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TECHNICAL NOTE

Characterization of nine microsatellite loci for a globally vulnerable species, Reeves's Pheasant (*Syrmaticus reevesii*)

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Abstract Reeves's Pheasant, *Syrmaticus reevesii,* is an endemic species of China. Due to habitat loss, poaching and human disturbance, its wild population has been decreased drastically and it is listed as a globally vulnerable species by IUCN/BirdLife/WPA (IUCN 2008). Here, we report nine new polymorphic microsatellite markers isolated from the Reeves's Pheasant. The number of alleles per locus varies between four and fourteen, with expected heterozygosity ranging from 0.349–0.776 ($n = 90$). These polymorphic loci provide a valuable tool for future population studies that relate to the conservation of this pheasant.

Keywords *Syrmaticus reevesii* · Microsatellite · Primers · Conservation

The Reeves's Pheasant (*Syrmaticus reevesii*) is an endemic gamebird that used to be widely distributed in temperate and subtropical forests of central and southwest China (Cheng et al. 1978). Due to habitat loss, poaching and human disturbance, the wild population has been decreasing dramatically. Therefore, it is listed as a globally vulnerable species by IUCN/BirdLife/WPA (IUCN 2008) and ranked as Grade II National Protected Wildlife in China (Zheng and Wang 1998). On the global scale, the previously continuous population of the pheasant was split into two isolated geographic subpopulations with a large gap in between as a consequence of habitat fragmentation. Concomitantly, the sizes of the subpopulations have been reduced by deforestation and poaching. Both empirical and theoretical evidences have proved a direct link between population size, demographic dynamics and genetic variation in the remaining small and isolated population (Frankham et al. 2002). It seems likely that some isolated subpopulations of Reeves's Pheasant are vulnerable to local extinction due to inbreeding depression, loss of genetic diversity, and environmental and demographic stochasticity.

Concerning the conservation genetics aspect, because Reeves's Pheasant shows both shrinking population size and a fragmented distribution range, it is a prerequisite and a primary objective to make estimates of genetic variation in the different populations. It is also critical to document dispersal patterns and population structures within the fragmented habitats. Knowledge of such aspects as well as delimitation of management units can only be derived from population genetics studies. Thus, there is a need to screen wild populations of the species. Moreover, the mating system of Reeves's Pheasant is still uncertain. The genetic approach is of great use for parentage studies of this pheasant. Microsatellites, also known as Simple Sequence Repeats (SSRs), show high degrees of polymorphism and are co-dominant markers, so therefore they are powerful tools to resolve those issues we must address about Reeve's Pheasant. Here, we describe the isolation and characteristics of nine novel microsatellite loci for Reeves's Pheasant.

We established a partial genomic library mainly based on selective hybridization protocols (Kijas et al. 1994). Genomic DNA was extracted from the blood of a captured

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Table 1 Nine polymorphic microsatellite loci isolated from Reeves's Pheasant

Locus GenBank	Primer sequence (5′–3′)	Repeat Motif	N_A	(size range)	H_O	H_E	Number of alleles at each locus across species						
							BEP ($N = 8$)	RNP ($N = 8$)	GP ($N = 4$)	BP ($N = 4$)	KP ($N = 4$)	CBP ($N = 4$)	YBT ($N = 8$)
SR01	F: ^{HEX}aacaaaagtcttcgggtcc	$(CA)_{18}$	7	122–136	0.453	0.480	2	6	3	N/A	6	N/A	2
FJ221371	R: tgtgcgtgtgcagcgaactcc												
SR03	F: ^{FAM}tctctgacgtatcgcatct	$(CA)_{11}$	5	299–307	0.525	0.624	N/A	8	4	2	1	1	N/A
FJ221373	R: acttcccctggtaaact												
SR08	F: ^{FAM}cgtcacatggctacacctt	$(CA)_{15}$	4	189–203	0.369	0.349	4	6	2	2	2	N/A	N/A
FJ221378	R: atcaagctgcgtatcct												
SR11	F: ^{FAM}atcaatatggactgctccgt	$(CA)_{11}AC(CA)_3$	11	198–218	0.773	0.759	N/A	7	5	2	6	2	3
FJ221381	R: tccttcaaggccaagtg												
SR13	F: ^{HEX}ttaatacaggcatgtgact	$(CA)_6$	4	151–159	0.550	0.488	N/A	N/A	1	N/A	N/A	N/A	3
FJ221383	R: cctgctttctctattggt												
SR17	F: agaagaccgcattgtt	$(GA)_3AA(GA)_5$	4	190–196	0.457	0.514	1	2	4	1	4	2	1
FJ221387	R: ^{FAM}agattgcacacgttagcca												
SR18	F: ^{FAM}tatgaaacagacttaatcc	$(CT)_2TCTA_5(CAA)_7$	4	183–195	0.402	0.390	N/A	2	2	N/A	2	N/A	N/A
FJ221388	R: tgcagcatttgagtaac												
SR19	F: ^{FAM}catggtatagatgcctgcgta	$(CA)_{10}TA(CA)_4$	11	250–270	0.714	0.776	2	5	3	2	2	2	2
FJ392394	R: cgcagcaggaggtccc												
SR20	F: ^{FAM}ccctacaattctgtgattc	$(CAA)_{23}$	14	169–214	0.670	0.737	1	2	3	2	4	3	7
FJ392395	R: agaacaaaatctgctaaaac												

H_O, observed heterozygosity; H_E, expected heterozygosity. N_A: Number of alleles. The annealing temperature was 58–47°C touch-down of the first 20 cycles, following by a constant 47°C for another 20 cycles. For the cross-species application, BEP: Brown eared-Pheasant *Crossoptilon mantchuricum*, RNP: Ring-necked Pheasant *Phasianus colchicus*, GP: Golden Pheasant *Chrysolophus pictus*, BP: Blood Pheasant *Ithaginis cruentus,* KP: Koklas Pheasant *Pucrasia macrolopha,* CBP: Chinese bamboo-partridge. YBT: Yellow-bellied tragopan *Tragopan caboti*. N/A: no amplification products

Reeves's Pheasant and was digested with the Saul3A restriction enzyme (Promega, Wisconsin, USA). Size-selected fragments (250–1000 bp) were ligated into a SAULA/SAULB linker (Hammond et al. 1998) and hybridized with 6 kinds of biotinylated oligonucleotide repeats($(CA)_{12}$, $(GA)_{12}$, $(AT)_{12}$, $(CG)_{12}$, $(AAC)_8$, $(AAT)_8$), followed by isolation with streptavidin-coated magnetic beads (Promega, Wisconsin, USA). The captured microsatellite DNA was then enriched by PCR with SAULA primer, and cloned into PBS-T plasmids (TianGen Biotech, Beijing, China) following the kit manufacturer's protocol. Colony PCR was conducted using M13/M13 reverse primers, together with one of the six probe primers. Plasmids from 108 clones with proper insertion length were sequenced in a single direction with M13 reverse primer from BGI Life Tech (Beijing, China).

After sequence alignment using the Mega 3.1 software, primers were designed from the two regions flanking the microsatellite repeat motif with the Oligo software version 6.54 (Molecular Biology Insights, Inc. Colorado, USA). We tested the PCR efficacy of each of the synthesized primer pairs, choosing 20 of them to label with either 6-FAM or HEX fluorescent dyes at the 5′-end in one of each pair.

In order to characterize these microsatellites, we used a total of 90 individuals sampled from three wild populations (populations of Dongzhai: 31°28′–32°09′N, 114°18′-114°30′E; Jinzhai: 30°10′-31°20′N, 115°20′-115°50′E; and Shennongjia: 31°15′-31°57′N, 109°58′-110°58′E) and one captured population in the Daxing breeding center of Beijing. PCR was carried out in a 10 μl mixture containing 100 ng DNA, 0.25 μM of each primer (one labeled with fluorescent dyes), 10× PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.75U *Taq* polymerase (All reagents were from Takara Bio, Tokyo, Japan). The thermal cycling protocol was denaturation at 94°C for 5 min, then a "touch-down" annealing process with temperature lowered 0.5°C per cycle (starting from 58°C, until a temperature of 47°C was reached). After the first 20 cycles, 20 additional cycles were run at a constant annealing temperature of 47°C, followed by a final extension of 10 min at 72°C. Fragment analysis was conducted on an ABI PRISM 3100 Genetic Analyzer using GeneMapper software (Applied Biosystems, Inc.) and the size of alleles was determined against the ROX-500 size standard (Applied Biosystems, Inc.).

Nine out of the 20 markers showed polymorphism, and the rest of them were only submitted to GenBank without being listed here. We used FSTAT version 2.9.3.2 (Goudet 2002) to calculate the heterozygosities, and to test for Hardy–Weinberg equilibrium (HWE) together with linkage-disequilibrium of the nine microsatellite loci. Results of the test were adjusted for multiple comparisons by applying sequential Bonferroni corrections (Rice 1989). At the same time, MicroChecker 2.2.3 (Van Oosterhout et al. 2004) was used to test the presence of null alleles.

Table 1 shows the characteristics of the nine polymorphic microsatellite markers. Among these, the number of alleles per locus varies between four and fourteen, with expected heterozygosity ranging from 0.349–0.776 ($n = 90$). After sequential Bonferroni correction (Rice 1989), no locus or either population deviated significantly from HWE (overall $P = 0.035$, adjusted (0.05) significance level was 0.00139). Also, no significant genotypic linkage-disequilibrium was observed between any pair of loci. The Cockerham estimator (Weir and Cockerham 1984) of Wright's FST (Table 2) together with "pairwise tests of differentiation" by exact G-test (Goudet et al. 1996) indicate significant population differentiation after standard Bonferroni corrections ($P < 0.001$ compared with adjusted significance level 0.00833). Furthermore, there is no evident inbreeding within wild populations (see FIS in Table 2), and these preliminary data infer higher genetic diversity in the Jinzhai and Shennongjia populations from the comparison of average Gd (Table 2). We also cross-amplified the same nine loci in various species, using identical reaction conditions and temperature ranges. The cross-amplification results are reported in Table 1.

For the noticeably lower frequency of microsatellites possessed by birds (Zane et al. 2002), nearly all loci isolated in our study own (CA)*n* motifs, indicating the relatively high frequency of (CA)*n* motifs among the whole bird genome. These microsatellite markers are beginning to be used in analyzing population genetics and paternity of wild populations, and they should contribute greatly to

Table 2 Parameters measured by nine microsatellite loci within or among populations

Populations	Sample size	Average Gd	Jinzhai	Daxing	Dongzhai	Shennongjia
Jinzhai	20	0.699	0.088*			
Daxing	12	0.391	0.1943	−0.019		
Dongzhai	38	0.531	0.0876	0.2844	0.026	
Shennongjia	20	0.650	0.0420	0.2629	0.0968	0.030

* F-statistics for Reeves's Pheasant populations, pair wise FST below the diagonal and FIS on the diagonal; Average Gd: Average gene diversity within populations

conservation studies of Reeves's Pheasant in the future. Furthermore, cross-species' tests infer much wider application of these primers in our preliminary experiments (Table 1). Because of limitations in our sample size (from four to eight at most), we can expect more polymorphisms at certain loci in some species, which emphasizes the potential benefits of these microsatellite loci for other studies.

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