while the average heterozygosity was 0.58.

Our microsatellite isolation method requires approximately one tenth of DNA (500 ng) than the original protocol of Fischer & Bachmann (1998). This made sample collection from live individuals less invasive, which is important for the study of an endangered species. Sequencing reactions were performed directly on the glassmilk-purified product of colony PCR, eliminating the need for minipreps.

Experiments are in progress to investigate the applicability of these markers to the gold and red strains, as well as to develop multiplex-PCRs for studies on genetic diversity of local Asian arowana brood stocks.

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Microsatellite variation in the freshwater schistosome-transmitting snail *Biomphalaria glabrata*

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Biomphalaria glabrata is a tropical freshwater snail and the main vector of schistosomiasis in South America and the Caribbean. This snail is a common inhabitant of slow flowing streams, drainage ditches and ponds from Brazil to Haiti. Reduced genetic variation has been previously reported in local populations of this species on the basis of allozyme studies (Mulvey & Vrijenhoek 1982), which may be related to large variation in population size. The mating system may also reduce variability because this species is a hermaphrodite that can self-fertilize. Microsatellite loci previously proved to be useful for producing variability in such situations (see Viard *et al*. 1997).

We characterized microsatellite loci in *B. glabrata*. DNA was extracted from foot tissue using the QIAmp Tissue kit (Qiagen). A partial genomic library (hereafter the classical library) and a GATA/GACA-rich library (hereafter the enriched library) were constructed following the protocols described by Estoup *et al*. (1993) and Billotte *et al*. (1999), respectively. Screening was performed following Waldbieser (1995). Positive colonies (1.4%, 23 and 19%, 19) were detected with $(GATA)_6$ and $(GACA)_5$ probes in the classical and enriched libraries, respectively (Table 1). Thus, the enrichment protocol yielded 13 times more tetranucleotide loci than the classical one. The classical library also yielded 2.2% (36) positive colonies for $(CT)_{10}$ and $(GT)_{10}$ probes. Only positive colonies with large inserts, as determined by polymerase chain reaction (PCR) with forward and reverse M13 primers, were sequenced. Ninety–100% of inserts sequenced contained at least one microsatellite (Table 1).

Table 1 The number of colonies screened, of positive colonies, of clones sequenced and of clones containing a microsatellite repeat are given for the two protocols used. Values obtained with $(CT)_{10}$ + $(GT)_{10}$/$(GATA)_6$ + $(GACA)_5$ probes are indicated for the classical library. The enriched library was screened with $(GATA)_6$/$(GACA)_5$ probes only

Library	Classical	Enriched
Colonies screened	1600	100
Positive colonies	36/23	19
Clones sequenced	20/10	12
Clones bearing microsatellites	19/10	11

Sixteen primer pairs were designed for sequences from both libraries and were tested using DNA from 30 individuals sampled in two populations from Central Venezuela (separated by 15 km). PCR amplifications contained 10 ng template DNA, 1.2 mM $MgCl_2$, 75 (M dNTP, 400 nM of reverse primer, 100 nM of forward primer radioactively labelled with γ^{33}P ATP, 0.25 Units of *Taq* DNA polymerase (Eurogentec), 75 mM Tris-HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20, in a final volume of 10 µL. PCR was performed using a PTC-100 thermal cycler (MJ Research): initial denaturation was at 94 °C for 1 min, followed by 30 cycles of 15 s at 94 °C, 30 s at the annealing temperature shown in Table 2, and 30 s at 72 °C. PCR products were run in 5% denaturing polyacrylamide gels and visualized by autoradiography. Allele sizes were determined from a sequence ladder run in parallel. Two loci failed to amplify correctly and were excluded from further analysis.

Details on loci diversity are shown in Table 2. Five loci from the classical library were monomorphic. Locus BgC6 showed different fixed allele for these populations and the loci BgC7 and BgC8 showed some variability within populations. On the other hand, all the loci from the enriched library were polymorphic, with up to nine alleles at loci

Table 2 Characterization of *Biomphalaria glabrata* microsatellite loci. BgC and BgE are loci detected in the classical and enriched genomic libraries, respectively. The core sequence of loci, primer sequences, annealing temperature (T_a °C) and GenBank accession nos are given for each locus. N_i within core sequences refers to strings of nucleotides unrelated to the repeated motif. Sixteen and 14 individuals were analysed in La Cuarta and Múcura populations. n_{all}, H_O and H_E refer to the number of alleles, observed and expected heterozygosities per population. The overall allele number (N_{all}) and size range (in bp) are also given

			La Cuarta			Múcura					
Locus	Core sequence/ Forward and reverse primers (5′–3′)	T_a	n_{all}	H_O	H_E	n_{all}	H_O	H_E	N_{all}	Size range	Accession no.
BgC6	$(CA)_4TT(CA)_2CT(CA)_5(TACA)_2$ f:GAGTCTGCGTTTAGCGTACAG r:TGCAGTGATTTGTTCCGTTC	58	1	0	0	1	0	0	2	302–304	AF216279
BgC7	$(AG)_3G(GA)_8GGGAGG(GA)_5$ f:AAACGGGATTGTGTGAATGG r:GCCCAGCAGCAGAGATTG	54	2	0.19	0.16	2	0.06	0.06	3	317–321	AF216280
BgC8	$(AG)_4N_{23}(AG)_8G(GA)_3N_{13}(AG)_5$ f:AGCCAGGACACCATGTTAGG r:GAAGCGAGCGTTTTGTTTTG	54	2	0.03	0.03	2	0.03	0.03	2	269–271	AF216274
BgE1	$(GT)_5(GA)_{15}(GACA)_6$ f:GATTGTAAGCTCAGGTGAATAGAAG r:ACACTCGAAAAACACACGAAC	54	1	0	0	2	0.04	0.04	2	133–148	AF216275
BgE2	$(GATA)_{18}TGGA(GATA)_9TAG(GATA)_5$ f:TTCCATATTCACGAACCAAC r:GGAACTTTGTGGAGACTGC	54	4	0.31*	0.33	4	0.25	0.24	7	310–398	AF216270
BgE3	$(GATA)_{25}$ f:GGCACCTTTTTCAATGTGG r:TTAGGGTTATTGTCTGTGAGGTTAG	60	5	0.22*	0.25	3	0.25	0.27	8	221–253	AF216269
BgE4	$(GATA)_{13}$ f:GTCAGGACTGTGTGTAAAAGGAAG r:AGAGGGCAGATGATGCAAAG	60	4	0.25	0.27	3	0.25	0.27	6	185–246	AF216272
BgE5	$(GATA)_{34}$ f:CAGCCTTAGCACCTCTAGTCG r:TCTCATGGAAGTGAAGCTGTG	60	6	0.28	0.35	4	0.14*	0.19	9	273–345	AF216271
BgE6	$(GATA)_{39}N_3(GATA)_{22}$ f:CAGCATTTACCACGAAGAGC r:CACCGCGCCTCTCTACTAC	54	5	0.20†	0.37	5	0.11*	0.16	9	328–526	AF216273

* and † in the H_O column refer to the exact tests for Hardy–Weinberg expectations significant at the 0.05 and 0.01 levels, respectively.

BgE5 and BgE6 (Table 2). The observed heterozygosity ranged from 0 (locus BgE1) to 0.31 (locus BgE2), both in La Cuarta. Departures from Hardy–Weinberg were evaluated using exact tests using the GENEPOP 3.1d package (Raymond & Rousset 1995). Loci BgE2, BgE3, BgE5 and BgE6 showed significant heterozygote deficiencies (Table 2) and this is most probably due to self-fertilization. Mean F_{IS} estimates for La Cuarta and Múcura populations were 0.18 and 0.06, respectively (see Raymond & Rousset 1995). This suggests that selfing rates are in the order of 0.31 for La Cuarta and 0.11 for Múcura (see Viard *et al*. 1997 and references therein). It was possible to test for genotypic disequilibria in 49 situations (pairs of loci within each population) using exact tests as implemented by GENEPOP. Only four pairs showed significant deviations from random expectations, but none were significant after Bonferroni correction.

In conclusion, at least eight polymorphic microsatellite loci suitable for population structure and mating system studies in natural populations of *B. glabrata* have been obtained. These loci, together with those previously described by Jones *et al*. (1999), could also be used for linkage analyses required by studies on the genetics of resistance of *B. glabrata* to the trematode parasite *Schistosoma mansoni*.

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