Molecular Ecology Resources (2008) **8**, 933–935 doi: 10.1111/j.1755-0998.2008.02122.x

PERMANENT GENETIC RESOURCES

Development of microsatellite markers for parentage analysis in the great tinamou (*Tinamus major*)

PATRICIA L. R. BRENNAN*† and CHAZ HYSENI*

Department of Ecology and Evolutionary Biology, Yale University, New Haven, 21 Sachem Street, CT 06511, USA, †Department of Animal and Plant Sciences, University of Sheffield, Alfred Denny Building, Western Bank, Sheffield S10 2TN, UK

Abstract

Eighteen microsatellite loci were isolated from great tinamous (*Tinamus major*), which are large terrestrial birds found in the Neotropics. These are the first primers developed for the Order Tinamiformes. Paternity analyses are possible because the levels of heterozygosity are sufficiently high (0.29–0.90).

Keywords: extra-pair paternity, microsatellites, parentage, tinamous

Received 4 November 2007; revision accepted 19 December 2007

Tinamous (order Tinamiformes) are one of the least-known groups of birds. All 46 species have a Neotropical distribution and are secretive and inconspicuous (Davies 2002). Little is known about their natural history and the few long-term studies that have been conducted (summarized in Cabot 1992), have not conducted genetic analyses of parentage. The primers published here are the first obtained for any tinamou species. Tinamous are the only order of birds where exclusive male parental care is found in all known species, while mating systems are varied (Handford & Mares 1985). Because females mate with multiple males, there is a cuckoldry risk for the incubating male; this risk can be compounded when males mate with multiple females that cannot be guarded. In great tinamous, males acquire three to eight eggs from one or more females (Brennan 2005), and to investigate parentage in their nests, we isolated microsatellite markers from blood samples obtained from individuals captured at La Selva Research Station in Costa Rica.

We collected feathers from incubating males and blood and tissue samples from all chicks in active nests. Blood was stored in lysis buffer in 1.5-mL tubes and kept frozen at –10 °C until DNA extraction. Feathers were cleaned and put in sealed plastic bags, which were kept refrigerated at 4 °C. Feather shafts were either frozen with liquid nitrogen and then macerated for DNA extraction, or they were cut in pieces < 1 mm with a clean blade. DNA was extracted using a DNeasy tissue kit (QIAGEN, Inc.).

Correspondence: Patricia L. R. Brennan, Fax: (203) 432-3854; E-mail: patricia.brennan@yale.edu

Eight microsatellite-enriched libraries were constructed by Genetic Identification Services (GIS) using pooled genomic DNA. Methods for DNA library construction, enrichment and screening were as described previously (Jones *et al*. 2002). Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV). Fragments in the size range of 300–750 bp were adapted and subjected to magnetic bead capture (CPG, Inc.), using biotinylated capture molecules.

Libraries were prepared in parallel using biotin-CA(15), -GA(15), -AAAC(8) and -TAGA(8) as capture molecules in a protocol provided by the manufacturer. A second set was performed using GA, AAG, CAG and CATC. Captured molecules were amplified and restricted with *Hin*dIII to remove the adapters. The resulting fragments were ligated into the *Hin*dIII site of pUC19. Recombinant molecules were electrophorated into *Escherichia coli* DH5alpha. Recombinant clones were selected at random for sequencing, and enrichment levels were expressed as the fraction of sequences that contained a microsatellite. Sequences were obtained on an ABI PRISM 377, using ABI PRISM *Taq* dye terminator cycle sequencing methodology (Applied Biosystems).

Primers were designed for 42 microsatellite-containing clones using DESIGNER PCR version 1.03 (Research Genetics, Inc.), and the 18 most promising ones were selected for further work. We genotyped 20 individuals selected from different nests and years to maximize the likelihood of picking up variable loci.

The optimal amplification reaction mix for all primer pairs consisted of 1× Biolase C buffer (from 10×stock supplied by manufacturer), 2 mM $MgCl_2$, 0.2 mM each dNTPs, 0.3 μM;

Table 1 Primer description and allelic variability of 18 loci developed for great tinamous (*Tinamus major*). Included are locus name, accession number, primer sequence, repeat motif, number of observed alleles (N_A), and number of individuals amplified (Ind), expected (H_E), and observed (H_O) heterozygosities, null allele estimate, fragment size range and Hardy–Weinberg (HW) *P* value

Locus/ accession no.	Primer sequence (5′–3′)	Repeat motif	N_A (Ind)	H_E	H_O	Null	Size range	HW *P* value
A1	AGCACATGAGCTTTTTAGAGC	GT	9	0.78	0.74	0.02	244–268	0.595
EU259625	GCACTTGACTTGCACAGTATG	(14)	(19)					
A11	AATTGGAAACGATGGTGTTAC	CA	10	0.85	0.29	0.30	202–238	0.000
EU259615	GCTCAAAATAATGAATGGACAG	(16)	(18)					
A104	AAGGAAGCTGCGACAAGTC	CA	13	0.87	0.79	0.04	299–333	0.001
EU259616	TAGCGGCCTAGCAGGTTAC	(18)	(19)					
A106	GAATTAGCCAGCATCTTTACAC	CA	13	0.89	0.78	0.06	156–186	0.147
EU259617	CCAAGTATTGTTTCAGTCAACC	(20)	(19)					
A108	CTCACGCCTTCATACAGAATAC	CA	8	0.78	0.63	0.09	183–203	0.180
EU259618	GTAAATAGGTCGCTGGTCAGTA	(21)	(19)					
A114	GGATGTCAGCAGACTTCAAAG	GT	5	0.64	0.50	0.09	184–202	0.094
EU259619	TGGAGGTAGTTTCACGCTTAC	(16)	(18)					
A118	CATTCAAGCCCATAAATATGAC	CA	4	0.64	0.59	0.03	119–127	0.171
EU259620	CATCCAGCCTTTGCATTAG	(12)	(17)					
E1	TCAGTGTCTTTCCATTGTCATC	GA	8	0.78	0.38	0.23	190–204	0.000
EU259621	CCCTCTCTCTGTAACTCTCCTG	(18)	(16)					
E4	GCAAAGTCTCAGTGCTTCTG	CT	8	0.75	0.72	0.02	285–299	0.014
EU259622	GAATCCTTCACCTCCCTAATC	(14)	(18)					
E101	CCCAGGGTTACCACAAGC	GA	11	0.85	0.83	0.01	194–226	0.579
EU259623	GCCCATACTCTCTCTTTCCC	(15)	(19)					
E105	TGCTGCCTGTCCACTTACTC	GA	14	0.88	0.84	0.02	202–236	0.340
EU259624	TCACGCTTCAGTTATGACCC	(22)	(19)					
E111	GCTTTCAGGATGCTGCTC	CT	8	0.72	0.79	-0.03	166–184	0.608
EU259626	CCCTTTAGTGTGCTGTAGGC	(14)	(19)					
E118	TGGCATTTGTAGAGTTGAATC	CT	9	0.65	0.53	0.08	234–282	0.071
EU259627	AAGTCCCCGTGGTGTTAC	(19)	(19)					
E119	TTGCCCATACTCTCTCTTTCC	CT	12	0.84	0.90	-0.03	201–233	0.952
EU259628	TGATTGCCCAGGTTACCAC	(14)	(20)					
H6	CTGACCACTCTTTCAACACTTC	CATC	5	0.7	0.56	0.09	163–191	0.318
EU259629	CACAGGACCCAGTGACTTC	(8)	(18)					
H8	CCCACTTCTACATGGCAGTG	GGAT	6	0.8	0.29	0.28	303–325	0.000
EU259630	GCTCATGCCGATTTTGTC	(11)	(17)					
H118	TCACCTCCTAATACCCTCTTG	GGAT	2	0.46	0.32	0.10	219–223	0.165
EU259631	CACCCATTCCTCCATCTC	6	(19)					
H123	AACCTTCCACCTGAGAACTTC	GGAT	9	0.85	0.82	0.02	255–299	0.141
EU259632	TGAGCCCTTTAGTGACCTACA	(15)	(18)					

0.025 U/μL Biolase C *Taq* polymerase, and 0.2 ng/μL template DNA in 10-μL final reaction volume. Samples were amplified in a PerkinElmer Cetus thermal cycler by an initial 3 min of denaturation at 94 °C, followed by 35 cycles of denaturation (94 °C, 40 s), annealing (55 °C, 40 s), and extension (72 °C, 30 s), with final extension time of 4 min at 72 °C.

Microsatellite loci were amplified in 10-μL reactions in the following reaction mix: $MgCl_2$, 2 mM; dNTPs (premixed), 0.2 mM each; primers, 0.3 μM each; Bio*Taq* DNA PolymeraseR (Bioline USA), 0.025 U/μL; template DNA, 0.2 ng/μL. Polymerase chain reaction (PCR) was conducted in a RoboCycler Gradient 96R thermocycler (Stratagene, Inc.) by an initial denaturation (94 °C, 3 min), followed by 35 cycles of denaturation (94 °C, 40 s), annealing (55 °C, 40 s), and extension (72 °C, 30 s), and a final extension at 72 °C for 4 min. PCR products were labelled using sequencing dyes NED, HEX or FAM (Applied Biosystems, Inc.). Amplification products were separated on polyacrylamide gels in an ABI PRISM 377 DNA sequencer and sized using GENOTYPER 2.5 software and Rox 400 HD size markers (Applied Biosystems, Inc.).

Genotypes of 20 individuals were analysed using IDENTITY 1.0 (Table 1). Null frequencies were high (≥ 0.1) in four loci (A11, E1, H8, H118). The same loci were not in Hardy–Weinberg equilibrium (Table 1). The overall exclusion probability for paternity analysis of all loci is 0.9999. Pairwise tests for linkage disequilibrium between loci performed in GENEPOP DOS version 3.4 (Raymond & Rousset 1995), revealed no significant deviations after correcting for multiple comparisons (Rice 1989). We are now using eight of these markers for parentage analysis in our sample of great tinamous from Costa Rica.

Before developing Tinamou primers, we tested primers OSM1, OSM4 (Kimwele *et al*. 1998), LIST002 and LIST011 (Kumari & Kemp 1998) developed from ostrich (*Struthio camelus*), which belongs to the sister taxa of tinamous. Although ostrich primers amplified great tinamou DNA, optimization of PCRs was not possible.

Acknowledgements

This work was funded through a National Science Foundation (NSF) postdoctoral grant to P.L.R.B. A previous effort to build a tinamou library at Cornell University did not result in publishable primers despite the assistance provided by Dr Steve Bogdanowicz, Dr Jennifer Calkins, Dr Susan McRae, Prof Kern Reeve and Dr Janet Sherman-Shellman. Funding for this earlier project was obtained from an NSF graduate fellowship award.

References

Brennan P (2005) *The breeding biology and ecology of great tinamous (Tinamus major): Female joint-nesting, extra-pair paternity and natural history*. PhD, Cornell University, Ithaca, New York.

Cabot J (1992) Tinamiformes. In: *Handbook of Birds of the World* (eds del Hoyo J, Elliot A, Sargatal J), pp. 112–144. Lynx Edicions, Barcelona, Spain.

Davies SJJF (2002) *Ratites and Tinamous. Tinamidae, Rheidae, Dromaiidae, Casuariidae, Apterygidae, Struthionidae*. Oxford University Press, Oxford, UK.

Handford P, Mares MA (1985) The mating system of ratites and tinamous: an evolutionary perspective. *Biological Journal of the Linnean Society*, **25**, 77–104.

Jones KC, Levine KL, Banks JD (2002) Characterization of 11 polymorphic tetranucleotide microsatellites for forensic applications in California elk (*Cervus elaphus canadensis*). *Molecular Ecology Notes*, **2**, 425–427.

Kimwele CN, Graves JA, Burke T, Hanotte O (1998) Development of microsatellite markers for parentage typing of chicks in the ostrich *Struthio camelus*. *Molecular Ecology*, **7**, 247–255.

Kumari P, Kemp J (1998) Polymorphic microsatellite markers in the ostrich (*Struthio camelus*). *Molecular Ecology*, **7**, 133–140.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenism. *Journal of Heredity*, **86**, 248–249.

Rice W (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.