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Thirty-one short red fox (*Vulpes vulpes*) microsatellite markers

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Thirty-one short red fox (*Vulpes vulpes*) microsatellite markers

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Running title: Red fox microsatellites

Abstract

Expanding populations of nonnative red foxes potentially threaten native red foxes with displacement or hybridization. Understanding and managing this problem requires a large suite of high-resolution nuclear markers capable of efficiently amplifying noninvasively acquired DNA. Here, we report on primers and multiplex assays for 31 short microsatellite loci and a sex marker based directly on red fox flanking sequence. Based on screening with 31 foxes, 31 microsatellite loci had an average of 6.3 alleles (range = 2-11) and expected heterozygosity of 0.68 (range = 0.14-0.86).

Our objective was to design primers and multiplexes to amplify short microsatellite (and one sex marker) amplicons in noninvasively acquired red-fox (*Vulpes vulpes*) DNA samples. We first designed external sequencing primers from dog flanking sequences around 37 published dog microsatellite loci and one sex marker, a portion of the canine amelogenin gene with homologues of differing length on X and Y chromosomes (Supplemental Information Table 1). We used the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu>; Karolchik et al. 2003) and PRIMER3 software (<http://frodo.wi.mit.edu/primer3/>) for this purpose.

Next, we used these external primers to amplify and sequence 4 red foxes at each locus. To insure that subsequently designed internal primers (i.e., genotyping primers) would be conserved across the species, we chose these foxes from distant portions of the species' range (Sweden, Idaho, California Sacramento Valley, and California nonnative). We extracted DNA from blood or muscle tissue with the Qiagen DNeasy tissue kit (Qiagen, Hilden, Germany). We PCR amplified fragments in 23 μ l reactions containing primers (0.2 μ M), 30-100 ng DNA, dNTPs (0.2mM), 1x Abgene PCR buffer IV, MgCl₂ (2.8 mM), 1x bovine serum albumen, and 1

U Abgene *Taq* polymerase. Thermal profile consisted of 94°C for 10 min, 85°C for 10 min (during which *taq* and buffer mix was added for a hot start), 16 cycles of 94°C for 1 min, 64°C to 48°C touchdown for 30 sec, 72°C for 30 sec., followed by 20 cycles of 94°C for 1 min, 48°C for 30 sec., 72°C for 30 sec., and a final extension for 4 min at 72°C. Products were purified using Millipore's Multiscreen PCR_{u96} filter kit and sequenced in both forward and reverse directions using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) reagents and protocols. Sequencing products were purified using Millipore Montage SEQ₉₆ cleanup kit and electrophoresed on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA.). Red fox fragments were aligned, both forward and reverse, and with the dog primer sequences in Sequencher 4.8 (Gene Codes Corp. 2007) and checked by eye to determine homology between species and conservation among red foxes. Sequences were accessioned in Genbank (Nos. GU179019--GU179058).

Based on these comparisons, genotyping primers were re-designed as necessary (using PRIMER 3 software). All candidate primer-pairs were assessed for multiplex combinations using program MultiPLX (Kaplinski et al. 2005). Ultimately, 31 loci (and 5 previously published markers; Wandeler and Funk 2005), plus the sex marker (K9-AMELO) were pooled into 5 multiplexes. Microsatellites were screened against 31 red foxes and the sex marker was tested on 25 females and 36 males, all from California. DNA was extracted as described above. Forward primers were fluorescently labeled (6-FAM, VIC, NED, PET; Applied Biosystems). Polymerase Chain Reaction was carried out using Qiagen multiplex PCR kit according to manufacturer recommended protocols (including Q-solution). Products were electrophoresed on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA. Alleles were scored relative to an internal size standard, Genescan 500 LIZ (Applied Biosystems), using Strand

version 2.2.30 (Veterinary Genetics Laboratory, University of California, Davis). We screened loci for polymorphism, linkage disequilibrium and deviations from Hardy-Weinberg equilibrium (HWE) using Genepop on the web (<http://genepop.curtin.edu.au/>), and (when loci were out of HWE) for evidence and estimated frequencies of null alleles using Microchecker (VanOosterhout et al. 2004).

For 6 of the 31 microsatellite loci, and the sex marker (K9-AMELO), no re-design of existing dog primers was necessary (Table 1). However, 25 microsatellite loci required at least one of the primers to be redesigned based directly on conserved red fox flanking sequence. Screening revealed three microsatellite loci to have significant deviations from Hardy-Weinberg equilibrium after sequential Bonferroni corrections (Table 1). These loci (C08.618, CPH18, FH2380) each showed evidence of a null allele, estimated at 20, 14, and 25% frequency, respectively. Additionally, 3 pairs of loci exhibited significant linkage disequilibrium after sequential Bonferroni corrections: REN247M2 and FH2088, CPH18 and AHT133, and AHT137 and FH2054. Microsatellite markers were highly polymorphic, with H_E averaging 0.68 (range = 0.14-0.86). The number of alleles per locus averaged 6.3 (range = 2—11). The sex marker accurately typed all 61 known-sex individuals with females homozygous for a 216 bp allele and males having one 202 bp and one 216 bp allele.

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Table 1. Characterization of 31 red fox microsatellite loci and one sex marker, indicating whether primers necessitated re-designing (N), fluorescent dye, multiplex set (M), and screening of 31 red foxes for number of alleles (NA), size range, expected heterozygosity (He), observed heterozygosity (Ho), and polymorphic information content (PIC).

Locus ^a	Repeat motif	Primer sequence	Dye	M	NA	Size range (bp) ^b	He	Ho	PIC
AHT121	(CA) ₂₂	F-TATTGCGAATGTCACTGCTT R-ATAGATACACTCTCTCTCCG	VIC	5	9	85-111	0.82	0.81	0.77
AHT133	(CA) ₂₃	F-AGCCCTGAGTTTGCTACATTT (N) R-GCAGGAAGAGGTGCAATCTC	PET	2	5	154-168	0.65	0.46	0.59
AHT137	(CA) ₂₁	F-TACAGAGCTCTTAAGTGGGTCC R-CCTTGCAAAGTGTCATTGCT	VIC	4	8	123-149	0.76	0.6	0.71
AHT171	(CA) ₁₃	F-AGGTGCAGAGCACTCACTCA R-CCTCAAACCCAGGTGAAGC (N)	PET	3	5	155-167	0.70	0.64	0.62
C01.424	(GT) ₁₁	F-AGCCTAGCTTACTGCCCTGG (N) R-TCCTTTGGTTTTTAGCAGGG	PET	1	4	179-185	0.69	0.77	0.63
C04.140	(GA) ₃ (GT) ₁₉	F-CAGAGGTGGCATAGGGTGAT R-TCGAAGCCCAGAGAATGACT	NED	1	8	139-161	0.83	0.83	0.79
C08.618	(GT) ₁₈	F-CAACCCAGGGTGGGAAGC R-GCCCAGAATCCATTGAGAAA (N)	6-FAM	2	7	190-202	0.83	0.48*	0.78
CPH2	(GT) ₁₉	F-TTCTGTTGTTATTGGCACCA (N) R-TTCTTGAGAACAGTGTCCTTCG	NED	2	8	93-111	0.79	0.71	0.74
CPH3	(TC) ₁₇	F-CAGGTTCAAATGATGTTTTAAG (N)	NED	4	7	158-174	0.74	0.56	0.69

		R-TTGACTGAAGGAGATGTGGTAA								
CPH7	(CA) ₁₂	F-ACACAACCTTTCCATAATACTTCCCA R-ATCAATGCTCTCCTCCCCAG	NED	3	4	154-176	0.40	0.35	0.34	
CPH8	(CA) ₂₁	F-TGCTCAATTGATTAGTCCGTCT (N) R-TAGATTTGATACCTCCCTGAGTCC	NED	5	8	168-188	0.76	0.61	0.72	
CPH11	(TA) ₈	F-GTTAATGTTTCTCCGATGTTTACA (N) R-GAAAGCCAAGCATGACTAGG	6-FAM	3	3	114-118	0.53	0.53	0.43	
CPH18	(AC) ₁₁ C (AC) ₁₂	F- CAGAGATACGTCTTGACACTAGCAGA R-GGCCATTCTGAAACACTTG (N)	6-FAM	3	8	168-192	0.72	0.52*	0.68	
CXX0279	(CA) ₉ G (AC) ₁₁	F-TGCTCAATGAAATAAGCCAGA (N) R-GAATTTGATTACTCTGGAAAACCTCC (N)	PET	4	5	95-107	0.67	0.50	0.61	
FH2001	compound	F-TCCTCCTCTTCTTTCCATTGG R-TGCATTCTTCTTGATGCTT (N)	6-FAM	2	4	132-150	0.22	0.15	0.20	
FH2004	(TTTC) ₄ (T) ₁₅	F-CTAAGTGGGGAGCCTCCTCT R-ACTGTGACCTACTGAGGTTGCA	VIC	1	3	212-218	0.57	0.63	0.49	
FH2010	(GAAT) ₉	F-GAATGGAACAGTTGAGCATGC (N)	6-FAM	1	5	216-236	0.70	0.64	0.63	

		R-CCCCTTACAGCTTCATTTTCC							
FH2054	compound	F-GCCTTACTCATTGCAGTTAGGG (N)	NED	2	7	142-198	0.75	0.82	0.71
		R-ATGCTGAGTTTTGAACTTTCCC							
FH2088	(ATGA)8 (ATAA)18	F-GAGCCTGCTTCTCCCTCTG (N)	VIC	1	4	115-131	0.62	0.54	0.56
		R-TAGGGCATGCATATAACCAGC							
FH2289	(CTT)2 (A)11	F-CATGGTCTCAGGATCCTAGGA	NED	1	2	209-213	0.14	0.07	0.12
		R-CTAAGCATTCTCTCTGATGGTCTT							
FH2328	compound	F-TCCAGGTAGTTTTCAGAAATGC (N)	VIC	2	9	131-167	0.83	0.84	0.79
		R-CAGCCTCAAGTCCCACAACCT (N)							
FH2380	(TTTC)14	F-TTCTGGAATTCCCTATTCATGG	6-FAM	1	6	149-181	0.73	0.33*	0.68
		R-CATTTTTAAAAGGAATGCTCTATCA (N)							
FH2457	compound	F-AACCTGGTACTTTGAATTTGCA	VIC	2	11	280-323	0.74	0.47	0.70
		R-AGAATGGATGGAAGCAAGGA (N)							
FH2848	(GT)16	F-CAAACCAACCCATTCCTC	VIC	3	6	221-235	0.74	0.71	0.69
		R-GTCACAAGGACTTTTTCTCCTA (N)							
		R-TTCCCATTTAATTGCTTCT (N)							
INU030	(CA)10	F-GGCTCTGTGCTCAAGTCTGT (N)	6-FAM	5	3	127-133	0.53	0.35	0.41
		R-CATTGAAAGGGAATGCTGGT							
INU055	(CA)13	F-CCAGGTGTCCCTATCCATCT (N)	NED	4	8	202-224	0.86	0.79	0.82
		R-GCACCCTTTGGGCTCCTTC							

REN105L03	(CA)21	F-GGTGCCTGACAAGATGGAAT (N) R-GAGATTGCTGCCCTTTTTACT (N)	VIC	5	9	161-177	0.84	0.73	0.80
REN162C04	(CA)22	F-AAAATCAGCATTATTCTTTCCACA (N) R-TGGCTGTATTCTTTGGCACA	VIC	4	8	174-188	0.75	0.63	0.69
REN169O18	(TG)22	F-TAGCAAAACCCCCAACTCAC (N) R-ACTGTGTGAGCCAATCCCTT	PET	4	6	187-199	0.79	0.76	0.75
REN247M23	(AC)18	F-TGACAACACCAAGGCTTTCC (N) R-AATCCAATCTGGGGATTGAA (N)	6-FAM	5	7	158-176	0.64	0.59	0.60
REN54P11	(GGT)2 (GT)20	F-TCCCTCTGGCTCAGTTAGAA (N) R-ATCAGCATCACCTGGGAACA (N)	PET	3	9	189-219	0.77	0.73	0.73
K9-AMELO	-- ^c	R-GACAAGGCCAGGCAATACAGT (N) F-GTGCCAGCTCAGCAGCCCGTGGT R-TCGGAGGCAGAGGTGGCTGTGGC	NED	3		202, 216			

^aPrimers for dog homologues were previously published as follows: AHT121, AHT133 (Holmes et al. 1995); CPH2, CPH3, CPH7, CPH8, CPH11, CPH18 (Fredholm and Winterø 1995); AHT137, AHT171, C01.424, C04.140, C08.618, FH2001, FH2004, FH2010, FH2054, FH2088, FH2289, FH2328, FH2380, FH2457, FH2848, REN105L03, REN162C04, REN169O18, REN247M23, REN54P11, REN64E19 (Breen et al. 2001); CXX0279 (Ostrander et al. 1993); INU005, INU030, INU055 (Ichikawa et al. 2002); K9-AMELO (E. Wictum, UC Davis, Veterinary Genetics Laboratory, unpublished data).

^bsize ranges (Min, Max) do not include the additional 18-bp stemming from the M13 tag.

^cK9-AMELO is a sex marker and does not contain microsatellites; Y-chromosome allele is 202bp and X-chromosome allele is 216 bp.

*Test for HWE indicated Sequential Bonferroni-corrected $P < 0.05$

Note: multiplexes also included the following additional loci from Wandeler and Funk (2005): CXX-468, CXX-462 (multiplex 3), AHT-142, CXX-374 (multiplex 4), CXX-402 (multiplex 5).

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