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Novel polymorphic microsatellites for the red-legged partridge (*Alectoris rufa*) and cross-species amplification in *Alectoris graeca*

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Abstract

The red-legged partridge, *Alectoris rufa*, is an endemic species of South-western Mediterranean, and the most popular game bird in the Iberian Peninsula. A total of 27 microsatellite loci was isolated from an enriched genomic library of *A. rufa*. Six perfect GT microsatellites were characterized and optimized in 45 individuals of *A. rufa*. All loci revealed high levels of polymorphism with a number of alleles that ranged from three to 13. Observed heterozygosity ranged from 0.2 to 0.6. Cross-species amplification showed that all loci were also polymorphic in rock partridge, *Alectoris graeca*. The new markers will be useful in determining hybridization between both species of *Alectoris*.

Keywords: red-legged partridge, game bird, hybridization, microsatellite, rock partridge

The red-legged partridge (*Alectoris rufa*) is an endemic species of Western Mediterranean. It occurs naturally throughout Iberian Peninsula, France, and northwestern Italy (Randi & Bernard-Laurent 1999), and constitutes an important prey species for a variety of endangered vertebrates. Once abundant, red-legged partridge populations have markedly declined in recent years because of human activities mostly related with habitat destruction and hunting (Potts 1980). The red-legged partridge is the most popular game bird in the Iberian Peninsula, and millions of captive-bred individuals are yearly released to reinforce wild populations (Negro *et al.* 2001). Hybridization of *A. rufa* with other species of the genus, including *A. graeca*, *A. chukar*, and *A. barbara*, is relatively common in the wild, but also deliberately induced by humans (Randi & Bernard-Laurent 1999; Negro *et al.* 2001). At present, the potential effects of reintroductions and hybridization on wild populations are largely unknown from a genetic perspective. Here, we report the first microsatellite loci available for examining population genetic structure and hybridization in *A. rufa*.

To obtain microsatellite sequences, a genomic library enriched for GT repeats was constructed based on the protocols of Ostrander *et al.* (1992) and Hamilton *et al.* (1999), with minor modifications. Briefly, genomic DNA was extracted from fresh muscle following a standard phenol-chloroform method. Purified DNA was simultaneously digested with *Rsa* I and *Pvu* II restriction enzymes (New England Biolabs), and ligated onto SNX oligonucleotide adaptors (Hamilton *et al.* 1999). (CA)₁₂ biotinylated probes were hybridized to digested DNA, and selectively retained using streptavidin

magnetic particles. Enriched DNA was subsequently PCR amplified using an SNX forward adaptor. Amplified enriched DNA was then ligated into a pGEM-T Easy vector (Promega), and transformed into *E. coli* XL-10 Gold ultra competent cells (Stratagene). Approximately 500 white recombinants were transferred onto Hybond-N+ nylon membrane (Amersham), and screened with a biotin-labelled oligonucleotide (CA)₁₂. Colonies that potentially contained microsatellites were detected by a chemiluminiscent method (CDP-Star detection kit, Sigma). Inserts of 30 positive clones (average length= 487 ± 148) were sequenced in an ABI Prism 3100 DNA Analyzer (Applied Biosystems) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (V3.0), and following manufacturer's instructions (Applied Biosystems).

A total of 27 out of the 30 positive clones contained microsatellites (GenBank accession numbers AY837847-AY837868). Of these, 13 were perfect, and 14 were compound. A total of six microsatellite loci containing perfect GT repeats were selected for further characterization (Table1). The corresponding PCR primers were designed using PRIMER3 software (Rozen & Skaletsky 1998). PCR amplifications were optimized for each locus, and consisted of 40 cycles of denaturation at 95°C for 45 s, annealing at 60-65 °C for 45s, and extension at 72°C for 1 min. Cycles were followed by a final extension at 72°C for 10 min. PCR reactions contained approximately 5 ng of sample DNA, 0.1 U of *Taq* DNA polymerase (Biotools), 0.5 µM of each primer, 200 µM of each dNTP, 1 mM MgCl₂ and 1x *Taq* buffer (Tris-HCl 67mM, pH 8.3, MgCl₂ 1.5 mM), in a total volume of 13 µl.

The six selected microsatellite loci were amplified in several individuals of *A. rufa* from a wild population in San Millán de Juarros, Burgos (Castilla–León; $N = 18$), and two breeding farms in North Central (Castilla-León; $N = 15$) and North East Spain (Catalonia; $N = 12$). Samples from the three populations were combined into a single data set, and analyzed globally. Locus designations, PCR primer sequences (and annealing temperatures), repeat motifs, range of allele sizes in base pairs, and observed and expected heterozygosities per locus are presented in Table 1. Observed and expected heterozygosities were estimated using GENETIX version 4.05 (Belkir et al. 2000). The probabilities of departure from Hardy-Weinberg equilibrium (P_{HW}), and of genotypic disequilibrium between pairs of microsatellites were assessed with GENEPOP version 3.3 (Raymond & Rousset 1995) using 5000 dememorizations, 500 batches and 500 iterations per batch. Sequential Bonferroni corrections (Rice 1989) were applied for all multiple tests performed simultaneously.

The number of identified alleles for the six scored loci ranged from 3 to 13 (average = 8). Observed heterozygosity ranged from 0.2 to 0.6. Genotypes at two loci (*ARU1.9* and *ARU1.27*) differed significantly from HW expectations. One pairwise test of genotypic disequilibrium (*ARU1.19* vs. *ARU1.29*) was statistically significant. The three sampled groups were also analyzed separately to explore microsatellite variation and genetic structure among different brooding (wild and farming) types. Levels of observed heterozygosity were higher in the wild population (mean observed heterozygosity per population, $H_o = 0.469 \pm 0.078$ and mean expected heterozygosity per population $H_E = 0.508 \pm 0.083$, respectively) and the farm in

Castilla – León ($H_o = 0.586 \pm 0.104$; $H_E = 0.551 \pm 0.096$). In contrast, the farm in Catalonia showed lower heterozygosity levels ($H_o = 0.313 \pm 0.271$, $H_E = 0.381 \pm 0.282$) that may be tentatively attributed to inbreeding, but larger samples sizes need to be analyzed to confirm this hypothesis.

All six microsatellite loci were also genotyped in six individuals of rock partridge (*A. graeca*) from Kavala (Greece) under the same PCR conditions designed for *A. rufa*, and all loci were found to be polymorphic in the cross-species amplification. We observed a similar allelic size distribution at five microsatellite loci between the two species, whereas the locus *ARU1.27* presented a disjointed distribution. This result may indicate possible exclusive alleles both for the red-legged and rock partridges, but larger samples sizes are required to fully confirm these results. Since the studied microsatellites are polymorphic in both species, and some of the alleles for locus *ARU1.27* may be likely diagnostic of the species, it is expected that the new nuclear markers will provide useful information for future studies on the population genetics of the red-legged partridge, and its hybridization with *A. graeca*, as well as help in the management of breeding farms.

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Table 1 Summary data for microsatellite developed from red-legged partridge (*Alectoris rufa*). N , number of individuals assayed; N_A , number of alleles detected per locus; H_O and H_E observed and expected heterozygosities, respectively. P_{HW} , refers to probability of Hardy-Weinberg equilibrium test.

Locus	Repeat motif	T» (¼ Primers sequence 5'-3'	N	N_A	Size range (bp)	H_O	H_E	P_{HW}	GenBank No.
<i>Aru1.9</i>	$T_{18}(GT)_{18}$	61 F: ACTTTAAACCTCCTGAGGGAATGT R: AGAAAGAAGAAAACCTTGTCCTCAA	45	13	110-142	0,644	0,711	0,000*	AY837852
<i>Aru1.19</i>	$(GT)_{11}TT(GT)_5$	60 F: TTATCATCATCTTTGGTCTGCTG R: AACACATATCTTGGTGTGGTTCA	44	6	188-200	0,568	0,651	0,052	AY837860
<i>Aru1.22</i>	$(GT)_9$	62 F: ATGTGAGTGTGTAAGGGGGAGT R: GACATCTGGCACATAAAAAATCAAG	45	3	114-120	0,400	0,343	0,672	AY837863
<i>Aru1.23</i>	$(GT)_{17}$	65 F: GTAACTTGCCCCCTGCTGTTC R: CTTCTCTGGGCAGCTGTGTC	45	4	177-185	0,244	0,258	0,636	AY837864
<i>Aru1.27</i>	$(GT)_{17}$	60 F: GTTCTGGCTTTAAAGAGCTTGG R: TGAGAATGCAGGACAGGAGATA	45	10	170-192	0,570	0,733	0,000*	AY837866
<i>Aru1.29</i>	$(GT)_{17}$	65 F: TATGTGTGTGTGCATCCATGTG R: ATGTCATCGAGAGCTCCGTAAT	43	12	148-174	0,674	0,729	0,044	AY837867

* $p < 0,05$ after Bonferroni correction