GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Development of novel polymorphic microsatellite markers for the silver fox (*Vulpes vulpes*)

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Genet. Mol. Res. 14 (2): 5890-5895 (2015)
Received July 21, 2014
Accepted January 21, 2015
Published June 1, 2015
DOI http://dx.doi.org/10.4238/2015.June.1.6

ABSTRACT. The silver fox (*Vulpes vulpes*), a coat color variant of the red fox, is one of the most important fur-bearing animals. To date, development of microsatellite loci for the silver fox has been limited and mainly based on cross-amplification by using canine SSR primers. In this study, 28 polymorphic microsatellite markers were isolated and identified for silver fox through the construction and screening of an $(AC)_n$-enriched library. The number of alleles per locus ranged from 2 to 8 based on 48 individuals tested. The expected and observed hetero-

zygosity and polymorphism information content per locus ranged from 0.2544 to 0.859, 0.2083 to 0.7917, and 0.2181 to 0.821, respectively. The polymorphic markers presented in this study may be useful for future analysis of the genetic diversity and population structure of farmed silver fox and wild red fox.

Key words: Silver fox; Microsatellite marker; Genetic polymorphism

INTRODUCTION

The red fox (*Vulpes vulpes*) belongs to the Canidae family and is the most widely distributed terrestrial carnivore in the world (Larivière and Pasitschniak-Arts, 1996). The silver fox, a farmed coat color variant of the red fox, has been domesticated for animal behavioral studies (Statham et al., 2011; Kukekova et al., 2012) and raised to provide fur for the clothing industry (Nowacka-Woszuk et al., 2013).

Microsatellites, also known as simple sequence repeats (SSRs), are short tandem repeats 1-6 bp in length (Zhao and Kochert, 1993). Microsatellite markers have been widely used in population genetic analysis due to their high degree of polymorphism, co-dominance, and their abundance in the eukaryotic genome (Sha et al., 2009; Ma and Chen, 2011). To date, development of microsatellite loci for the silver fox has been very limited and mainly based on cross-species amplification with canine SSR primers (Kukekova et al., 2004; Sacks and Louie, 2008). In the present study, we developed 28 polymorphic microsatellite markers for silver fox from a microsatellite enriched library.

MATERIAL AND METHODS

Genomic DNA from the muscle tissue of 48 farmed silver foxes was isolated using the standard proteinase K/phenol extraction protocol (Sambrook and Russel, 2001). A partial DNA library enriched for $(AC)_n$ motifs was constructed as described by Novelli et al. (2006) with modifications. Briefly, the genomic DNA from a male individual was digested with the *Sau*3A I restriction enzyme. Fragments ranging from 300 to 1000 bp were recovered and ligated with adapters (Linker1: 5'-PO_4-GATCGCAGAATTCGCACGAGTA CTAC-3'; Linker2: 5'-GTAGTACTCGTGCGAATTCTGC-3'). The fragments were enriched by hybridizing to an $(AC)_{13}$ biotin-labeled probe and separated with streptavidin magnetic beads (Promega, Madison, USA). The amplified products from the Linker2 primer were cloned into a pMD18-T vector (Takara, Dalian, China) and transformed into *E. coli* DH5α competent cells. Clones containing inserts were sequenced by Sangon Biotech (Shanghai, China).

Primer pairs were designed according to the flanking DNA sequences of the repetitive region using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA). A M13 (-21) tail (5'-TGTAAAACGACGGCCAGT-3') was added to all the for-

ward primers (Schuelke, 2000). The universal M13 (-21) primer was fluorescently labeled (FAM, HEX, or TAMARD). To evaluate the PCR primers and amplification conditions, preliminary analyses were first conducted with a sample of 4 individuals. The polymorphic information for those loci that showed specific amplification patterns was assessed in 48 farmed silver foxes following the protocol provided by Schuelke (2000) with modifications. PCR was conducted in a total reaction volume of 25 μL containing approximately 10 ng genomic DNA, 1X *Taq* polymerase buffer with Mg^{2+}, 0.1 mM of each dNTP, 0.5 U *Taq* polymerase (Takara), 0.25 μM M13 (-21) tailed forward primer, 1 μM M13 (-21) fluorescently labeled tag primer, and 1 μM reverse primer. PCR amplification was conducted on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with the following condition: 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 20 min.

The fluorescently labeled products were separated on an ABI 3730 DNA sequencer in conjunction with the GeneScan-500 internal size standard (Applied Biosystems). Allele size was estimated using the GeneMapper® software version 4.0 (Applied Biosystems). The polymorphic parameters for each locus, including number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E) and the polymorphism information content (PIC), were assessed using the CERVUS 2.0 software (Marshall et al., 1998). Deviations from Hardy-Weinberg equilibrium were evaluated using the GENEPOP software (Raymond and Rousset, 1995).

RESULTS AND DISCUSSION

Sequencing analysis revealed that out of 142 recombinant clones, 113 clones contained more than five CA or TG tandem repeats. Each colony was given a name consisting of the prefix VVM (*V. vulpes* microsatellite) followed by a number. The number of CA or TG repeats in these clones ranged from 4 to 20. Of the 113 clones, 78 had perfect motifs, 20 had imperfect motifs, and 15 had compound repeat motifs.

Fifty-eight primer sets were designed for microsatellite sequences, which contained at least 8 repeats and possessed sufficient flanking sequences suitable for primer design. Thirty-three loci produced specific products while other primers showed multi-banded patterns or non-specific amplification. Of these 33 loci, 28 exhibited polymorphisms in the 48 individuals tested. The primer sequences, motif information, number of alleles, PCR product size, and the GenBank accession No. of the 28 loci are shown in Table 1. The number of alleles per locus ranged from 2 to 8. The H_E and H_O per locus ranged from 0.2544 to 0.859 with a mean of 0.6371 and from 0.2083 to 0.7917 with a mean of 0.5856, respectively. The PIC ranged from 0.2181 to 0.821 with a mean of 0.5683. None of the loci showed significant deviations from Hardy-Weinberg equilibrium in the population tested.

In summary, the 28 polymorphic microsatellite loci described in the present study will provide useful tools to estimate the population genetic structure and diversity of the farmed silver fox and wild red fox in the future.

Table 1. Characteristics of the 28 polymorphic microsatellite markers developed for the silver fox.

Locus	Primer sequences (5' - 3')	Repeat motif	N_A	Size range* (bp)	H_E	H_O	PIC	Accession No.
VVM 148	F: CCTAACTTCCAACCTGAAATACTCT R: GATTTTATTACTACATGTTCCCTTG	$(TG)_{11}(AC)_5$	4	135-145	0.6002	0.5833	0.5047	JN831722
VVM 219	F: ACAAGGGGCATAACCTGGAAGT R: TCCCAGATATCAAGACTCCCTAG	$(TG)_{19}(AG)_{13}$	6	143-166	0.5816	0.4583	0.5373	JN831723
VVM 33	F: CAATCAATCTGAGCACCACAATC R: TAGATGAGGGGAATGTGAGGAAC	$(TG)_{12}$	5	164-184	0.6735	0.6400	0.6037	JN831724
VVM 812	F: GCAAATGGCAACATCTCCTT R: ATGGAAGCAGCCCAAGTGTG	$(AC)_{18}$	5	142-176	0.5434	0.4583	0.4891	JN831725
VVM 85	F: GATAGTAGCAATTAAGTTTTCCCAG R: TTGAGACCATGAGGAGGTAGGA	$(AC)_{16}$	6	156-170	0.6996	0.5600	0.6435	JN831726
VVM 509	F: GGTTGCTGGTAACAGTAACAAGACA R: GAGTGCTTTCATTCTTAGGGAGTG	$(AC)_9$	2	319-327	0.5027	0.5417	0.3711	JN831727
VVM192	F: GTGTCCTTGCTAACAAAATGCTG R: CCACCTTTAGATGAGATTCTGTTTC	$(CA)_{17}$	3	316-322	0.5293	0.4583	0.4624	JN831728
VVM 224	F: TTGGAAAGCATCTAGTTCAGTCA R: CTCAGCCTCTCTTAAAATGGTTC	$(CA)_{14}$	4	198-204	0.6754	0.7391	0.5931	JN831729
VVM 39	F: ACTACGGCTTTCATAATAGCCT R: TGTATACCCCTCTGCATGGTT	$(TG)_{19}$	2	195-205	0.5106	0.5833	0.3750	JN831730
VVM 104	F: TTTGACCGAGGAGTTAGTGATGC R: CTAAGTCAGCCTTGGTTTTCACA	$(TG)_{17}(AG)_{14}$	4	201-209	0.6950	0.6667	0.6218	JN831731
VVM 838	F: CTTCCTTGGTCCCAGAGTCAG R: AGCGATGTCACCTTCCGAGA	$(TG)_3TT(TG)_{14}TT(TG)_5$	7	191-223	0.4326	0.3750	0.4079	JN831732
VVM 25	F: AAGGGGCACAGGTCTAAGCA R: CATGTTGTAGCAAATAGCAGGA	$(CA)_{18}$	5	205-213	0.6871	0.5833	0.6178	JN831733
VVM 831	F: CAAGCGTTAGTAGCAGGATTTTC R: AGAGGCTCATCACTTGGGACA	$(TG)_{12}$	3	300-311	0.6161	0.5000	0.5298	JN831734
VVM 100	F: CCTCGTGAAACTTTATTAACCAACA R: TGCTGAAGGAAGAAAAGAGGTC	$(CA)_{17}$	4	285-294	0.6587	0.6250	0.5876	JN831735
VVM 190	F: ACATTTGAGGGTCAGTGTAAGAG R: CATAATGTCACTCCAGCAACC	$(TG)_{17}$	3	227-231	0.6693	0.5833	0.5817	JN831736
VVM 246	F: ATCTGGTTCTTATTTTTGCTCTGA R: GAAAGACTGAAGAAATCACAGGACT	$(TG)_{17}$	4	226-238	0.7261	0.6667	0.6547	JN831737
VVM 63	F: AAGTCCTTTGCGTGGTTCTTCTG R: TCGACTGCACTCTAGCCAACTCT	$(AAAT)_5AAG(TG)_{17}$	7	232-244	0.8590	0.7917	0.8210	JN831738
VVM 128	F: TGGCAAGAGGAGCAGACATTTC R: TGAAGGTAGGAACAATCCCCAC	$(GT)_{15}$	2	240-250	0.4317	0.4583	0.3741	JN831739
VVM 529	F: GGCAGTAAAATGTGAAACAACTAATG R: ATCTTTGCTCTTCCTTAAACCCA	$(TG)_5C(GT)_{15}$	7	272-294	0.8440	0.7500	0.8028	JN831740
VVM 189	F: AGTTTAAGGTTGTACAGATTTGAGTT R: GTAATGTTCCAGACAGGAGGATGT	$(TG)_{19}$	6	244-260	0.7828	0.7500	0.7346	JN831741

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Table 1. Continued.

Locus	Primer sequences (5' - 3')	Repeat motif	N_A	Size range* (bp)	H_E	H_O	PIC	Accession No.
VVM 235	F: CCTTCTTGTTTCCTGTTAGATGCA R: GTCTGTCTCTCAACACACTCATAAC	$(TG)_{11}$	3	255-259	0.5505	0.5000	0.4817	JN831742
VVM 508	F: GATACTGAAGGGGAACTCCATAC R: TCTGTCAACACCTCAAAGATAGC	$(TG)_{20}(AG)_{14}$	4	252-262	0.6711	0.6667	0.5873	JN831743
VVM 238	F: CATCTGCTCTATGTATGTGGGTC R: TTGCGTTGCCTGAGGCTTTC	$(AC)_{16}AATG(CA)_5$	5	247-265	0.7473	0.6667	0.6869	JN831744
VVM 81	F: ACTGAATTGCATGGACTCTGAGA R: GCTGAATGGATGAAAGGTTGAC	$(GT)_{17}A(TG)_4$	5	272-292	0.7828	0.6250	0.7293	JN831745
VVM 844	F: TGTGTGTCTATGTGTCTGCTTTGA R: GCCAGGGAAAGTGAGCAGAG	$(TG)_{20}$	8	263-291	0.7863	0.7500	0.7357	JN831746
VVM 213	F: AGGAGTGGGCTTGCTGTTTG R: CTTAGGTTCTCTTAGTTTTGTTGGT	$(AC)_{18}$	2	273-275	0.4965	0.4167	0.3680	JN831747
VVM 124	F: TGAACACGCCCTCTGCTACAC R: TCTCCTGGTATTCCTGTGCCT	$(CA)_{12}$	2	272-274	0.2544	0.2083	0.2181	JN831748
VVM 828	F: GACTATGACAATGGGACTGTAAGGT R: CTCTAACTTTGCCAATGGTGAA	$(TG)_{17}$	7	270-288	0.8324	0.7917	0.7923	JN831749

F = forward; R = reverse; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content; *size ranges include the additional 18 bp from the M13 (-21) tag.

ACKNOWLEDGMENTS

Research supported by projects of the National Natural Science Foundation of China (#31072018).

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