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PRIMER NOTE

# Isolation and characterization of microsatellites in European alpine marmots (*Marmota marmota*)

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**Abstract**

**For future investigations of the mating system of a highly social mammal (*Marmota marmota*), we identified 16 new microsatellites using an enrichment protocol. Five loci were revealed to be polymorphic. The polymorphism was rather low (two to six alleles among 24 individuals). However, these markers, added to the other six published microsatellites for *M. marmota* and *Spermophilus citellus*, will help to assess dispersal patterns and test for genetic monogamy in alpine marmots from the European Alps.**

*Keywords*: enrichment, extra-pair copulation, microsatellite cloning, monogamy, paternity assignment, sciurid mammals

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Alpine marmots are one of the most social species of the genus *Marmota* (Arnold 1990). They live in family groups and are socially monogamous.

Monogamy has been assumed to be rare among mammals and has been described for only 3–5% of all mammals species. Recent studies using molecular markers such as microsatellites have demonstrated that extra-pair copulations do occur in presumably monogamous mammals (Foltz 1981; Agren *et al*. 1989). In the alpine marmots, a long-term study of a population in the French Alps has revealed that extra-pair paternity is not rare (Goossens *et al*. 1998).

In order to obtain a better understanding of the mating system of the alpine marmot, we need to identify the males that perform extra-pair copulation and sire extra-pair young. Such a study requires a large number of polymorphic markers, such as microsatellites, to guarantee a reasonable confidence level of paternity assignment. Consequently, we have developed new microsatellites for *Marmota marmota*.

We used an enrichment protocol followed by a polymerase chain reaction (PCR) screening (Paetkau 1999). Genomic DNA was digested with *Rsa*I (Boehringer Mannheim). Size-selected fragments (300–600 bp) were then ligated in M13 phage and cloned in XL1-Blue competent bacteria (Stratagene). This allowed us to obtain single-stranded DNA, which was probed with one synthetic oligonucleotide labelled with biotin (OligoExpress). We used $(CA)_{10}$, $(CT)_{10}$ and $(ATT)_{10}$ motifs. Each hybridization reaction was then mixed with streptavidin-coated magnetic beads (Dynal MPC®-E) to allow biotin–streptavidin binding. Hybridized fragments were retained through the biotin–streptavidin bead bond in a magnetic particle concentrator (Dynal MPC®-E), while unhybridized fragments were removed with the supernatant fluid. Competent cells were transformed with the enrichment mix after having broken the biotin–streptavidin bond by increasing the temperature to 85 °C. Resulting clones were then screened by PCR using the universal pUC/M13 forward and the microsatellite sequence as primers [$(CA)_{10}$, $(CT)_{10}$ and $(ATT)_{10}$]. Positive clones were subsequently sequenced using the PE-Applied Biosystems Big Dye Terminator Kit on an ABI 377 DNA sequencer with the universal pUC/M13 forward and reverse primers. Primers for PCR were designed for 16 microsatellites using OLIGO software (National Biosciences, Inc.).

PCR amplifications were prepared in a 12 μL volume containing about 20 ng template DNA, 0.1 mM of each dNTP, 0.4 μM of each primer (the forward primer was fluorescent labelled, Genset Oligo), 2.5 mM $MgCl_2$ (2 mM $MgCl_2$ for *MA91* and *MA58*), 0.6 U Ampli*Taq* Gold

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**Table 1** Characteristics of optimized microsatellites tested on 24 individuals

| Locus | Microsatellite sequence in clone | Primers sequences (5′ → 3′) | Allele size (bp) range | *Tm* (°C) | No. of cycles (*n*) | $H_O$ | $H_E$ | $N_a$ | GenBank accession numbers |
|---|---|---|---|---|---|---|---|---|---|
| MA001 | $(CA)_{15}C_4(CA)_6$ | AGGGGAACAGAACCAAAAGG<br>GTTTCTTCCAGGGACAAAGCACCATC | 306–310 | 65–55* | 10 + 35 | 0.458 | 0.58 | 3 | AY197780 |
| MA018 | $(CA)_{13}$ | ATCCGTCCAATAAAGAAATTC<br>GTTTCTTGTGGCTCAGTGGTCAGATG | 296–298 | 65–55* | 10 + 35 | 0.166 | 0.329 | 2 | AY197781 |
| MA002 | $(CA)_{21}$ | CATTTAGACGCACATTTTG<br>GGGATGGAGAATGAGGAAG | 274–283 | 65–55* | 10 + 35 | 0.625 | 0.68 | 4 | AY197782 |
| MA066 | $(CA)_{14}$ | AATATGTTAAGGCAGTTCTAGC<br>GTTTCTTCCTGATATGGAAAGATGATGT | 234–244 | 55–45* | 10 + 35 | 0.666 | 0.486 | 2 | AY197783 |
| MA091 | $(CA)_{20}$ | CCTGTGTGAGTCCTGGAGTC<br>AGCCATTTAGGTTACATCTGC | 163–182 | 60–50* | 10 + 35 | 0.666 | 0.590 | 6 | AY197784 |

*Tm*: Annealing temperature. *: starting from the first indicated temperature (left), the annealing temperature is decreased by 1° C per cycle for the first 10 cycles and is subsequently stabilized for 35 cycles at the second indicated temperature. $H_O$: observed heterozygosity. $H_E$: unbiased expected heterozygosity (Nei 1978). $N_a$: number of alleles.

Polymerase (Applied Biosystems) and 1× *Taq* buffer (containing 100 mM Tris-HCl, pH 8.3, 500 mM KCl, according to the manufacturers' specification; Applied Biosystems). Amplifications were performed in a GeneAmp PCR System 2400 (Applied Biosystems) with the following cycling conditions: 10 min at 95 °C, *n* (number of cycles) cycles composed of 30 s denaturing at 95 °C, 30 s annealing at *Tm* (annealing temperature), 30 s extension at 72 °C, and 7 min at 72 °C, to assure complete extension (see Table 1 for *Tm* and *n*). Loci that did not amplify after having been tested with four annealing temperatures were considered useless. Amplified fragments were then loaded on 5% Long Ranger polyacrylamide gel (Fric) and electrophoresis was run for 3 h on an automated sequencer ABI 377™ (Applied Biosystems) using the size standard, ROX 350, to determine allele sizes. Microsatellite patterns were examined with GENOTYPER 2.0 (Applied Biosystem).

Sixteen loci were tested of which five were found to be polymorphic. These microsatellites were tested on 24 individuals from the Sassiere population being studied for its mating system in the French Alps (Vanoise National Park). The observed heterozygosity ranged from 0.17 to 0.67, and the number of alleles ranged from two to six per locus. Genetic analysis using GENEPOP (Raymond & Rousset 1995) showed that loci are in Hardy–Weinberg proportions except for *MA018* (deficit of heterozygotes, $P < 0.05$), and that there is no linkage disequilibrium between different microsatellites.

The proportion of amplifiable and polymorphic microsatellites was rather low (31.25%), and the polymorphism was not high, although this study involved only one population. Nonetheless, these new microsatellites, combined with other microsatellites already published for *M. marmota* (Hanslik & Kruckenhauser 2000), could help improve our understanding of the mating system of the alpine marmot, for example, by identifying males involved in extra-pair paternities. Moreover, these new microsatellites will facilitate studies of population structure and gene flow, and can potentially be used for research concerning other Sciurid species.

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