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TECHNICAL NOTE

Characterization of 13 polymorphic microsatellite loci in the European pine marten *Martes martes*

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Abstract A set of 13 polymorphic microsatellite markers were isolated and characterized from a genomic library enriched for dinucleotide repeats in the European pine marten *Martes martes*. Microsatellite loci amplification was tested on a panel of 12 tissue samples and 9 distinct hair samples collected from either road-killed or trapped animals in Tuscany, Italy. Allelic diversity was 6 and the number of alleles per locus ranged from 2 to 10. Mean observed and expected heterozygosity were 0.610 (range: 0.238–0.905) and 0.698 (range: 0.400–0.856), respectively. This novel set of microsatellite loci will be particularly useful for non-invasive genetic studies to assess population distribution and patterns of population structure and dispersal of *M. martes* in woodlands and fragmented habitats.

Keywords Pine marten · Mustelidae · Microsatellites · Population genetics · Non-invasive genetics

The European pine marten (*Martes martes*) is a medium-sized carnivore of the family *Mustelidae*. It is found in Europe, including the major Mediterranean islands, with the exception of most of the Iberian Peninsula and Greece, and parts of Belgium and the Netherlands, with limited distribution in the UK (Proulx et al. 2004). Habitat fragmentation and human disturbance have caused a significant decline in European populations, which are now protected under the EU habitat directive. In Italy, pine martens live mainly in high canopy coniferous and deciduous forests. They are solitary animals, meeting only to breed in late spring or early summer, with relatively low population densities. Pine martens are also difficult to observe and few studies have been conducted on the status, distribution and dynamics of Italian populations (e.g. Manzo et al. unpublished). We developed a set of 13 polymorphic microsatellite markers to elucidate patterns of colonization and dispersal of pine martens across woodlands and to gain a greater understanding of their distribution in Tuscany through non-invasive genetic analysis based on samples collected using hair traps.

A genomic library enriched for dinucleotide repeats was constructed using a pooled sample of genomic DNA extracted from muscle tissue of two road-killed pine martens with a standard phenol–chloroform procedure. Approximately 5 μg of DNA was digested with *Rsa*I and ligated to a double-stranded adaptor made of Oligo A (5′-GGC CAG AGA CCC CAA GCT TCG-3′) and Oligo B (5′ PO4- GAT CCG AAG CTT GGG GTC TCT GGC C -3′) oligonucleotides (Edwards et al. 1996). DNA fragments between 500 and 1,500 bp were eluted from a 1.5% agarose gel and hybridized to a mixture of $(CA)_{14}$ and $(GA)_{14}$ biotinylated probes at a final concentration of 4 μM each and 1 mg of streptavidin-coated magnetic beads (Roche). Microsatellite enriched DNA was recovered in a magnetic stand (Dynabeads), PCR amplified using the Expand Long Template Enzyme mix (Roche) and cloned using the StabyCloning kit (Delphi Genetics) as described in Ciofi et al. (2009). A total of 288 clones were harvested, boiled for 10 min in DNase/Rnase-free distilled water and cycle-sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were

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Table 1 Characteristics of 13 microsatellite loci developed for the European pine marten

Locus	Genbank accession number	Repeat motif	Primer sequence (5′-3′)	T_a (°C)	N_a	Allele size range (bp)	H_E	H_O
Mar02	HM626371	$(CA)_{17}$	F: FAM-CCCTCCTTTTCTTTTTCTTTCC	50	3	150–156	0.624	0.571
			R: CCGTTCTCTGAGTGAAATGC					
Mar06	HM626372	$(CA)_{14}(CT)_{13}$	F: NED-TGTTCAAACCAGGATTACAGC	50	4	219–227	0.680	0.381*
			R: CAAACATTCCCCCAGACG					
Mar08	HM626373	$(CA)_{22}$	F: FAM-CCCTTTAGTTGGCACAGTCC	55	7	144–158	0.826	0.905
			R: CTTTGGCATGAGTCATTTGG					
Mar14	HM626374	$(TC)_{11}AT(CA)_{15}$	F: FAM-GGATTCATGCAGTCAAGAACAG	52	3	223–229	0.608	0.476
			R: CTCTGGGTTGTGAGATCAAGC					
Mar15	HM626375	$(CT)_{12}(CA)_{11}$	F: VIC-TAAGTGGTCCCATCCACACC	55	2	181–183	0.400	0.238
			R: CCAAATGGACATGTAATGTAGGC					
Mar19	HM626376	$(CT)_{15}(CA)_8$	F: FAM-GAAGTAGTCCAAGTGTCCATCG	55	3	185–197	0.573	0.333
			R: TTGTCTTTCCCTGACTTATTTGG					
Mar21	HM626377	$(CA)_{24}$	F: NED-ACATGCATACCTCCCAGACC	55	8	159–183	0.844	0.714
			R: TTTGCTTCCTCCATCTCTCC					
Mar36	HM626378	$(CA)_{24}$	F: PET-TGAGTTTGGTGGGAGAGAGG	55	8	218–248	0.856	0.714
			R: TTCACTGCCAATATTATCTTCTCAAG					
Mar43	HM626379	$(CA)_{26}$	F: VIC-CTTGTCACCCCAGGAGAGG	55	7	123–169	0.573	0.524
			R: CCTAAGCCCAAATCTAAGTGC					
Mar53	HM626380	$(CA)_{18}$	F: NED-TCTCCAGCATTTACCTTTACCC	52	7	238–254	0.777	0.850
			R: GAACAGCCAACCCCATACC					
Mar56	HM626381	$(CA)_{21}$	F: NED-TCTGCACTTAACCCCTCTCC	55	5	209–217	0.715	0.619
			R: AGGGCCATTTGTCTCTTGC					
Mar58	HM626382	$(CA)_{12}G(CA)_7$	F: PET-GTCCCAAATGTTGCACTGG	50	10	231–257	0.813	0.762
			R: CAAAAGACAGGGAGGTGTGG					
Mar64	HM626383	$(CA)_{21}$	F: PET-GGCCCCAAAGTCTTACAGTTC	55	6	171–191	0.784	0.619
			R: CGTTTTGAATCATGCTGTGG					

Fluorescent dyes are reported for each forward primer sequence. T_a, annealing temperature; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; * significant heterozigosity deficiency ($P < 0.01$) prior to Bonferroni correction

resolved by capillary electrophoresis in an Applied Biosystems 3100 genetic analyzer and screened for the presence of microsatellites using OLIGOFACTORY (Schretter and Milinkovitch 2006). Sequences were edited using CODONCODE ALIGNER (CodonCode Corporation). One hundred and thirty recombinant DNA inserts had microsatellite sequences, of which 65 had sufficient flanking regions to design primers for PCR amplification. Primers were designed using default parameters in PRIMER3PLUS (Untergasser et al. 2007) and screened for polymorphism on 1% agarose gel using 11 tissue samples from road-killed pine martens and three hair samples plucked from live-trapped animals. Of the 22 primer pairs which amplified consistently, 16 were selected for genotyping using a panel of 21 samples (12 tissues and 9 hairs from different animals) from the province of Siena, Italy. PCR amplification was performed using forward primers labelled with FAM, HEX, PET and VIC fluorescent dyes (Applied Biosystems) in 10 µl total volume containing 10 ng of DNA, 1× reaction buffer, 1.5 mM $MgCl_2$, 300 µM of each dNTP, 0.5 µM of each primer and 0.5 U of Taq DNA polymerase (Invitrogen). Thermal profiles consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 40 s at 94°C, annealing for 40 s at locus-specific temperature (Table 1) and extension of 60 s at 72°C, with a final extension step of 5 min at 72°C. The amplicons were resolved on an Applied Biosystems 3100 genetic analyser and allele sizes scored against a GeneScan 500 LIZ size standard (Applied Biosystems) using GENEMAPPER 4.0. Microsatellite genotypes from hair samples were obtained using a multiple-tube approach (Taberlet et al. 1996). After three PCR replicates, an allele was accepted when scored at least twice. Samples were scored as homozygotes after eight positive and consistent amplifications of the same allele.

Three microsatellite loci were monomorphic. For each of the other 13 polymorphic loci, scoring error due to stuttering, allele dropout and evidence for null alleles were tested using MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004). Observed and expected heterozygosity, departure from Hardy–Weinberg equilibrium and genotypic linkage disequilibrium were assessed using GENEPOP version 4.0 (Rousset 2008). The mean number of alleles per locus was 6 (range 2–10) and mean observed and expected heterozygosity were 0.610 and 0.698, respectively (Table 1). No significant linkage disequilibrium and no evidence of scoring errors due to stuttering or large allele dropout were observed. Departure from Hardy–Weinberg equilibrium was detected at locus Mar06 ($P < 0.01$), which had evidence of null alleles. This deviation was not significant after applying a sequential Bonferroni correction to adjust critical probability values for multiple tests (Sokal and Rohlf 1994). This set of polymorphic microsatellite markers will be useful to assess patterns of population structure and dispersal of European pine martens in woodlands and fragmented habitats. Positive PCR and allele scoring of hair samples is of particular relevance for populations genetic studies and presence/absence surveys based on non-invasive sampling which have so far relied upon cross-specific marker amplification (e.g. Mullins et al. 2010).

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