Supplementary Note

Conditions for M13-Tagged Primers

M13-tagged primers were tested in two male Novosibirsk foxes in an initial screening phase without fluorescence (i.e. using M13 without an attached fluorescent probe). The conditions used here were for a reaction at 15 µL total volume that included 7.5 µL of GoTAQ master mix (Promega, Madison, WI) and primers at Concentration A (0.15 µL of fluorescently tagged M13 at 20 mM, 0.15 µL of the reverse primer at 20 mM, and 0.15 µL of the M13-tagged fluorescent primer at 10 mM). The standard M13 protocol was used on the thermocycler, as described below.

Results were visualized on an acrylamide gel and visually screened for primer-dimers. In cases where the bands were faint, alternative conditions were tested using a gradient. This resulted in the selection of modified protocols VVY7 and VVY13, as described below. Primers for which the PCR failed were excluded from further analysis.

In the second phase of screening, the fluorescently tagged M13 was used and the products were sent for fragment analysis. In cases where peaks were low, the PCR was re-run at the same volume but with primers at Concentration B (0.30 µL of fluorescently tagged M13 at 20 mM, 0.30 µL of the reverse primer at 20 mM, 0.30 µL of the M13-tagged fluorescent primer at 10 mM). The products were then sent to the Keck Center at the University of Illinois at Urbana-Champaign for fragment analysis and evaluated to determine whether peaks were higher.

Thermocycler Protocols

Standard M13 Protocol:

96° 2’
96° 20” 56° 20” 72° 40” (30 cycles)
96° 20” 53° 20” 72° 40” (8 cycles)
72° 20’
10° ∞’

Touchdown M13 Protocol:

96° 2’
96° 20” 64° 20” 72° 40”
96° 20” 63° 20” 72° 40”
96° 20” 62° 20” 72° 40”
…
96° 20” 45° 20” 72° 40”
72° 20’
10° ∞’

Special Protocol for VVY7:

96° 2’
96° 20” 54° 20” 72° 40” (30 cycles)

96° 20” 53° 20” 72° 40” (8 cycles)
72° 20’
10° ∞’

Special Protocol for VVY13:

96° 2’
96° 20” 57.7° 20” 72° 40” (30 cycles)
96° 20” 53° 20” 72° 40” (8 cycles)
72° 20’
10° ∞’

Optimized Conditions

Marker	Concentration (A or B)	Thermocycler Protocol	Fluorescent Dye
VVY3	A	Touchdown	6-FAM
VVY5	A	Standard	NED
VVY7	A	Special Protocol VVY7	NED
VVY8	A	Standard	6-FAM
VVY10*	B	Standard	6-FAM
VVY11	B	Standard	6-FAM
VVY13	A	Special Protocol VVY13	NED
VVY14	A	Standard	6-FAM
VVY15	A	Standard	6-FAM
VVY16	B	Standard	NED
VVY17	A	Standard	6-FAM

*Use of M13 with these primers is not recommended due to excessive banding.

Primers from the Literature

The primers used by Statham et al. (2014) to amplify two microsatellites, other than the fact that a 1-nucleotide change was made to their DogY30_R to match the fox reference genome sequence, were analyzed under the following conditions:

Marker	Concentration (A or B)	Thermocycler Procedure	Fluorescent Dye
Y29	2	Touchdown	NED
Y30	2	Standard	NED

Analysis of VVY10

The forward primer Ib1F was re-ordered with a VIC fluorescent tag already attached (ThermoFisher Scientific, Waltham, MA) to eliminate need for the M13-tag. The PCR conditions were 15 µL total volume with 7.5 µL of GoTAQ master mix (Promega, Madison, WI) and 0.3 µL of each of the tagged forward primer and the reverse primer at 10 mM. While more than one peak was still observed using the directly tagged primers, this shift rendered the genotypes much easier to call across foxes and therefore is recommended for future studies.

Scoring VVY5 and VVY10

At two loci, multiple genotypes were scored at a single locus in males. Zero genotypes were identified in female controls, suggesting that all copies are Y-specific.

VVY5: Up to two alleles observed per fox. Fragments observed to be "short" (less than 225 bp) were coded as VVY5a and any "long" fragments (longer than 227 bp) were coded as VVY5b. This system did not work in every case, because 226-bp fragments co-occurred with both long (230 bp, haplotype O) and short fragments (218 bp in haplotypes H and J and 222 bp in haplotypes I, Q, S and T). However, because fragments of 226 bp occurred only in animals at which two peaks amplified for this primer set, 226 was coded as either short or long according to the other length observed at this locus. Only a single copy was observed in animals with Great Britain haplotypes (including the Maryland fox with haplotype B), suggesting the possibility that the duplication occurred in the North American fox lineage in allopatry. However, there is also only a single copy observed at this locus for the Novosibirsk haplotype N3, which is how haplotype N3 is distinguished from haplotype N2.

VVY10: Up to 3 bands appeared even with the primers directly tagged with fluorescence. These are coded as VVY10a (298-300 bp), VVY10b (302-307 bp, except in *V. lagopus*) and VVY10c (308-314 bp). In foxes carrying haplotypes in the Great Britain cluster (including B, which is find in a Maryland fox), either zero or one allele was called for VVY10. All foxes from the North American cluster carried three distinct alleles at this locus, with the exception of the fox with haplotype N3, for which two alleles were identified.

Additional Canid Outgroups

In addition to the Arctic fox (*Vulpes lagopus*), domestic dog (*Canis lupus familiaris*), grey wolf (*Canis lupus*), and red wolf (*Canis rufus*) were also tested as possible outgroups. Dog samples were from a Rough Collie, an Elkhound, a Shetland Sheep Dog, and a Golden Retriever collected from experimental populations maintained at the University of Pennsylvania and from pet animals. Wolf samples were supplied by the Wolf Park in Battleground, IN. The red wolf sample came from the Rosamond Gifford Zoo in Syracuse, NY. Samples were extracted with a DNeasy Blood and Tissue kit (Qiagen, CA).

Genotyping of the *Canis* species failed for all samples at VVY3, VVY13, and VVY17. None of the loci appeared to be duplicated. Across the remaining 10 loci, three haplotypes were identified (Table 1). Haplotype Canis_1 was found in three grey wolves and the Shetland Sheep Dog. Haplotype Canis_2 was found only in the Golden Retriever. Haplotype Canis_3 was found in the red wolf and two colony dogs (Rough Collie and Elkhound).

Table 1: Genotypes across all markers for the three canine haplotypes identified. Only one allele was observed in the canine species at the loci that are putatively duplicated in some fox (VVY5 and VVY10). Asterisk indicates the only marker for which the M13 tag is not included in the length (i.e. all other markers have an 18-bp addition from the M13).

Haplotype	VVY5	VVY7	VVY8	VVY10*	VVY11	VVY14	VVY15	VVY16	Y29	Y30
Canis_1	204	251	110	307	290	152	175	218	193	399
Canis_2	204	251	110	307	290	152	167	218	?	?

Canis_3	204	251	110	307	290	152	175	218	195	399

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