deviations from HWE revealed no significant departures ($P > 0.05$) at eight loci; conversely, a significant excess of homozygotes was observed at one locus (ST2-41). The use of MICRO-CHECKER software (Van Oosterhout *et al.* 2004) indicated that the significant deviations from HWE at this locus may be the result of null alleles, although no scoring errors were found associated with stuttering bands or large allele dropout. ARLEQUIN was also used to test for gametic disequilibrium by application of a Bonferroni correction for multiple comparisons (Rice 1989). No evidence of gametic disequilibrium was detected for any pairs of loci.

The relatively small number of useful loci for *S. terebrans*, resulting from an initially promising set of positive clones, suggests that the isolation of suitable microsatellite loci for this group of Crustaceans is not easy, as also demonstrated by the small number of simple sequence repeats isolated in isopods thus far (Grandjean *et al.* 2005; Verne *et al.* 2006; Haye & Marchant 2007, 2008; Leese *et al.* 2008). Nevertheless, we are confident that the loci characterized in this study are suitable to infer parentage, relatedness and phylogeographic patterns in this taxon, which probably includes some cryptic species.

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Characterization of Mauritius parakeet (*Psittacula eques*) microsatellite loci and their cross-utility in other parrots (Psittacidae, Aves)

CLAIRE RAISIN,*†‡§ DEBORAH A. DAWSON,* ANDREW G. GREENWOOD,‡ CARL G. JONES¶§ and JIM J. GROOMBRIDGE†

**NERC Molecular Genetics Facility, Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK, †Durrell Institute of Conservation & Ecology, University of Kent, Canterbury CT2 7NZ, UK, ‡International Zoo Veterinary Group, Keighley Business Centre, South Street, Keighley, W. Yorkshire BD21 1AG, UK, §Mauritian Wildlife Foundation, Grannum Road, Vacoas, Mauritius, ¶Durrell Wildlife Conservation Trust, Les Augres Manor, Trinity, Jersey JE3 5BP, UK*

Abstract

We characterized 21 polymorphic microsatellite loci in the endangered Mauritius parakeet (*Psittacula eques*). Loci were isolated from a Mauritius parakeet genomic library that had been enriched separately for eight different repeat motifs. Loci were characterized in up to

Correspondence: Jim Groombridge, Fax: +44 (0)1227 827289; E-mail: j.groombridge@kent.ac.uk

43 putatively unrelated Mauritius parakeets from a single population inhabiting the Black River Gorges National Park, Mauritius. Each locus displayed between three and nine alleles, with the observed heterozygosity ranging between 0.39 and 0.96. All loci were tested in 10 other parrot species. Despite testing few individuals, between seven and 21 loci were polymorphic in each of seven species tested.

Keywords: AVES, cross-species utility, Mauritius parakeet, microsatellite, parrot, Psittacidae,

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The Mauritius parakeet (*Psittacula eques*) is the only endemic psittacine remaining in the Mascarene Islands and is classified as endangered (IUCN 2008). The population declined to less than 20 individuals (including only three known breeding females) in the early 1980s but currently numbers approximately 360, all of which are found in the Black River Gorges National Park, South West Mauritius (Malham *et al*. 2006). We have characterized a set of polymorphic microsatellite loci for the Mauritius parakeet.

Blood was collected from 43 Mauritius parakeet individuals and stored in 70–90% ethanol at room temperature. For library construction, genomic DNA was extracted from blood using a DNeasy Blood and Tissue Kit (QIAGEN Ltd). Mauritius parakeet microsatellites were isolated from an enriched genomic library prepared essentially as Jones *et al*. (2002) by Genetic Identification Services. The pooled genomic DNA of three Mauritius parakeet individuals (one male and two females) was digested with blunt ended-restriction enzymes (*Alu*I, *Dra*I, *Fsp*I, *Hpa*I, *Msc*I, *Nae*I). Fragments (350–700 bp) were ligated to the double-stranded linker sequence 5′-AAGCTTTCGTTTTACAACGTCGTGG-3′ (which contains a *Hin*dIII site at the 5′ end) and hybridized separately against one of eight target repeat sequences: $(CA)_{15}$, $(GA)_{15}$, $(AAT)_{15}$, $(ATG)_{12}$, $(CAG)_{10}$, $(CATC)_8$, $(TAGA)_8$ and $(TGAC)_8$. The enriched fragments were digested at the *Hin*dIII site of the linker and then ligated into pUC19-*Hin*dIII. Colonies were not screened for repeat motifs but directly sequenced. A total of 104 clones were sequenced in the forward orientation using an M13-based forward primer (5′-AGGAAACAGC TATGACCATG-3′). Primer sets were designed for 48 loci using DesignerPCR version 1.03 (Research Genetics, Inc.) and used to amplify eight unrelated Mauritius parakeet individuals to assess polymorphism. When initially amplified in eight unrelated individuals and screened on agarose gel, 35 loci appeared polymorphic, eight monomorphic and five failed to amplify or were unscorable (unpublished data). Twenty-one polymorphic loci (enriched for $(CA)_{15}$, $(GA)_{15}$, $(CATC)_8$ and $(TAGA)_8$ motifs) were selected for further optimization.

Genomic DNA was extracted from blood using ammonium acetate following the method used by Nicholls *et al*. (2000) and used for PCR. A range of annealing temperatures (50 °C–68 °C) were tested and the temperature producing the cleanest and strongest PCR product when observed on an 0.8% agarose gel stained with ethidium bromide was selected for PCR. Each locus was amplified separately. Each 2 μL PCR contained approximately 10 ng of genomic DNA with 0.2 μM of each primer and 1 μL QIAGEN multiplex PCR mix (QIAGEN Inc.). PCR amplification was performed using a DNA Engine Tetrad 2 thermal cycler (MJ Research, Bio-Rad). The PCR profile used was 95 °C for 15 min; then 40 cycles of 94 °C for 30 s, T_a °C (Table 1) for 90 s, 72 °C for 90 s, followed by one cycle of 10 min at 72 °C. Genotypes were scored on an ABI 3730 DNA Analyser with GeneScan ROX-500 size standard using GeneMapper software (Applied Biosystems). Each locus was loaded on the ABI 3730 separately. Multiplexing was not performed pre- or post-PCR amplification.

All 21 loci were polymorphic and the number of alleles observed when tested in 24–43 individuals from the recovered population ranged from three to nine (Table 1). The DNA sequences of the 21 polymorphic loci were confirmed to be free of vector and unique using BLASTN version 2.2.4 (Altschul *et al*. 1997). Loci were checked for sex linkage by genotyping individuals of known sex (15 females and 14 males; sex based on morphology). Two loci (Peq16 and Peq21) were found to be *Z*-linked, therefore only males were characterized at these loci as females were always homozygous.

Observed and expected heterozygosities, and null allele frequencies were calculated using Cervus version 2.0 (Marshall *et al*. 1998). Tests for departures from Hardy–Weinberg and linkage equilibrium were conducted using a Markov chain method implemented in GenePop version 3.4 (Raymond & Rousset 1995). One locus (Peq08) deviated from Hardy–Weinberg equilibrium and also displayed a high predicted null allele frequency (Table 1).

Based on sequence homology, 15 of the 21 loci could be assigned a chromosome location in the zebra finch (*Taeniopygia guttata*) genome assembly (compiled by the Washington University School of Medicine Genome Sequencing Centre, http://genome.wustl.edu/genome.cgi?GENOME=Taeniopygia%20guttata&SECTION=assemblies) and 11 loci were assigned a chicken (*Gallus gallus*) chromosome location (using a WU-BLAST with distant homologies settings implemented on the ENSEMBL webpage www.ensembl.org/Gallus_gallus/index.html; see Dawson *et al*. 2006; Table 1, Fig. 1). Loci Peq16 and Peq21 were assigned to the zebra finch *Z* chromosome (Table 1, Fig. 1).

Fifteen known family groups (both parents and at least one offspring) were genotyped and used to test for linkage

Table 1 Characterization of Mauritius parakeet Psittacula eques microsatellite loci (Psittacidae, Aves)

Locus	EMBL accession number	Chr. location (see footnote and text)	Chr. location (bp)	BLAST P(N)	Repeat motif	Fluoro-label (F)	Primer sequences (5′–3′)	Optimised T_a (°C)	*n*	*A*	Exp. allele size (bp)‡	Obs. allele sizes (bp)	H_O	H_E	P_{HWE}	Est. null allele freq.
Peq01	FM865414	Tgu2	61550289	3E-28	$(CA)_{13}$	HEX	F: AGGCTTAACAGATGTAGGACAC R: TGTGCTTTTCCATCACAAG	63	42	6	202	189–209	0.67	0.58	0.87	–0.16
Peq02	FM865415	No strong hits			$(GATG)_{10}$	6-FAM	F: CTTTGAGCACCCACACAAC R: GTGTCGCAACCTCCTTATG	66	43	3	145	144–153	0.47	0.53	0.77	0.04
Peq03	FM865416	Tgu1	79810332	4E-18	$(TAGA)_{12}(CAGA)_2$ $(TAGA)_1(CAGA)_1$	6-FAM	F: GGATGTTCAGTTCTCTTGTTTC R: ATGGTTCTCCTGTGTCTATCAG	66	42	7	287	274–300	0.76	0.76	0.20	0.09
Peq04	FM865417	Tgu4	40782899	2E-38	$(TAGG)_3(TAGA)_{10}$	HEX	F: GGATTTCTCATGCCTAAAATG R: TGACAAGTCCACCAAAAATATC	56	43	5	287	285–306	0.65	0.63	0.37	0.04
Peq05	FM865418	Tgu1	39968225	1E-41	$(CA)_{11}$	6-FAM	F: GGAATTGTAGGTTTTAATGCAC R: AGCTCATAAACAGCCATATCTC	56	42	4	133	125–131	0.57	0.60	0.52	–0.01
Peq06	FM865419	Tgu4	43705460	7E-42	$(TG)_{13}$	HEX	F: CAAAAGAGCATCAATGGTATG R: GCTAACACCCCCCTATCC	59	41	7	216	213–244	0.83	0.79	0.89	–0.03
Peq07	FM865420	Tgu4	59562728	4E-13	$(CA)_{11}$	HEX	F: AAACAAACATACCCACAGAAAC R: GGAGGATAAGCAGAACTTGAG	66	42	3	128	114–124	0.40	0.34	0.64	–0.06
Peq08†	FM865421	Gga27	2668172	9E-15	$(GATG)_9$	6-FAM	F: AGTCGGGAACAGTTTCATTAG R: GACATGATGCTGACACAGATAG	58	42	9	255	235–265¶	0.48	0.68	0.00	0.12
Peq09	FM865422	Tgu2	154849943	1E-24	$(CATC)_{10}CACC$ $(CATC)_6$	HEX	F: GGTGGGTTGTGTGAAAGAA R: GGACTGTGGATGGAGAGGT	56	41	6	218	206–242	0.81	0.80	0.78	0.04
Peq10	FM865423	Tgu3	17147094	2E-48	$(TAGA)_{14}$	6-FAM	F: ACCCTTCCAAGAGAGTTTTAAG R: GGATCTTTCCTTTATCTGTGTG	59	42	6	133	109–129	0.76	0.80	0.35	0.01
Peq11	FM865424	Tgu3	30982572	4E-31	$(TCTA)_{14}TCTTA$ $(TCTA)_2$	6-FAM	F: CTCAAGGAGAATCTGAAGTCTG R: TGGGAGGTTAGAGTGAAAAAC	56	42	8	282	253–281€	0.81	0.81	0.64	0.06
Peq12	FM865425	Tgu2§	154288876	1E-33	$(TAGA)_{11}$	HEX	F: CACTCGGGATTCTGTGTTAG R: CCTTAGTCATCGTGCTTGTAG	59	41	6	279	271–291	0.90	0.82	0.95	0.00
Peq13	FM865426	No strong hits			$(TG)_8T(TG)_7$	HEX	F: GGAACATTCCAAAGCTCAAAGT R: TTGCTGCACTGTAGTTCTGTGA	55	24	4	125	114–122	0.67	0.65	0.97	–0.04
Peq14	FM865427	None			$(CA)_{12}$	NED	F: GGCTACCATCCTATTTGAAAGA R: ACGCTGGATCATAACAGAGTAA	58	23	4	219	209–225	0.39	0.34	1.00	–0.10
Peq15	FM865428	Tgu12	6379632	6E-41	$(CA)_{14}$	NED	F: CTTCATTTTCCTGTGCAGTCTA R: GGAGTTTTTTGTCGTTTTCTGT	59	23	9	206	205–221€	0.96	0.86	0.31	–0.07
Peq16	FM865429	TguZ	43664061	1E-16	$(GATG)_8$	6-FAM	F: GCTCCTAAGCCTTTGTAAAGAC R: CATGTTTGCTTGTCCAGTTTAT	63	14 (males)	3	136	131–149	0.50	0.43	1.00	—
Peq17	FM865430	No strong hits			$(CATC)_{13}$	HEX	F: ACCTCACAGACACAAAAGGATA R: GAAATGATAGGCAACAGACAGT	62	22	7	228	196–224	0.85	0.78	0.60	–0.07
Peq18	FM865431	Gga10	641761	7E-19	$(TAGA)_2T(TAGA)_1$ $TAA(TAGA)_3$	HEX	F: AGCAAGTAGATTTGGTCGTG R: TTCTCCCAGAGCACACAC	59	24	6	170	155–179	0.78	0.73	0.92	0.00
Peq19	FM865432	Tgu1A	22948103	3E-15	$(TCTA)_7TC(CATC)_{10}$ $TATC(CATC)_1$	6-FAM	F: GAGCCTCCTACCATAGTTGC R: CTTCAAATTCAGCAAACTTGTC	58	23	5	230	202–234	0.87	0.79	0.68	–0.07
Peq20	FM865433	Tgu20	2718287	1E-08	$(TCTA)_6$ & $(ATCA)_4$ $ATCTA(TCTA)_5$	6-FAM	F: GATGCAAATGTTACAGCAGTGT R: AACCCAATCTGTCTGAGAACAG	61	23	4	235	223–235	0.74	0.71	0.51	–0.04
Peq21	FM865434	TguZ	612947	9E-13	$(TAGA)_3T(TAGA)_2$ $TTGA(TAGA)_1T$ $(TAGA)_1TAGG$ $(TAGA)_{10}$	6-FAM	F: CCCAGAGTGGTAGAGAAAGAT R: GCTGTGTTCAAAAGGGTATG	59	14 (males)	5	180	159–179	0.56	0.55	0.72	—

Chromosome location: more loci could be assigned a location in the zebra finch genome than the chicken genome (15 vs. 11) and no chromosome assignments conflicted; therefore, when the same chromosome was assigned in both genomes only the location in zebra finch is presented. Five loci were assigned a location of the zebra finch genome only (Peq01, Peq03, Peq07, Peq16 and Peq20) and on two occasions, a location could only be assigned in the chicken genome (Peq08 and Peq18). Tgu, zebra finch chromosome; Gga, chicken chromosome. Peq16 and Peq21 are *Z*-linked loci and therefore were characterized in male individuals only. †Deviation from Hardy–Weinberg equilibrium as identified by GenePop version 3.4 (Raymond & Rousset 1995; $P < 0.001$). T_a, annealing temperature; *n*, number of unrelated Mauritius parakeet individuals genotyped from the population inhabiting Black River Gorges National Park, South West Mauritius. *A*, number of alleles; ‡expected allele size based on length of the Mauritius parakeet sequenced clone; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HWE}, Hardy–Weinberg equilibrium test *P* value as identified by GenePop version 3.4; §multiple other weaker hits also detected; ¶some observed allele sizes increased by only 1 bp and may be difficult to score.

Table 2 Allele sizes observed in other species when amplified using Mauritius parakeet (*Psittacula eques*) microsatellite loci*

Locus	Ringneck parakeet (Rose–ringed) *Psittacula krameri* *n* = 10 (number of alleles)	Longtailed parakeet *Psittacula longicauda* *n* = 1	Derbyan parakeet *Psittacula derbiana* *n* = 1	Moustached parakeet *Psittacula alexandri* *n* = 2	Blossom parakeet *Psittacula roseata* *n* = 1	Slaty–headed parakeet *Psittacula himalayana himalayana* *n* = 1	Alexandrine parakeet *Psittacula eupatria* *n* = 2	Eclectus parrot *Eclectus roratus roratus* *n* = 2	Black–lored parrot *Tanygnathus gramineus* *n* = 1	Vasa parrot *Coracopsis vasa* *n* = 2	Number of parrot species polymorphic	Blue crane *Anthropoides paradisea* *n* = 1	Chicken *Gallus gallus* *n* = 1	Crocodile *Crocodylus porosus* *n* = 1
Peq01	189–203 (7)	X	199, 203	184, 195, 210	195, 199	197	195, 199	193, 205, 209	195, 203	194	7	X	195	X
Peq02	144–190 (4)	149, 157	X	X	X	X	X	X	X	X	2	X	X	X
Peq03	282–306 (7)	X	X	X	X	X	X	X	X	X	0	X	X	X
Peq04	297, 317 (2)	194, 219	190	211, 219	213, 223	192, 202, 282	189, 213	104, 108§	208, 219	192	8	176	295	173
Peq05	121–125 (3)	123	120	116	124, 126	122	123, 125	136, 138	120	120, 131	5	131	X	X
Peq06	217–238 (5)	203	203	200	X	236	234, 238	201	203	X	2	X	X	X
Peq07	122–126 (3)	112	X	116, 128	110	X	X	168, 172	X	173, 175	4	X	X	X
Peq08	251–259 (3)	272, 277	276, 280	263, 288	251, 259	X	251, 255	259, 279, 291	235	255, 263	8	X	X	X
Peq09	195–235 (6)	242, 246	X	257, 261, 265	234, 258	X	191, 222	175	X	X	5	X	X	X
Peq10	117–129 (4)	X	109, 125	132, 136	X	113, 129	121, 125	100	113, 129	113	6	113, 129	X	113, 129
Peq11	241–273 (7)	308, 343§	264	239, 243, 267, 270	268	264	249, 256, 260, 264	279, 287	272, 276	240, 248, 252	7	X	X	X
Peq12	275–295 (4)	243, 332	283, 337	322, 348	X	X	291, 299	234, 270	284, 308	X	7	X	X	X
Peq13	108–123 (7)	109	114, 116	116	X	104, 127	108	104, 108	108	100	4	116, 122	116, 123	123
Peq14	222, 226 (2)	X	X	X	X	X	X	X	X	X	1	X	X	X
Peq15	188–217 (3)	X	X	X	X	X	X	X	X	X	1	X	X	X
Peq16	135–144 (3)	134	135	131, 135	161	210§	144, 153	131, 140, 144	126	X	4	X	X	X
Peq17	189–223 (6)	233, 241	201, 205	201, 205, 208	234	X	217, 221	205, 209	213, 217	X	7	128	X	X
Peq18	162–178 (4)	X	X	X	X	X	X	X	X	X	1	X	X	X
Peq19	218–234 (4)	207, 231	266, 286	261, 265, 277	230	257	242	225, 281	258, 270	246	6	X	X	X
Peq20	214–242 (6)	X	X	X	X	229, 245	259, 267	X	X	X	3	X	X	X
Peq21	168–188 (3)	248§	166, 180	170	180	X	184	146, 151	180	180	3	180	180	180
# Amp	21	13	14	15	11	10	15	15	13	10		6	4	4
# Poly	21	8	8	11	5	4	12	12	7	4		2	1	1

*All loci were amplified using the same PCR program and reaction constituents as used with Mauritius parakeet *Psittacula eques* individuals (see text). *n*, number of individuals tested; X, did not amplify; #Amp, number of loci amplifying per species; #Poly, number of loci polymorphic per species. §, the difference in observed and expected allele sizes suggests an alternative locus may be amplifying to that which was originally cloned and sequenced in the Mauritius parakeet.

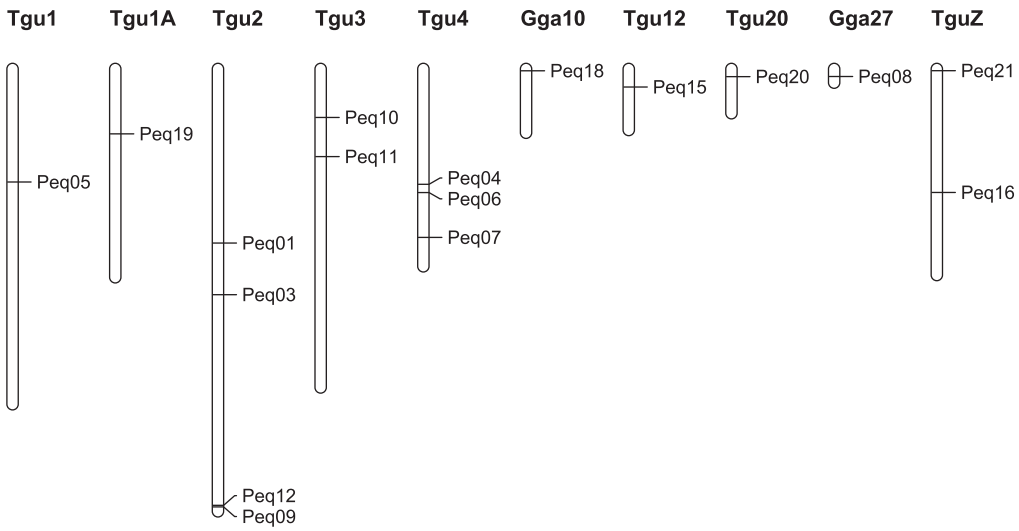

Fig. 1 Chromosome locations in the zebra finch (*Taeniopygia guttata*) and chicken (*Gallus gallus*) genome of 15 microsatellite loci characterized in the Mauritius parakeet (*Psittacula eques*).

using CriMap software (Lander & Green 1987). Loci were also checked for linkage disequilibrium in unrelated individuals using GenePop version 3.4 and applying a sequential Bonferroni correction (Rice 1989). One pair of loci was assigned to closely neighbouring locations on the same zebra finch chromosome (Peq09 and Peq12, located 0.5 Mb apart on Tgu2; Table 1, Fig. 1) and displayed both linkage (LOD = 10.24) and linkage disequilibrium. The second nearest known neighbouring pair of loci, Peq04 and Peq06 were located 3 Mb apart on Tgu4 and did not display either significant linkage or linkage disequilibrium. Significant linkage was detected between Peq03 and Peq05 (LOD = 4.16) although these loci mapped to different zebra finch chromosomes; these loci were not in linkage disequilibrium. Linkage disequilibrium was additionally indicated between Peq08 and Peq09, which were assigned to different avian chromosomes.

All 21 loci were tested in 13 other species including 10 parrot species (Table 2). Between seven and 21 loci were polymorphic in each of seven parrot species despite testing only one to two individuals. Six loci show particular potential for genetic studies of other parrot species. These six loci were each polymorphic in at least seven other parrot species (Peq01, Peq04, Peq08, Peq11, Peq12 and Peq17).

We have successfully characterized 21 polymorphic microsatellite markers for the Mauritius parakeet. These loci will be used to study the genetic variability of the existent population and the species' mating system.

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