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Combined use of maternal, paternal and bi-parental genetic markers for the identification of wolf–dog hybrids

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The identification of hybrids is often a subject of primary concern for the development of conservation and management strategies, but can be difficult when the hybridizing species are closely related and do not possess diagnostic genetic markers. However, the combined use of mitochondrial DNA (mtDNA), autosomal and Y chromosome genetic markers may allow the identification of hybrids and of the direction of hybridization. We used these three types of markers to genetically characterize one possible wolf–dog hybrid in the endangered Scandinavian wolf population. We first characterized the variability of mtDNA and Y chromosome markers in Scandinavian wolves as well as in neighboring wolf populations and in dogs. While the mtDNA data suggested that the target sample could correspond to a wolf, its Y chromosome type had not been observed before in Scandinavian wolves. We compared the genotype of the target sample at 18 autosomal microsatellite markers with those expected in pure specimens and in hybrids using assignment tests. The combined results led to the conclusion that the animal was a hybrid between a Scandinavian female wolf and a male dog. This finding confirms that inter-specific hybridization between wolves and dogs can occur in natural wolf populations. A possible correlation between hybridization and wolf population density and disturbance deserves further research.
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Introduction

Hybridization is a natural process that can lead to speciation. It is also an undesirable issue threatening the genetic integrity of endangered species (Arnold, 1997). Detecting the degree or extent of hybridization between species is thus important for evolutionary studies of speciation processes, as well as for conservation biology studies of species potentially in genetic peril. Moreover, being able to detect individual cases of hybridization may be important from a management perspective. Studies on hybridizing species and populations have increasingly sought to use genetic markers that are unique for each taxon (Saetre *et al*, 2001), in some cases combined with morphological characters (Beaumont *et al*, 2001). Also, hybrid populations have been compared to pure populations to infer the degree of gene flow (Reich *et al*, 1999; Madrigal *et al*, 2001). However, given that hybridization is most likely between closely related taxa, in many cases differentiation between hybridizing populations may be primarily in the form of allele frequency differences rather than the frequent occurrence of private alleles. Identifying individual hybrids in such cases may be particularly problematic. The issue of potential hybridization between wolves (*Canis lupus*) and dogs (*C. familiaris*) represents an example of this situation.

Hybridization can occur between many species of the canid family (Gray, 1954; Lehman *et al*, 1991; Mercure *et al*, 1993; Roy *et al*, 1996; Wayne and Brown, 2001) and sometimes threatens the survival of endangered canid species or populations (Nowak, 1979; Wayne and Jenks, 1991; Gottelli *et al*, 1994; Roy *et al*, 1994). The close relationship between wolves and dogs, a consequence of their recent divergence (Vilà *et al*, 1997), suggests that hybridization between these species could be especially common since reproductive isolation may not be completely developed. Wolves coexist with dogs across most of their range.

Wolf populations in Eurasia have become increasingly fragmented during the last centuries (Mech, 1970; Wayne *et al*, 1992). Their numbers have dramatically decreased and in most areas of Europe only small populations survive in close contact with increasing numbers of humans and domestic dogs (Promberger and Schröder, 1992). It is under these conditions that hybridization between wolves and dogs is most likely to occur (Boitani, 1983; Bibikov, 1988; Blanco *et al*, 1992). Boitani (1984) hypothesized that the recovery of wolf populations in Italy could have been the result of hybridization with dogs, and Butler (1994) suggested that

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European wolf populations could be composed mainly of hybrids.

Despite these concerns, a recent review of genetic evidence has suggested that wolf–dog hybridization may not be a threat even in small, endangered wolf populations near human settlements (Vilà and Wayne, 1999). Specifically, the analysis of mitochondrial DNA (mtDNA) suggests that hybridization between wolves and dogs is uncommon, that is, there is no clear evidence of introgression of dog mtDNA into wolf populations, except a few cases in an east European wolf population (Randi *et al*, 2000). However, this infrequent presence of dog mtDNA haplotypes in wolves only implies that offspring of crosses between female dogs and male wolves are uncommon or do not back-cross into wolf populations. The use of mtDNA cannot provide any information about introgression of hybrids of crosses between a male dog and a female wolf. However, pairs composed of a female wolf and a male dog have been observed in Russia, Israel, Italy and Spain (Ryabov, 1985; Randi *et al*, 1993; Vilà and Wayne, 1999; however, see Randi *et al*, 2000) and some recent studies involving nuclear markers have shown that hybridization occasionally occurs in the wild (Andersone *et al*, 2002; Randi and Lucchini, 2002). More detailed genetic studies using a variety of genetic markers and in different populations are thus necessary to conclusively address the issue of wolf–dog hybridization and to understand its directionality and frequency of occurrence. As a result of the current fragmentation of the wolf distribution range into more or less small patches (Promberger and Schröder, 1992), the detection of these inter-specific crosses may be especially troublesome in areas where the arrival of wolves from other populations – likely to be genetically differentiated to some degree – may occur.

Hybridization with dogs could potentially be expected for Scandinavian (Swedish+Norwegian) wolves. This wolf population, presumed extinct during the 1970s, was founded by a very small number of individuals in the early 1980s (Wabakken *et al*, 2001), and by the winter 2001–2002 was about 92–107 animals (Aronson *et al*, 2002). In 1999, a presumed juvenile wolf was found road-killed in southern Norway, close to Oslo. The uncommon morphology of the animal gave rise to questions about its possible hybrid origin. In this study we combined the use of mtDNA, autosomal and Y chromosome markers to analyze the identity of this juvenile canid and we attempt to genetically characterize it as either a pure Scandinavian wolf, a migrant from Finland or Russia, a domestic dog, or a first-generation hybrid between any of these groups.

Materials and methods

Samples

The study focused on two samples from the county of Østfold in southern Norway: sample A was blood from a juvenile individual killed by a car in October 1999 and sample B constituted snow with urine and blood collected at the end of the previous winter, in March 1999. Sample A is derived from the suspected hybrid, while sample B was assumed to correspond to the alpha female in estrus from a wolf pack close to the site where sample A was killed. In the winter of 1998/99, when sample B was collected, she was in estrus but snow tracking suggested that she was not yet paired to a male. Apparently, she was the only wolf in the area. However, during spring 1999 she was sighted with a male wolf and in the summer a litter of at least four pups was detected (Terje Bø, personal communication). As far as is known, this was the first time that this female was breeding.

Samples A and B were analyzed together with DNA samples extracted from muscular tissue of wolves from Scandinavia collected after 1980 ($n=25$), Finland ($n=23$), northwest Russia ($n=24$) Latvia ($n=8$) and Estonia ($n=23$), as well as of 44 domestic dogs. The dog samples correspond to pure-bred Huskies, Eskimo dogs, Akita, Elkhound, Wolfspitz, Great Pyrenees, Kuvasz and German Shepherd dogs. Although the dog samples originated from the USA, we assume that members of the same breeds in different continents will still be more similar to each other than to different populations of wolves. A separate set of 38 male pure-bred Scandinavian dogs from diverse breeds was also genotyped for Y chromosome markers.

Laboratory procedures

DNA was isolated using variations on phenol–chloroform extraction methods (Sambrook *et al*, 1989). For sample B, snow containing urine and blood was centrifuged for over 30 min to concentrate cells before attempting DNA isolation.

Amplification of a 350 base pairs (bp) fragment of the mtDNA control region I was performed via the polymerase chain reaction (PCR) using primers Thr-L 15926 and DL-H 16340 (modified from Kocher *et al*, 1989). PCR conditions and profile were as described in Vilà *et al* (1999). PCR products were sequenced using Big Dye Terminator cycle sequencing chemistry on an ABI 377 instrument (Perkin Elmer), following protocols provided by the manufacturer. Sequences were aligned using the program CLUSTAL W (Higgins *et al*, 1992) and checked by eye. All sequences were compared to each other and to sequences available in GenBank and databases previously developed (based on Ellegren *et al*, 1996; Okumura *et al*, 1996; Taberlet *et al*, 1996; Tsuda *et al*, 1997; Vilà *et al*, 1997, 1999; Pilgrim *et al*, 1998; Randi *et al*, 2000), using the program PAUP*4.0b8 (Swofford, 1998).

A total of eighteen autosomal microsatellites developed for dogs were selected for this study: c2001, c2010, c2017, c2054, c2079, c2088 and c2096 (Francisco *et al*, 1996), vWF (Shibuya *et al*, 1994), u213, u250 and u253 (Ostrander *et al*, 1993), and PEZ01, PEZ03, PEZ05, PEZ06, PEZ08, PEZ12 and PEZ20 (Perkin Elmer, Zoogen; see dog genome map at http://www.fhcrc.org/science/dog_genome/dog.html). In addition, one highly polymorphic Y chromosome microsatellite, MS41B (Sundqvist *et al*, 2001), was analyzed. This marker was only genotyped in the additional set of 38 pure-bred male dogs and the target samples, and the results were compared to results published by Sundqvist *et al* (2001) for north European wolves. PCR products, including one fluorescently labeled primer, were run on an ABI 377 instrument (Perkin Elmer) following protocols provided by the manufacturer. PCR primers, conditions and profile were essentially as in the original reports. The alleles observed for each microsatellite were sized and scored using the software Genescan 3.1 and Genotyper 2.1 (Perkin Elmer). Owing to the small amount and low quality of DNA

extracted from sample B only a limited number of microsatellite amplifications could be successfully performed for this individual.

Data analysis

To study the likelihood of finding one of the observed autosomal genotypes in each one of the reference populations, we used an assignment test (Paetkau *et al*, 1995, 1998; Waser and Strobeck, 1998). This calculates the log likelihood of finding a certain genotype combination in each population and assigns the individual to the population for which it has the highest likelihood. From the moderate number of genotypes gathered from each population ($n = 23$–44), we cannot expect the samples to represent most of the variability in the populations, although the allele frequencies should be well represented. To characterize how well an individual genotype did fit into the distribution of genotypes expected from each population, we generated 1000 synthetic genotypes taking random alleles for each locus according to their frequency. Similarly, we generated populations of 1000 synthetic genotypes of hybrids between dogs and Scandinavian wolves, and between dogs and wolves from neighboring populations (see Thulin, 2000). In these cases, the synthetic genotypes contained one allele derived from each of the two parent populations at each locus. We then calculated the likelihood of assignment to the Scandinavian wolf population. If the likelihood of assignment of a target sample was outside the range observed for the 1000 synthetic genotype combinations, we assumed that the sample did not belong to this population. To standardize the likelihood estimates, the log likelihood of assignment of the target sample to the wolf population was subtracted from the log likelihoods of the synthetic genotypes. After standardizing, the likelihood for the target sample becomes zero. If the value zero lies outside the distribution of assignment likelihoods for the synthetic population (or inside the 2.5% margins at each side of the distribution), the hypothesis that the target sample belongs to that population should be rejected. Since the number of microsatellites successfully scored was different for each target sample, the analyses were redone for each of the target samples including only the loci successfully amplified.

As a complement to the assignment test, we also used a model-based genetic mixture analysis developed by Pritchard *et al* (2000), which is implemented in the program Structure (available at http://www.stats.ox.ac.uk/~pritch/software.html). This program is based on a Bayesian approach and we used it to identify two groups ($K = 2$) in a sample composed of Scandinavian wolves and domestic dogs. Besides this initial classification of each individual sample, we used Structure to estimate the probability that each sample represented an immigrant or had a parent or grandparent that was an immigrant.

Assuming that the female of sample B is the mother of sample A (see below), we deduced the composition of paternally contributed alleles. We constructed a synthetic genotype homozygous for those alleles and calculated its assignment likelihood to different populations. Thus, the likelihood for the paternal haplotype is the square root of the likelihood for the synthetic homozygous individual.

Results

Mitochondrial DNA sequences

The Scandinavian wolf population is fixed for a mtDNA haplotype H1 (Ellegren *et al*, 1996). This variant is also the most common in neighboring populations, present in about 65% of north European wolves, although it is not fixed in any of them (Table 1). Four different haplotypes were observed in Estonia and Finland, and five in Russia. Haplotype H1 has not been reported in domestic dogs (Okumura *et al*, 1996; Tsuda *et al*, 1997; Vilà *et al*, 1997; and complete GenBank searches). Both samples A and B were found to carry the H1 mtDNA haplotype. We thus conclude that the suspected hybrid was either a pure wolf or represented a hybrid with wolf ancestry in the maternal line. However, the geographical origin of this ancestry cannot be revealed by the mtDNA data.

Y chromosome microsatellite

Table 2 shows the alleles observed in one Y chromosome microsatellite (MS41-B) in male wolves from northern Europe and in 38 male dogs. Nine alleles have been observed in wolves: eight of them in the Baltic States (Estonia and Latvia), six in Russia and four in Finland. A total of eight alleles were observed in our sample of domestic dogs, including the two alleles found in Scandinavian wolves and almost all of the alleles observed in other wolf populations.

Among the two target samples, the Y chromosome microsatellite was successfully amplified in sample A only, confirming that this came from a male and supporting the notion that sample B was a female. The allele identified (*2 2 2*) was not found in Scandinavian wolves, but has been seen in other North European wolf populations and in dogs. Thus, this result does not discriminate between a wolf or a dog as the father of sample A. However, it suggests that the father was not a Scandinavian wolf.

Autosomal microsatellites

An assignment test comparing wolves from the Scandinavian population and dogs clearly shows that the allelic distributions allow for distinguishing between them (Figure 1; all dogs are located above the diagonal, indicating a higher likelihood of being dogs than wolves, whereas all wolves are below the diagonal). Figure 1 also includes the target samples. Sample A lies

Table 1 mtDNA haplotypes in wolves from northern Europe and in the target samples

	Populations				*Sample A*	*Sample B*
	Scandinavia	*Finland*	*Russia*	*Estonia*		
H1	25	10	6	19	X	X
H2		2				
H3			3			
H4		10	12	1		
H5			2	1		
H6		1				
H7			1	2		

Table 2 Y chromosome microsatellite alleles (locus MS41-B) observed in male wolves from northern Europe (data from Sundqvist *et al*, 2001), pure-bred dogs, and in the target sample A

MS41-B alleles (bp):	*212*	*214*	*216*	*218*	*220*	*222*	*224*	*226*	*228*
Scandinavia		3			9				
Finland			6	5		2			3
Russia			8	13	1	1	1		2
Baltic States (Estonia+Latvia)	1	5	5	5		5	4	3	3
Dogs		6	2	8	7	3	5	3	4
Sample A						X			

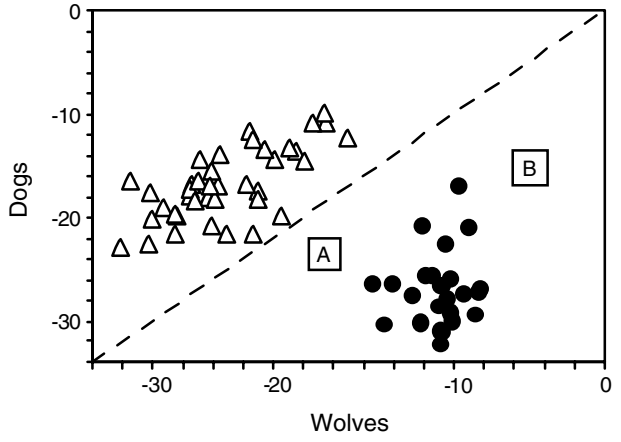

Figure 1 Log likelihood of assignment for dogs (open triangles) and Scandinavian wolves (black circles). The log likelihoods for the two target samples (A, B) are also indicated.

between the distributions of dogs and wolves, a position that would be expected for a wolf–dog hybrid. Sample B appears at the limit of the distribution of wolves. This sample has the highest likelihood, among all animals, of assignment to the Scandinavian wolf population; this extreme position is likely to be a consequence of the low number of microsatellites successfully scored for this individual (11) because of the low quality of the sample (drop of blood in snow). Its likelihood of being a wolf is clearly higher than the likelihood of being a dog.

To analyze if the target samples differed significantly from the distribution of expected haplotypes for either Scandinavian wolves, dogs or F_1 hybrids, we studied the distribution of the log likelihood of assignment to the Scandinavian wolf population of three groups: 1000 synthetic hybrids, 1000 synthetic dogs and 1000 synthetic Scandinavian wolves. Figure 2 (left) shows that the genotype combination of sample A is significantly different from that expected for pure dogs or wolves, but is inside the distribution for F_1 hybrids. Figure 2 (right) indicates that the genotype of sample B is outside the expected distribution for hybrids or dogs, but inside the distribution expected for Scandinavian wolves. A similar analysis shows that none of the target samples can be identified as a wolf immigrant from Finland or Russia (Table 3). We also tested if the assignment likelihoods of samples A and B were outside the expected distribution for an F_1 hybrid between a dog and an immigrant. The target samples were outside the distributions in both cases and thus this possibility could be excluded as well (analyses not shown).

The allelic composition of the two target samples is indicated in Table 4. For 10 out of 11 loci for which genotyping was successful for both samples A and B, sample B is compatible with being the parent of sample A. However, one locus (c2079) excludes this possibility: sample A is heterozygote for alleles 275 and 283, whereas B is homozygote for allele 271. We consider a technical artifact to be the most likely explanation for this non-congruence and that sample B is indeed parent to sample A. The quality and quantity of the DNA extracted from the thawed snow (sample B) might have been so low that allelic dropout has occurred. Allelic dropout, the accidental lack of amplification of one allele, is more common in samples of poor quality (Taberlet *et al*, 1999). This idea lends support from the fact that seven loci failed to amplify for sample B and possibly also from the fact that 10 out of 11 (91%) of the amplifying loci appeared homozygous. The average observed heterozygosity for all Scandinavian wolves for the 18 microsatellite markers was 0.65 (SD = 0.16) and, consequently, for 11 loci typed for sample B we would expect to have around seven heterozygous loci. Unfortunately, the small amount of DNA obtained for sample B did not allow for further amplifications that may have confirmed allelic dropout.

In order to further test if sample B could correspond to the mother of A, we tried to assess how likely is to find a wolf in Scandinavia that is as similar to A as sample B is. We generated 10 000 simulated genotypes for the 11 microsatellite loci successfully amplified in B using the allele frequencies observed in the Scandinavian wolf population. For each locus, we assume that dropout could lead to the amplification by PCR of only one of the alleles, resulting in a false homozygote if the locus was heterozygote. We used a frequency of allelic dropout of 18%, as observed by Lucchini *et al* (2002), for autosomal microsatellites on other low-quality samples of wolves (scats). In spite of the extremely low genetic diversity that characterizes the Scandinavian wolf population (Ellegren *et al*, 1996; Ellegren, 1999), over 90.5% of the simulated genotypes could be excluded as possible parents of A at two or more loci. Sample B, instead, shows incompatibility at only one locus. The evidence that the genotype of B – in spite of the mismatch at locus c2079 (Table 4) – is highly similar to A, together with the evidence provided by field observations suggesting that it could have been the only wolf in the area during the winter before A was born, supports the notion that B could be the mother of A.

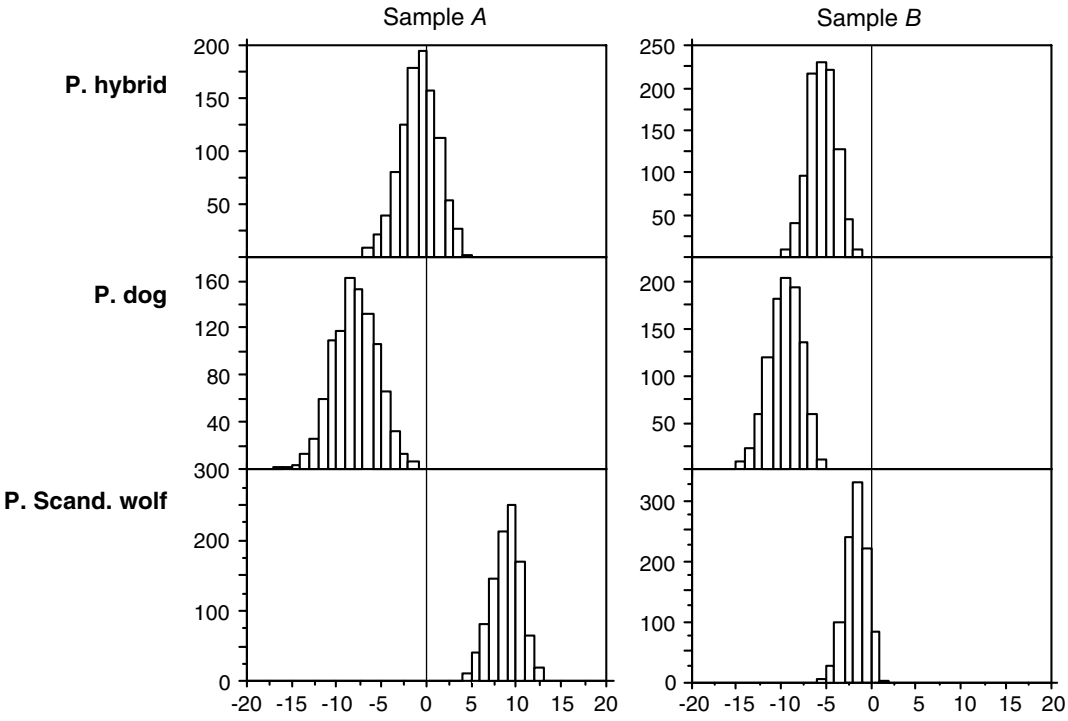

Figure 2 Distribution of the log likelihood of assignment to the Scandinavian wolf population of 1000 synthetic genotypes corresponding to dogs, Scandinavian wolves and F_1 hybrids between dogs and wolves. Values are standardized by subtracting the log likelihood calculated for each target sample. If the value 0 (corresponding to the target sample) is outside the distribution, we can conclude that the genotype of the target sample is unlikely to occur in the dog, wolf or hybrid population.

Table 3 Proportion (*P*) of 1000 synthetic genotypes in which the likelihood of assignment to the respective wolf population is lower than the likelihood of assignment observed for the target samples (A, B and for the synthetic father, see text)

Synthetic population	*Assigned to*	*Sample A*		*Sample B*		*Synthetic father*	
		P	*N*	*P*	*N*	*P*	*N*
1000 Scandinavian Wolves	Scandinavian wolf population	0.000	18	**0.917**	11	0.000	13
1000 dogs	Scandinavian wolf population	1.000	14	1.000	8	**0.256**	10
1000 F_1 hybrids[a]	Scandinavian wolf population	**0.648**	14	1.000	8	0.000	10
1000 Finnish wolves	Finnish wolf population	0.000	18	0.000	11	0.000	13
1000 Russian wolves	Russian wolf population	0.000	18	0.002	11	0.000	13

[a]F_1 Hybrid: Dog × Scandinavian wolf. Figures shown in bold indicate those tests where the sample could not be excluded from the simulated distribution. *N* is the number of microsatellite loci considered for the analysis.

Making the tentative assumption that sample B represents the mother of sample A, we determined the paternally contributed allele at 13 loci (Table 4). Two of the alleles found in A and assumed to come from the father (allele 131 at locus c2088 and 104 at PEZ05) have not been observed before in the Scandinavian wolf population, and other alleles are present in very low frequencies. These alleles were present in wolves from other populations and in dogs. As above, the origin of the paternal haplotype was assessed by comparison with synthetic genotypes (Table 3). The likelihood of obtaining this haplotype from the Scandinavian, Finnish or Russian wolf population is extremely low and outside their expected distribution. Also, this haplotype is not expected from a hybrid between a Scandinavian wolf and a domestic dog. However, the likelihood for the paternal haplotype falls inside the distribution for pure dogs.

Additional support for these results was provided by the model-based method of Pritchard *et al* (2000). All Scandinavian wolves had a probability of at least 0.95 of being classified as pure wolves (the probability was higher than 0.99 for 92% of the wolves). Similarly, all dogs but one had a probability higher than 0.95 of being genetically identified as pure dogs. The target sample B, in spite of its incomplete genotype, had a probability of 0.998 corresponding to a pure Scandinavian wolf. On the other hand, the corresponding probability for sample A was only 0.264. For this sample, the probability of having one dog as parent was 0.402 and the probability of having it as a grandparent was 0.334. The probability of assignment to the dog population was 0.000. Consequently, sample A was likely to have a hybrid origin (probability = 0.402+ 0.334 = 0.736).

Table 4 Microsatellite alleles identified for each target sample

Locus	*Sample A*	*Sample B*	*Paternal allele for sample A*
c2001	149/153	153/153	149
c2010	225/237		
c2017	258/266		
c2054	148/152	148/148	152
c2079	275/283	271/271	
c2088	131/135	127/135	131
c2096	95/103	95/95	103
PEZ01	112/120	120/120	112
PEZ03	132/138	138/138	132
PEZ05	96/104	96/96	104
PEZ06	174/174	174/174	174
PEZ08	238/238		238
PEZ12	272/272		272
PEZ20	177/177	177/177	177
u213	159/162		
u250	126/138		
u253	106/112	106/106	112
VWF	157/157		157

The last column indicates alleles that could be identified as coming from the father of A assuming that B is the mother.

Discussion

The absence of species-specific genetic markers seemingly makes the identification of hybrids difficult, but the recent development of methods aimed at identifying inter-population migrants based on the initial characterization of allelic distributions in the parent populations (species) offers new means for hybrid identification (Paetkau *et al*, 1995; Pritchard *et al*, 2000). In addition, the combined use of autosomal markers and both paternally and maternally inherited markers may allow the direction of hybridization events to be determined. However, such precise knowledge on hybridization has so far not been possible to derive owing to a general lack of polymorphic Y chromosome markers. This study therefore represents one of the first applications of Y chromosome polymorphisms, together with mtDNA and autosomal markers, to study hybridization in nature (cf Evans *et al*, 2001). The combined use of the markers allowed us to conclude that a hybridization event between dog and wolf had occurred in the endangered Scandinavian wolf population. The direction of hybridization was a male dog paired with a female wolf, the latter coming from the Scandinavian wolf population. Indeed, Vilà and Wayne (1999) suggested that if wolves and dogs would hybridize, the most likely direction is male dog crossing with female wolf. However, the lack of observable effects on the wolf populations led these authors to suggest that survival of hybrid pups could be difficult because dog fathers are less likely to help to raise the offspring and because their integration into wolf packs could be difficult (see also Randi and Lucchini, 2002).

An important consequence from our results is the confirmation, with compelling genetic evidence, that hybridization between wolves and dogs does occasionally occur in the wild and that hybrids can be successfully raised. However, as all 25 Scandinavian wolves included in the study are clearly differentiated from domestic dogs, that is, they do not show signs of recent hybridization, it indicates that hybridization may be an uncommon event. Our results agree with recent studies suggesting that this hybridization occasionally takes place across Europe but may be fairly uncommon (in Bulgaria, Randi *et al*, 2000; in Latvia, Andersone *et al*, 2002; in Italy, Randi and Lucchini, 2002; in Spain, Vilà and Llaneza, personal observation).

The generation of synthetic genotypes for both pure specimens and hybrids allowed an intuitive representation of the variability that can be expected in each population group. This method allowed us to infer that the genotype of the target sample A would be very uncommon for pure dogs or Scandinavian wolves. The generation of synthetic genotypes is dependent on a fairly accurate knowledge of the allelic frequencies. The low genetic variability of Scandinavian wolves (Ellegren *et al*, 1996; Ellegren, 1999) simplifies the estimation of the allele frequencies, but this can be a harder task for dogs. The strong genetic fragmentation of dogs into breeds may limit the power of hybridization tests like the one we present here. Modern breeding practices imply the almost complete reproductive isolation between breeds, each of them with a small effective population size, leading to fast inter-breed differentiation owing to genetic drift (Lingaas *et al*, 1996; Zajc *et al*, 1997; Wilton *et al*, 1999). The selection of local dogs belonging to the breeds that could be most likely to hybridize could increase the resolution of the test, allowing for an increase in power that could enhance the likelihood of detecting F_2 hybrids and backcrosses.

The birth of a litter had been detected in the area where the individual corresponding to sample A was killed. During autumn 1999, five pups were observed. The killed animal was assumed to be one of these pups. Direct observation of the litter had suggested that these animals could be of hybrid origin. The determination of the hybrid status of sample A confirmed the suspicion and led to the management decision to remove its presumed siblings. As a result of the management efforts, two of them were killed by government officials. Another one is believed to have been illegally killed, and the last one is unaccounted for (Terje Bø, personal communication). This action should have reduced the chances of dog genes introgressing into the wolf population.

Further research is necessary in order to confirm if fragmented and low-density wolf populations that coexist with a larger number of domestic dogs are at high risk of hybridization, as suggested (Boitani, 1983; Blanco *et al*, 1992; Andersone *et al*, 2002). If this is shown to be the case, actions that could result in the decrease of the density of already threatened wolf populations, or in the disruption of social groups, should be avoided.

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