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PRIMER NOTES

Isolation of microsatellite markers in *Astatoreochromis alluaudi* and their cross-species amplifications in other African cichlids

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The three Great Lakes of East Africa, Lakes Victoria, Malawi, and Tanganyika, are second to none in cichlid species richness and the speed at which those species have evolved (Fryer & Iles 1972). Nevertheless, the evolutionary histories of the endemic Lake Victoria Region (LVR) haplochromine cichlids have been poorly understood, primarily due to a lack of sufficiently variable genetic markers (Sage *et al*. 1984; Meyer *et al*. 1990). To ameliorate this problem, a size-selected partial genomic library with an average insert size of 400 bp from a LVR cichlid, *Astatoreochromis alluaudi*, was constructed and screened as previously described (Paetkau & Strobeck 1994). Approximately 8910 recombinant plaques were screened using a mixture of 5′-biotinylated $(GT)_{12}$ and $(CT)_{12}$ probes, and ≈ 22 000 recombinant plaques were screened using a mixture of 5′-biotinylated $(ACC)_7$ and $(AGC)_7$ probes. Approximately 1.8% (158/8910) of the recombinant plaques screened for dinucleotide microsatellites are putative positive clones, and about 0.6% (127/22 000) are putative positive clones for trinucleotide microsatellites. We define putative positive clones as clones that showed positive signals after the first round of screening. A total of 45 putative positive clones were bi-directionally sequenced using GIBCO BRL double-stranded DNA (dsDNA) Cycle Sequencing System and the M13/pUC18 universal primers, and 42 of them con-

Table 1 Microsatellite core sequences, primer sequences and PCR conditions*

Locus	Repeat motif	Primer sequences (5' – 3')	Size range (bp)	No. of alleles	H_E	H_O	Accession number	*Tm*	No. of cycles
OSU09d	$(TG)_{20}(CGT)_{14}$	CCTCTGTAGTGATGTTTAATCTCTGT TGACACTGCACTTACTTGGCT	145–213	22	0.94	1.00	U66810	60 °C	28
OSU12t	$(NGC)_{13}$	TCAAACACCCACAGCCTTCA CGGTGATTGCTGTTGATACTGA	107	1	0.00	0.00	U66809	60 °C	22
OSU13d	$(GT)_{25}$	TAAGCTGATAGGAACCCAAC ACTCCTATTTTGTTATTTTTGTGA	105–151	20	0.93	0.94	U66811	58 °C	30
OSU16d	$(GT)_{10}$	GGCGAATGGTGGGTCAAG ATGTTGCTTGCCGCTGC	72–76	3	0.21	0.23	U66812	58 °C	32
OSU19d	$(GT)_{47}$	CAGTGCTTTGGTGGTGCT CATGACGTCTTTCAATAAGGAT	128–178	18	0.91	0.93	U66813	55 °C	30
OSU19dN		GATCACTTGTCAGTGCTTTG GATAGAACACTTAGAGTGCAGG						55 °C	30
OSU19t	$(CA)_{11}(ANC)_{12}$	TGAAGGACAAAGCAGGACTG TGCCCGAACCTTTTTATTTA	126–142	10	0.78	0.81	U66814	60 °C	28
OSU20d	$(GT)_{47}$	GAAGTGGGATTTGCAGCTTG CATGCTTACAAAGAACAGGGTTAC	151–211	26	0.94	0.86	U66815	60 °C	30
OSU20dN		ACACCTGGGTGAGACTGGC TTAGAGCGTGTCACACAGCAT						58 °C	31
OSU21d	$(GT)_6GC(GT)_4$	GCCGCTCAGAGTTTGGTG AGGCATGTGTCAGTTCATCCT	146–156	2	0.06	0.00	U66816	60 °C	22
OSU22d	$(GT)_{41}$	TGAAATCAAATACTAGAGCAAATA GGAGTTTAAAAATGATGCGT	107–173	23	0.94	1.00	U66817	55 °C	32
OSU22dN		GATCACTTTTTCCCTTTTA ATTCATACAACTACTGGCAC						50 °C	30

*PCR conditions are defined by PCR cycle numbers and annealing temperature (*Tm*). Estemates of size range (in base pairs), number of alleles, H_E (expected heterozygosity), and H_O (observed heterozygosity) are based on 31 individuals of *A. alluaudi* sampled from Lake Victoria using both primer sets OSUxxx and OSUxxxN.

tained the expected repeat motifs. The $(GT)_n$ motifs were estimated to occur at an average interval of 24 kb in the *A. alluaudi* genome.

Primers were designed for 15 positive clones using the computer program OLIGO (Rychlik & Rhoads 1989). Genomic DNA was extracted from the alcohol-preserved muscle tissues using either the standard phenol–chloroform extraction protocol or the NaOH extraction protocol (Zhang *et al*. 1994). For PCR analysis, each forward primer was end-labelled with [γ^{32}P]-ATP using T4 polynucleotide kinase. PCR was carried out in 5 or 10 μL of a mixture containing 20–30 ng of DNA template, 1.5 pmol of each primer, 100 μM dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM (KCl), and 0.5 U of *Taq* polymerase (GIBCO BRL). Amplification was achieved in either a Perkin-Elmer thermal cycler or an Ericomp Twin Block thermal cycler by running the appropriate number of cycles of 45 s at 95 °C, 45 s at the appropriate annealing temperature, and 30 s at 74 °C (Table 1). This main PCR profile was preceded by a long denaturation cycle at 95 °C for 5 min, and followed by a long extension cycle at 74 °C for 6 min. Multiplex PCR was performed for markers OSU12t and OSU21d. PCR products were resolved in 6% or 8% polyacrylamide sequencing gels, and their sizes were determined by running several pUC18 plasmid control sequences in adjacent lanes. Nine of the 15 primer pairs consistently amplified individuals of *A. alluaudi* (Table 1). Because the initial population screening on *A. alluaudi* suggested that genotypic distributions were significantly different from the Hardy–Weinberg expectations at three loci (OSU19d, OSU20d, and OSU22d), extra pairs of PCR primers (listed as OSUxxxN in Table 1) were designed to test for the presence of null alleles due to variation in primer sequences.

Samples of 10 additional African cichlid species, including seven LVR haplochromine species (Table 2), and one

Table 2 Genetic variability of microsatellites in LVR cichlid species

Species	OSU09d	OSU12t	OSU16d	OSU19d	OSU19t	OSU20d	OSU21d	Average
A. velifer								
N (*n*)	70 (7)	67 (4)	66 (27)	66 (28)	70 (25)	65 (28)	69 (3)	68 (17.4)
H_E	0.37	0.38	0.91	0.95	0.93	0.93	0.23	0.67
H_O	0.34	0.36	0.71	0.80	0.84	0.45	0.14	0.52
H. 'ruby'								
N (*n*)	18 (6)	19 (2)	19 (26)	19 (16)	17 (20)	18 (22)	19 (2)	18 (13.6)
H_E	0.34	0.10	0.95	0.92	0.91	0.94	0.33	0.64
H_O	0.39	0.11	1.00	1.00	0.71	0.67	0.32	0.60
P. 'rock kribensis'								
N (*n*)	31 (3)	31 (2)	31 (20)	30 (16)	29 (24)	30 (22)	31 (2)	30 (12.7)
H_E	0.47	0.12	0.87	0.88	0.87	0.92	0.41	0.65
H_O	0.55	0.13	0.61	0.87	0.76	0.50	0.39	0.54
P. 'black para'								
N (*n*)	22 (2)	23 (3)	23 (23)	23 (21)	21 (18)	20 (19)	23 (2)	12.6
H_E	0.09	0.20	0.94	0.93	0.87	0.90	0.16	0.58
H_O	0.09	0.17	0.91	1.00	0.71	0.75	0.09	0.53
P. sp.								
N (*n*)	16 (4)	16 (2)	16 (16)	16 (14)	16 (16)	16 (16)	16 (2)	16 (10.0)
H_E	0.33	0.22	0.92	0.91	0.90	0.90	0.30	0.64
H_O	0.38	0.00	0.69	0.63	0.88	0.75	0.13	0.49
Y. laparogramma								
N (*n*)	10 (4)	9 (2)	10 (12)	9 (10)	9 (12)	10 (13)	9 (1)	9 (7.7)
H_E	0.57	0.48	0.89	0.87	0.90	0.91	0.00	0.66
H_O	0.70	0.33	0.70	0.67	0.67	0.80	0.00	0.55
Y. fusiformis								
N (*n*)	30 (6)	29 (2)	29 (23)	29 (20)	30 (26)	30 (23)	29 (2)	29 (14.7)
H_E	0.32	0.21	0.94	0.93	0.88	0.94	0.03	0.61
H_O	0.27	0.17	0.86	0.72	0.80	0.63	0.03	0.50
Average								
N (*n*)	28 (4.6)	28 (2.4)	28 (21.0)	27 (17.9)	27 (20.4)	27 (20.4)	28 (2.0)	28 (12.7)
H_E	0.36	0.23	0.92	0.91	0.90	0.92	0.22	0.64
H_O	0.33	0.18	0.78	0.81	0.77	0.65	0.16	0.53

N, sample sizes; *n*, number of observed alleles; H_E, expected heterozygosity; H_O, observed heterozygosity.
For genus name, *A.* = *Astatotilapia*, *H.* = *Haplochromis*, *P.* = *Paralabidochromis*, and *Y.* = *Yssichromis*.

individual each from the two tilapiines species, *Oreochromis niloticus* and *O. esculentus*, as well as from a Lake Tanganyika congener of *A. alluaudi*, *A. straeleni*, were used to test cross-species amplifications of the nine primer pairs (OSUxxx in Table 1). All the nine primer pairs amplified *A. straeleni*, while in the more distantly related tilapiine cichlids, only five primer pairs (OSU09d, 12t, 16d, 20d, and 21d) produced clear PCR amplification products with typical microsatellite 'shadow' bands. Seven pairs of primers consistently amplified the presumptive homologous loci in the seven other LVR haplochromine species tested, and produced scorable bands. In sharp contrast to both allozyme markers (Sage *et al*. 1984) and DNA sequences (Meyer *et al*. 1990), these microsatellite markers detected substantial amounts of genetic variation within each species, with the average number of observed alleles ranging from 7.7 to 17.4 per locus, and the average expected heterozygosity (Nei 1987) being estimated to be from 0.58 to 0.67 (Table 2). These results suggest that microsatellites may be sufficiently variable to be useful in both population genetic analyses and phylogenetic studies of the closely related members of the Lake Victoria haplochromine species flock.

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Characterization of six polymorphic microsatellite markers in gilthead seabream, *Sparus aurata* (Linnaeus 1758)

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The gilthead seabream (*Sparus aurata*) is one of the most important species for fisheries and aquaculture in the Mediterranean Sea. It is a protandrous hermaphroditic euryhaline species, very common in the Mediterranean and Adriatic seas and one of the most important farmed fish in Europe. Therefore, the evaluation of the genetic variability of the species' populations is of great importance for fishery management. On the other hand, selection for improved characteristics of economic importance such as growth or disease resistance is of great interest in the aquaculture industry. Breeding experiments in marine aquaculture tend to be very laborious and expensive, in terms of facilities and personnel. In such experiments, progeny from different families must be reared at separate tanks in many replicates in order to detect and correct for among-tanks variance. Microsatellite DNA markers can facilitate such breeding experiments, reducing the effort and the facilities needed. The application of microsatellites allows procedures such as 'common rearing' to be followed. The latter reduces the environmental variance caused by the replicates. Here we report on the isolation and characterization of six microsatellite markers in the gilthead seabream.

High-molecular-weight genomic DNA was isolated from fish blood stored in 90% ethanol. Total DNA extraction was performed following the proteinase K/salt extraction technique (Pogson *et al*. 1995). Approximately 50 μg of DNA of a single individual was digested with three different restriction enzymes (*Hae*III, *Rsa*I and *Hin*cII). The restriction fragments were separated on a 1% low-melting-point agarose gel and a range between 300 and 800 bp were phenol extracted and ligated into a pUC18/*Sma*I/BAP vector (Pharmacia). Plasmids were transformed into *Escherichia coli* competent cells (MAX efficiency DH5α, Gibco BRL) which subsequently were plated onto Luria–Bertani (LB) agar plates containing 50 μg/mL ampicillin. Transformed cells were grown overnight at 37 °C and blotted on Hybond-N membranes which were hybridized with an end-labelled [g^{32}P]-ATP synthetic oligonucleotide $(GT)_{15}$ probe (Sambrook *et al*. 1989). Positive clones were isolated and cultured. Plasmid DNA was extracted using the alkaline lysis method (Sambrook *et al*. 1989) and the inserts were sequenced using the

Table 1 Characteristics of six microsatellite loci of the gilthead sea bream for $n = 32$ unrelated individuals

Locus	Primers (5'→3')	Repeat sequence	T_a (°C)	Size range of alleles (bp)	No. of alleles	H_E	H_O	EMBL Accession no.
SaGT1	F:AGGAGTTTAGTGCTCAATC R:TGTCCTGACTTCTTAAAACC	$(TG)_{25}C(GT)_8$	52	118–172	22	0.955	0.969	Y17265
SaGT41b	F:GGAGGCACTTGCAAGTGCAG R:AGATGGCAGAGGTCTGGTAG	$(AC)_{13}$	52	148–192	18	0.926	0.875	Y17262
SaGT26	F:GCCTCTCAACCGTATGTAG R:TGGTGATATTTATGCATCTAG	$(CA)_8CT(GT)_{31}$	52	223–257	13	0.896	0.938	Y17266
SaGT32	F:GAGCAGACACCAGTGCATG R:GATAATGGCCAAAAGTCACTG	$(CA)_{32}$	52	148–182	16	0.928	1.000	Y17264
SaGT41a	F:TCAAAGACAGATGGAGCTGG R:GTCACATCAGTCTGCACTTG	$(TG)_{27}C(GT)_7$	52	91 – > 200*	26	0.952	0.969	Y17263
SaGT31	F:CATAATTGTTCTCGTCAGGG R:GCAGCCAACACAAATAAGTC	$(TG)_{18}$	54	236–242	4	0.481	0.500	Y18103

*The allele before the one coded as > 200 bp was at a length of 159 bp.
T_a, annealing temperature; H_E, expected heterozygosity; H_O, observed heterozygosity.

Sequenase kit (USB) from both ends (Sanger *et al*. 1977). The microsatellite-containing regions of the inserts were identified and the flanking sequences were used to design the primers (Table 1).

Genomic DNA of 32 individuals was used for DNA typing. A small fraction (0.4 pmols) of one of the primers was end-labelled with [γ^{32}P]-ATP and used in the mixture of the PCR reaction. The PCR reactions were carried out in 10 μL, which contained 10–100 ng of genomic DNA, 1× reaction buffer (10 mM Tris-HCl pH 8.3, 1 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin), 200 μM of a mixture of the four dNTPs, 0.25 units of *Taq* polymerase (BRL) and 6 pmols of primers (for the one used in the labelling reaction 5.6 pmols of 'cold' and 0.4 pmols of 'hot'). The program profile set in a thermocycler (Perkin-Elmer 9600) was: 30 cycles for 60 s at 94 °, 30 s for the annealing temperature (Table 1) and 30 s at 72 °C.

Upon the completion of the PCR, an equal volume of loading buffer (10 ml) was added to each reaction and then the denaturation step was followed at 95 °C for 5 min. Six to seven ml of each reaction was loaded on a 6% denaturing polyacrylamide gel (7 M urea). The gels were run at 60 W for 2.5–3.5 h (depending on the product size), lifted, dried, and exposed to an X-ray film. Microsatellite alleles were sized relative to a sequencing ladder of single-stranded M13 mp18 DNA.

The six microsatellite markers revealed a considerably high amount of polymorphism (Table 1). The number of alleles per locus ranged from 4 to 26. The observed heterozygosity was 0.5 for SaGT31, 0.875 for SaGT41b, 0.938 for SaGT26, 0.969 for SaGT1 and SaGT41a and 1 for SaGT32 (average heterozygosity 0.875). If we ignore the less-polymorphic SaGT31 the average heterozygosity increases to 0.95 which can be considered among the highest reported values compared to other teleost fishes (Brooker *et al*. 1994; Galbusera *et al*. 1996).

Four of the markers (SaGT1, SaGT41b, SaGT26, and SaGT32) were used to profile 900 offspring of these 32 parents. Assuming codominant Mendelian inheritance, the offspring were successfully traced back to their parents in all cases. As the biology of the species does not permit direct crosses (obligatory group spawning) the above can be regarded as indirect proof of their codominant Mendelian inheritance.

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Microsatellite markers for the onychophoran *Euperipatoides rowelli*

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Phylum Onychophora (peripatus, velvet worms) are phylogenetically basal to arthropods, and inhabit moist environments in many circumtropical areas and Gondwanan landmasses. They are morphologically conservative, have high levels of local endemism and cryptic speciation, and diverse reproductive biologies (Briscoe & Tait 1995).

We are studying *Euperipatoides rowelli* (Reid 1996) as a model of population processes at increasing spatial scale from local patterns of mating through to regional phylogeography. This onychophoran is common in rotting logs in forests of southeastern New South Wales (NSW), Australia (Reid 1996). Allozymes identify regional cryptic species, but are unusually invariant at finer scales (Tait *et al*. 1995), and therefore we cloned microsatellites which could be expected to be more variable.

Approximately 20 000 recombinant colonies were screened with CA and GA polymers in two independent *E. rowelli* size-selected genomic libraries (following Taylor *et al*. 1994). Sequences were obtained from 22 dinucleotide microsatellite-containing plasmids. Primers were designed for 16 dinucleotide regions (the remainder were unsuitable because of the characteristics of the flanking regions) and three mononucleotide ones. Despite extensive optimization of all of these primer pairs, only five useable loci were obtained. To obtain PCR template, DNA from ≈ 20 mg of peripatus was extracted by salting-out (Sunnucks & Hales 1996), modified to remove skin (which contains PCR inhibitors) by dipping in extraction buffer and agitation with clean forceps on paper towel. DNA was dissolved in 60–80 μL of TE to ≈ 50 ng/μL. With specified exceptions, loci were screened by standard acrylamide gel isotopic microsatellite PCR in 10 μL volumes, containing 0.5 U *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2 mM $MgCl_2$, 200 μM of dGTP, dCTP, and dTTP, 20 μM of dATP, 10 pmol of each primer, ≈ 50 ng of DNA and 0.05 μL of [α^{33}P]-dATP at 1000 Ci/mmol. The PCR profile was 94 °C for 3 min, five cycles of 'touchdown' (anneal first cycle at 62 °C for loci P6, P23, and 'P17low', 54 °C for 'P17free' and P18, and decrease 2 °C per cycle for the next four) denature 94 °C for 15 s, extend 72 °C for 45 s, then 29 cycles annealing at 55 °C, and a final 2 min extension at 72 °C. Cycling was performed in a MJ Research PTC100 thermocycler. Other assay details follow Sunnucks *et al*. (1996).

Under standard conditions, only loci P6 and P23 gave scoreable polymorphic patterns of the expected size (Table 1). (These loci work well in duplex if the primer concentrations of P6 are doubled.) Primers for P17 gave poor amplification of the $(CA)_{14}$ 170 bp target, but yielded two other polymorphic loci: 'P17low' and 'P17free' (there is no linkage disequilibrium between these, $P > 0.05$ in all populations). Shorter P17 primers (Table 1) give better results for P17low. P18 could not be made to amplify until we added 0.01% final Tween 20 and Nonidet P40 detergents, with which the locus is scoreable. Consistency with Mendelian inheritance of the five loci has been confirmed in mother–offspring and population samples.

Some loci do not amplify at all in some populations (Table 1), probably because *E. rowelli* is a species complex: adjacent populations can differ by 20% or more in mtDNA COI sequence (P. Sunnucks, unpublished). P6 and P23 have nonamplifying alleles, including in the population from which they were cloned.

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Table 1 Details of polymorphic *Euperipatoides rowelli* microsatellites.

Locus	Motif	Size range (bp)	Primer sequence (5'-3')	Tot.	No. of alleles (No. of individuals) {H_O/H_E}				
					F	N	S	B	M
P06	$(CA)_{12}$	158–172	ATGTGATTTCCATTACGCTA ATGGGTTCACAACAATGACT	9	n (36) {0/0}	2 (276) {0.33/0.51}	8 (361) {0.58/0.70}	3 (19) {0.21/0.47}	n (8) {0/0}
'P17 low'	?	97–114	TTCCATCCCAGGTTCCA GTATCAAAAGACATGGACAG	4	2 (46) {0.09/0.12}	2 (266) {0.18/0.15}	2 (205) {0.27/0.50}	1 (24) {0/0}	3 (8) {0.88/0.63}
'P17 free'	?	116–255	TGTTTCCATCCCAGGTTCCA AGTGTATCAAAAGACATGGACAG	7	1 (6) {0/0}	3 (39) {0.46/0.55}	5 (37) {0.41/0.62}	1 (6) {0/0}	3 (6) {0.83/0.65}
P18	$(TG)_{14}T_4G_{13}$	148–165	CATGCAAGAAAGGTGTGGAC CTGACCCTGAAAAGCATCCA	18	–	11 (50) {0.54/0.83}	16 (34) {0.74/0.91}	–	–
P23	$T_{12}(TC)_8$	128–145	TTACTTCCCTTGCTTTGAATCT ATGCCGACCAATGTTGTTAT	8	1 (32) {0/0}	3 (268) {0.14/0.49}	4 (360) {0.23/0.69}	3 (19) {0.05/0.57}	n (8) {0/0}

n, nonamplifying; –, not assayed.

Locations: F, Forbes Creek; N, north Tallaganda State Forest (TSF); S, southern TSF; B, Badja SF; M, Monga SF: probably cryptic species. H_O, observed heterozygosity (direct count); H_E, Nei's unbiased expected heterozygosity; Tot., total number of alleles per locus. GenBank Accession nos AF109350–AF109353.

Characterization of polymorphic brown lemur (*Eulemur fulvus*) microsatellite loci and their amplification in the family Lemuridae

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Lemurs are prosimian primates endemic to Madagascar. All extant lemur taxa are considered threatened as a direct result of human-induced habitat degradation and fragmentation, with potential genetic repercussions (Mills & Allendorf 1996). Fine-scale genetic variation has in the past been quantified in lemurs using techniques such as allozymes (Tomiuk *et al*. 1997), with amounts of variation too low to obtain unique individual genotypes, and RAPDs (i.e. Rabarivola 1998), with questionable reproducibility of results (Perez *et al*. 1998). Identification of genetically unique populations for management purposes (Scheffrahn *et al*. 1998), development of genetically sound breeding programmes, and better understanding of lemur social structure are among the conservation-related reasons for interest in quantifying lemur genetic diversity. Here, we describe the development of 12 microsatellite loci in brown lemurs (*Eulemur fulvus*) and test them for amplification and variation in 10 species or subspecies of the family Lemuridae.

Several small insert (200–500 bp) red-fronted lemur (*Eulemur fulvus rufus*) genomic libraries were created to isolate microsatellites. Approximately 30 μg of genomic DNA was isolated from blood using a standard phenol–chloroform extraction protocol, and digested to completion with *Sau*3AI (Gibco BRL). Small insert fragments were excised from a 1% agarose gel, purified using QIAquick™ spin columns (QIAGEN), and ligated into *Bam*HI-digested dephosphorylated M13mp18RF vector (Gibco BRL). Phages were transfected into *Escherichia coli* DH5α F′ by either of two methods: the 18 °C procedure (Inoue *et al*. 1990), or electroporation (*E. coli* Pulser™, Bio-Rad). Over 20 000 recombinant clones were screened for microsatellites using four biotinylated oligonucleotide probes: $(GT)_{11}$ $(AAGG)_4$ $(GC)_6$ $(GA)_7$. Approximately 200 positive clones were identified using the BluGENE™ nonradioactive detection kit (Gibco BRL). Inserts from 36 of the strongest positive clone inserts were amplified as in Zheng *et al*. (1995). PCR products were isolated via QIAquick™ spin columns (Qiagen), and sequenced on an ABI 377 DNA Sequencer using a dRhodamine Terminator Cycle Sequencing kit (Perkin-

Table 1 Characteristics of *Eulemur fulvus* microsatellites ($n = 33$ individuals). Underlined primer sequences are pigtails (Brownstein *et al*. 1995), added to promote adenylated PCR product

Locus	Size range (bp)	Repeat type (and length)	No. of alleles	H_O	H_E	Primer sequences (5' to 3')	Accession no.
Efr02	118–122	$(TG)_{14}$	3	0.212	0.449	AATGTGTAAAAATTCCTTCT GTGTCCTGGTGACAGAGTAAG	AF104088
Efr05	118–151	$(TG)_{15}$	9	0.818	0.735	TGTCTCTGTCTTTCCTACT GTGTAAAATGTCTTCTGTATTCA	AF104089
Efr08	112–160	$(AT)_{16}GTAT(GT)_9$	13	0.756	0.839	AAAGACAAGGGGTATG GTTTGCATTTGAGGAATCA	AF104090
Efr09	91–105	$(GT)_{15}$	6	0.394	0.788	AATTTCAGGTATGCGTGT GTTTGGGGCAAGTTGAATAG	AF104091
Efr24	92–101	$(GT)_{17}$	8	0.697	0.814	CCCAATCTCCCTGAGTTTGAG GTTTAGCCTGGGCAACATAGTGAG	AF104092
Efr26	252–253	$(GT)_4(GC)_2(GT)_3$	2	0.091	0.088	AGGGATGTTTGGGAACTCTG GTTTGGCAGGCTAGGCTGTCAG	AF104093
Efr30	309–325	$(TG)_{19}$	5	0.606	0.637	GACTACAACTTTTCCATCTG GTGTCATTGCTGATTATTTCTGAT	AF104094
Efr37	219–235	$(TG)_3(AT)_2(GT)_9$	8	0.788	0.760	CCACATTTACTACAGCACTATC GTTTATTTCTCTATCCAGGGTTG	AF104095
Efr53	170	$(GAT)_4GACCAT(GAT)_3$	1	0	0	GCATGTAAGGCACCTAGCA GTTTATCTCCTGACTAATTGGCACA	AF104097
Efr56	241–251	$(GTGC)_2(GT)_{16}$	5	0.727	0.704	CCACCTTAGCATATTTAGCAT GTTTGATGTTCGGAACTGAGAG	AF104098
Efr81	182–196	$(CT)_3(GT)_{11}$	5	0.636	0.761	TCGTTATTGAGCATATTCA GTGTCGGTTCTTTACCAATG	AF104099
Efr88	96–106	$(TG)_{14}$	5	0.8181	0.703	ATCTAAACCACTCAGCTATAAAG GTTTGATCCCAGGAGAGCTTT	AF104100

Table 2 Cross-species amplification of 12 brown lemur microsatellite loci. PCR product size ranges at each locus are given, followed by the number of different alleles in brackets. Absence of PCR product is expressed as –; a multibanding pattern is denoted by 'M'

	Red-fronted lemur *Eulemur fulvus rufus* $N = 8$	Collared lemur *Eulemur fulvus collaris* $N = 6$	Black lemur *Eulemur macaco macaco* $N = 4$	Blue-eyed lemur *Eulemur macaco flavifrons* $N = 4$	Crowned lemur *Eulemur coronatus* $N = 4$	Red-bellied lemur *Eulemur rubiventer* $N = 4$	Black & White Ruffed lemur *Varecia variegata variegata* $N = 4$	Golden Bamboo lemur *Hapalemur aureus* $N = 3$	Greater Bamboo lemur *Hapalemur simus* $N = 2$	Ring-tailed lemur *Lemur catta* $N = 1$
Efr02	116–126 (5)	118–122 (2)	116–120 (3)	118–122 (3)	118 (1)	106 (1)	116–132 (6)	M	125–141 (3)	126–128 (2)
Efr05	118–151 (7)	122–126 (3)	114–128 (3)	114–126 (4)	118–122 (4)	118–122 (3)	118 (1)	118–135 (3)	114 (1)	116 (1)
Efr08	111–137 (10)	121–137 (6)	97 (1)	97 (1)	124–130 (4)	M	–	101 (1)	101 (1)	99 (1)
Efr09	91–101 (3)	95–101 (4)	93–99 (3)	93–101 (4)	99–103 (3)	108–112 (3)	105–107 (2)	97–116 (4)	M	91–101 (2)
Efr24	94–121 (6)	98–104 (3)	90–96 (4)	90–94 (3)	79–90 (3)	84–94 (3)	M	M	M	–
Efr26	251–253 (2)	252–253 (2)	252 (1)	252 (1)	252 (1)	251–252 (2)	252–263 (2)	252–253 (2)	252–253 (2)	252 (1)
Efr30	309–330 (8)	309–313 (3)	305–315 (4)	305–313 (4)	307–312 (3)	305–313 (4)	308–320 (2)	305 (1)	294 (1)	292 (1)
Efr37	221–237 (7)	225–234 (4)	223 (1)	223 (1)	206–214 (2)	–	212 (1)	234 (1)	223–228 (2)	–
Efr53	170 (1)	170 (1)	166–169 (2)	166–169 (2)	169–173 (2)	168–169 (2)	165 (1)	169–172 (2)	175 (1)	175 (1)
Efr56	235–251 (6)	249–251 (2)	243–255 (3)	245–255 (3)	247–251 (3)	245 (1)	235 (1)	235–237 (2)	233 (1)	233 (1)
Efr81	182–194 (6)	182 (1)	189 (1)	189 (1)	190–196 (3)	193–201 (4)	–	183 (1)	185 (1)	183 (1)
Efr88	98–106 (5)	102–106 (3)	84–107 (6)	94–110 (5)	88 (1)	88–107 (2)	90 (1)	94–102 (2)	88–107 (3)	90 (1)

Elmer). Primer sets were designed using OLIGO software (National Biosciences, Version 5.1) for 21 sequences containing microsatellites; one primer per set was pigtailed to promote preferential amplification of adenylated PCR product (Brownstein *et al*. 1996), to aid in correct identification of genotypes. The primers were synthesized on a model 391 DNA synthesizer (Applied Biosystems), with one primer of each set fluorescently labelled with 6-HEX, 6-TET, or 6-FAM dyes (Perkin-Elmer).

To assess amplification and preliminary variability of these prospective microsatellite loci, genomic DNA was isolated from five to 100 plucked hairs from each of five brown lemurs (*E. fulvus*), using the QIAamp™ spin column tissue protocol (Qiagen). Twenty-five μL PCR reactions were conducted, each containing 17 μL of the resulting 200 μL DNA extraction, 0.16 μM (4 pmols) of each primer, 120 μM dNTPs, 2 mM $MgCl_2$, 0.5 U of *Taq* DNA polymerase (isolated as in Engelke *et al*. 1990) and 10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 50 mM KCl and 0.16 mg/mL BSA. Reactions were placed into a 9600 thermal cycler (Perkin-Elmer) heated to 94 °C, with the following cycling conditions: 1 min at 94 °C, three cycles of 30 s at 94 °C, 20 s at 54 °C, and 5 s at 72 °C, followed by 33 cycles of 15 s at 94 °C, 20 s at 54 °C, and 1 s at 72 °C, followed by a final extension of 72 °C for 30 s. PCR products were run with an internal lane standard on a model 377 DNA sequencer, and product sizes quantified using Genescan and Genotyper software (Applied Biosystems).

Of the loci tested, 12 yielded easily quantifiable genotypes. To examine the genetic variation at these microsatellite loci, genotypes were obtained for 33 individuals from a closed population of brown lemurs at Berenty Reserve, Madagascar. These results are summarised in Table 1. One locus (Efr26), while exhibiting little size variation, was found to contain considerable intraspecific sequence variation in the regions flanking the microsatellite (Jekielek *et al*. 1999). All the loci were tested for amplification and variation in 10 taxa from the family Lemuridae, with PCR conditions identical to those above. The results of these cross-species amplifications are summarized in Table 2.

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Characterization of hypervariable microsatellites in the cooperatively breeding white-browed sparrow weaver *Plocepasser mahali*

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Single-locus microsatellite markers have advantages for studying genetic relationships within family groups. Unlike multilocus DNA fingerprints (Danforth & Freeman-Gallant 1996; McRae 1996), for any given locus, bands can accurately be assigned as either paternal and maternal when at least one parent is known. Thus, even an offspring that is the product of an incestuous mating, such as between mother and son, can be identified as such when for a given autosomal locus the offspring matches two alleles from its mother (the dominant female) and none from the dominant male. Paternity can confidently be assigned to the dominant male when the offspring's paternal allele is matched only with the dominant male's genotype and not that of the helper son. To ensure paternity exclusion of the father or son by at least one locus requires about seven loci, each with six alleles to achieve theoretically an exclusion in 99% of cases.

We cloned microsatellite loci to look at genetic relationships within cooperatively breeding white-browed sparrow weaver social groups. DNA was extracted from the blood samples of four presumed unrelated individual white-browed sparrow weavers using a phenol–chloroform extraction procedure (Sambrook *et al*. 1989). A 300–800 bp partial genomic library was constructed by ligating *Alu*I-digested sparrow weaver DNA into a pUC18 *Sma*I/BAP (blunt phosphorylated) plasmid vector which was used to transform competent cells (Gibco BRL). The library was probed with $(AC/GT)_n$ $(AAC)_n$ and $(GCT)_n$ synthetic dinucleotide polymers using high washing stringency (Allen *et al*. 1995). DNA from the 16 positively hybridizing colonies was sequenced by ABI PRISM^{TM} dye terminator cycle sequencing (Perkin-Elmer). PCR primers were designed using Primer3 software (Rozen & Skaletsky 1996-1997), from flanking sequences for the microsatellite sequences with pure arrays of greater than 10 repeats of the motif.

To test the primers, DNA was extracted (as above) from 375 individual white-browed sparrow weavers from a breeding population at Okosongomingo Farm near the Waterberg Plateau Park in Namibia (20.8S, 17.0E). Adults were sexed in the field by bill colour, and extensive data have been collected on their social group membership and breeding behaviour (S. B. McRae, unpublished). We also investigated whether the primers would amplify across three other species: the confamilial sociable weaver *Philetarius socius* ($N = 6$) and Southern masked weaver *Ploceus tæneopterus* ($N = 7$), and the non-passerine common moorhen *Gallinula chloropus* ($N = 6$).

PCR amplifications were performed in a total volume of 10 μL using a Touchdown thermal cycler (Hybaid). Each reaction contained the following: ≈ 10 ng of template genomic DNA, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.04 mM dCTP, 0.01 μL of (0.1μCi) [γ^{32}P]-dCTP, 5 pmol of each primer and 1 unit *Taq* polymerase (Promega). The general PCR profile consisted of one cycle of 94 °C for 2 min, followed by seven cycles of 92 °C for 40 s, 54 °C for 1 min, and 72 °C for 40 s, and then 25 cycles of 89 °C for 30 s, 56 °C for 1 min, and 72 °C for 20 s. PCR products were denatured and resolved by electrophoresis on polyacrylamide gels (Sequagel-6). The gels were dried and exposed to film at – 70 °C for 1–3 days.

The characteristics of eight independent primer sets (GenBank Accession nos AF130431–AF130438) are summarized in Table 1. Each microsatellite locus was highly variable (mean allele number = 14), and exhibited a Mendelian pattern of inheritance, except for WBSW10, which shows an inheritance pattern indicative of sex linkage to the Z chromosome.

Cross-species amplification was successful among the two other ploceid weaver species, with five and six loci being variable for sociable and masked weavers, respectively (Table 2). Variable PCR products have also been amplified for another ploceid weaver, the red-billed quelea *Quelea quelea* using six of the primer pairs (Dallimer 1999). The primers did

Table 1 Characterization of eight pairs of primers for amplifying white-browed sparrow weaver microsatellite loci, with expected (H_E) and observed (H_O) levels of heterozygosity based on a population of 375 individuals from Okosongomingo farm, Namibia

Primer name	Sequence (5'-3')	Repeat	Product length	(range)	Allele number	H_E	H_O
WBSW1f	TAT TTT ATG CTC TGC CCA GTT G	$(TG)_{15}$	174	(172–190)	11	0.91	0.82
WBSW1r	TAG GCA TTG CCA AGG TTA ATC						
WBSW2f	AAG GTC ACT GTG CAT CTT GC	$(TG)_3TA(TG)_{11}$	233	(227–241)	6	0.83	0.57
WBSW2r	GCA GAC TTG ATA GAT CTT CAC TGT AA						
WBSW4f	TAC CAC TTG GTC CTC TGG CT	$(AC)_{21}$	183	(165–207)	16	0.94	0.80
WBSW4r	GGT TAT GCT ACA AAC TGG TCA C						
WBSW7f	TCT GGA GTT CTG GGA CCT GT	$(CA)_{16}$	153	(141–165)	13	0.92	0.68
WBSW7r	CTC ACT CAA CAG CAG GAC CA						
WBSW8f	TGT GGA AGC AGG GTT AAA GG	$(TG)_{17}(CGT)_{19}$	168	(122–198)	20	0.95	0.88
WBSW8r	CAG CCT GGT ACT ATT TGC CTG						
WBSW9f	TTG AGT GGC TAA TTT TGT GAA GG	$(GT)_{21}$	127	(104–138)	15	0.93	0.82
WBSW9r	TCT TAT GTC CCC CAT TTG GA						
WBSW10f	CCG AAT GTG TTT CGT GAA TG	$(TG)_3(TA)_5(TG)_{17}$	193	(179–215)	14	0.87	0.75*
WBSW10r	GAT GCA ATC CAT AAG TCC AC						
WBSW11f	TGA AAA TCC CAG GTC CCT ATT	$(AC)_{15}...(GT)_6$	193	(183–213)	14	0.93	0.73
WBSW11r	CCA CAT CTT TTT CCA CAG CA						

* Z = linked locus, H_E and H_O based on field-identified males only (N = 146).

Table 2 Cross-species amplification with eight white-browed sparrow weaver microsatellite loci. Figures indicate the number of alleles detected

Species	N	WBSW1	WBSW2	WBSW4	WBSW7	WBSW8	WBSW9	WBSW10	WBSW11
Sociable weaver *Philetarius socius*	6	M	–	2	2	7	5	3	M
Southern masked weaver *Ploceus tæniopterus*	7	5	2	5	3	–	8	M	9
Common moorhen *Gallinula chloropus*	6	–	–	–	–	–	–	M*	–

–, no fragments amplified in expected size range; M, monomorphic; *, amplified for females only.

not produce variable PCR products of the expected size for the moorhen. However, the sex-linked WBSW10 locus produced a monomorphic product for three female individuals and no product for the three males, suggesting that they amplified a product from the avian W sex chromosome in this species. Such sex-linked microsatellites may be useful for determining sex of individuals of monomorphic species and of immature animals, or as a check for more commonly used molecular sexing methods (Griffiths *et al*. 1998).

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Isolation and characterization of microsatellite loci in the bushcricket *Ephippiger ephippiger* (Orthoptera: Tettigoniidae)

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As with many insects, female Tettigoniids are polyandrous and store sperm. The threat of sperm competition is therefore high. When gametes from different males are competing to fertilize the eggs of a female, mating success may not accurately reflect the fertilization success of a male (Parker 1970). Previous studies of insects have used the sterile male technique (Parker 1970) to assign paternity; however, a number of problems with this method have been highlighted (Zimmering & Fowler 1966; Parker 1970; He *et al*. 1995). Therefore, in order to assess paternity polymorphic nuclear markers are required. Microsatellites are highly polymorphic and are ideal for this purpose. We have developed a set of primers for polymorphic microsatellite loci in the Tettigoniid *Ephippiger ephippiger* that will allow us to elucidate the sperm utilization pattern in this species.

A microsatellite library enriched for CA repeats was constructed from genomic DNA pooled from 20 individuals from Montpellier (Hérault France), using a Biotin/Avidin enrichment protocol (Hammond *et al*. 1998). This enrichment method involves hybridization extraction of target repeats from size-selected DNA using biotinylated CA_{48} oligonucleotide. The probe/genomic DNA hybrids are then extracted from solution with Vectrex Avidin (Vector Laboratories) which is complementary to biotin. Genomic DNA was extracted from hind femur muscle tissue following the animal tissue protocol of the Puregene DNA isolation kit (Gentra Systems, Inc.).

Nineteen positive clones were identified out of ≈ 2000 screened. These were sequenced on an ABI 377 automated sequencer (Perkin-Elmer). Eighteen clones contained uninterrupted repeat sequences of 10 or more dinucleotide pairs. Ten had flanking regions unsuitable for primer design. Primers were designed for the remaining eight using the program Primer3 (Rozen & Skaletsky 1996–1997)

The microsatellite loci of 20 *E. ephippiger* individuals were amplified on a G.R.I. PTC 100 thermal cycler to evaluate polymorphism and heterozygosity, and DNA was extracted as before. The amplification reaction contained ≈ 25 ng of template DNA, 1× buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25 °C), 1.0% Triton®X-100, an optimized concentration of $MgCl_2$ (see Table 1), 0.1 mM of each dNTP, 25 pmol of each primer and 0.5 units of *Taq* DNA polymerase in a volume of 25 μL. The amplification program was 94 °C for 3 min, then 35 cycles of a denaturation temperature of 94 °C for 20 s, the optimized annealing temperature (see Table 1) for 30 s and primer extension at 72 °C for 30 s. A final extension was carried out at 72 °C for 10 min. The PCR fragments were resolved on a 6% denaturing polyacrylamide gel by vertical electrophoresis at 30–50 mA for ≈ 8 h and visualized by silver staining (Promega) according to the manufacturer's instructions.

Six loci were polymorphic and had a mean allele number of 6.8 (range 5–9). The average observed heterozygosity was 0.56 (range 0.48–0.77) (Table 1).

We also examined the conservation of the six polymorphic microsatellite loci in 11 closely related Tettigonids (Tettigoniidae: Ephippigerinae) (Table 2). Touchdown PCR (Don *et al*. 1991) was used to check for cross-species amplifi-

Table 1 Characterization of six microsatellite loci in *Ephippiger ephippiger*

Locus	Approx. length of PCR product (bp)	Primer sequence (5'-3')	Repeat motif	T_a (°C)	$MgCl_2$ (mM)	No. of alleles	H_O	H_E	GenBank accession no.
BC52	129	F:CACTGATGAGGTTGGAAGGG R:TGCTCCAACACATGTCATCC	$(CA)_{15}$	59	1.0	5	0.77	0.00	AFO91021
BC67	124	F:CCCTACCAATGATGAGGGTG R:GTTCATTCCAATGACCAGGG	$(CA)_{15}$	59	1.5	4	0.67	0.83	AFO91026
BC69	165	F:AAACCATCTCTGTCCGCATC R:TTTTGGACTCGAACTCACCC	$(CT)_7(CA)_{13}$	59	1.5	6	0.77	0.86	AFO91027
BC90	244	F:GGAAGCTTGGGATAGCGAGT R:TTGGAATTAGCCAATGAGGG	$(CA)_{12}$	58	1.0	5	0.65	0.78	AFO91028
BC299	278	F:AACAGTTCCACGCTCTTGTG R:ACGCCCACTCAGTTTTATGG	$(CA)_{14}$	56	1.5	4	0.65	0.84	AFO91022
BC633	288	F:CGCAACAATACTGCCAAAAA R:TGTACCGGTTAAAATTTGCG	$(CT)_{15}(CA)_{10}$	59	1.5	4	0.48	0.66	AFO91024

H_O, observed heterozygosities; H_E, expected heterozygosities.

Table 2 Cross-species amplification of six Bushcricket loci

Species	Locus					
	BC299	BC633	BC52	BC69	BC90	BC 67
Genus: *Ephippiger*						
Ephippiger ephippiger	+	+	+	+	+	+
Ephippiger areolaria	+	–	+	+	+	+
Ephippiger ephippiger	+	+	+	+	+	+
Ephippiger perforatus	–	–	+	+	+	+
Ephippiger ruffoi	+	–	–	+	+	+
Ephippiger terrestris	?	+	?	+	+	+
Genus: non-*Ephippiger*						
Ephippigerida zappapteri	–	–	–	+	+	+
Steropleurus brunnerii	–	–	–	+	+	+
Steropleurus catalaunicus	–	+	+	+	+	+
Steropleurus perezi	–	–	–	–	+	+
Steropleurus stali	+	–	–	+	+	+
Uromenus rugosicolis	?	+	+	+	+	+

+, amplification; – nonamplification.
? = untested.

cation. The loci were amplified using the same conditions as above except that 30 cycles were used instead of 35, 15 cycles were at the annealing temperatures stated (Table 1) followed by 15 cycles at 2 °C below the initial annealing temperature. Table 2 shows that the conservation of these loci between the species examined is extensive. However, the extent of polymorphism is not known.

As the levels of microsatellite polymorphism suggest that microsatellites consist of neutral DNA (Schlotterer *et al*. 1991; Rico *et al*. 1996), it is assumed that microsatellite flanking regions are neutral (Schlotterer *et al*. 1991). If this is the case then microsatellite primers designed for one species would be unlikely to amplify in distantly related taxa because of the divergence of flanking regions over time (Schlotterer *et al*. 1991; Primmer *et al*. 1996; and Chapuisat 1996, but see Rico *et al*. 1996). It is therefore interesting to note that, among loci which vary in amplification, the probability of successful amplification is higher within the genus *Ephippiger* $\chi^2 = 5.351$, 1d.f., $P = 0.021$ (see Table 2).

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