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Short Communication

Detection of an East European wolf haplotype puzzles mitochondrial DNA monomorphism of the Italian wolf population

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ABSTRACT

Southern European wolves suffered from reiterated population declines during glacial periods and historically due to human persecution. Differently from other European wolf populations, a single mitochondrial DNA (mtDNA) control region haplotype (W14) has been so far described in the Italian wolves, although no intensive genetic sampling has ever been conducted in historical source populations from central and southern Italy. Using non-invasive genetic techniques, we report the occurrence of an unexpected mtDNA haplotype (W16) in the wolf population of the Abruzzo, Lazio and Molise National Park (PNALM), central Italy. This haplotype, detected in three out of 90 faecal samples from the PNALM, was previously reported in wolves from the North Carpathians, Slovakia and the Balkans only. Microsatellite analysis and molecular sex determination confirmed that the W16 samples belonged to three distinct wolves. Although alternative explanations can be formulated for the origin of this mtDNA haplotype in the otherwise monomorphic Italian wolf population, assignment procedures indicated the likely admixed ancestry of one W16 sample with East European wolves. Anthropogenic introgression with dogs has been detected in the Italian wolf population using nuclear DNA microsatellites, but no population-wide genetic survey had previously reported a mtDNA control region variant in Italian wolves. Our findings strongly suggest that, in addition to wolf × dog hybridization, captive-released wolves or wolf × dog hybrids may successfully interbreed with wolves in the wild, and that human-mediated introgression may occur even in well established protected areas.

Non-invasive molecular techniques have become essential tools in the study and conservation of endangered wildlife. Although several sources of error may invalidate results from non-invasive samples (Taberlet et al. 1999; Paetkau 2003; Pompanon et al. 2005), the possibility of deriving genetic data from hairs, scats or urine has significantly enhanced our ability to study demography, behaviour and conservation status of populations, as well as occurrence and levels of hybridization (Allendorf et al. 2001; Schwartz et al. 2006).

Italian wolves (*Canis lupus*) experienced prolonged population contraction and subsequent genetic isolation, first as a consequence of landscape changes during the last Pleistocene glaciation (Lucchini et al. 2004), and more recently from heavy persecution occurred until the 1970s (Boitani 1992). Since then, legal protection of remnant populations in the south-central Apennines (Zimen and Boitani 1975) and an increase in prey abundance led to the natural recovery of wolves in Italy and the re-colonization of the western Alps (Fabbri et al. 2007). Genetic surveys based on tissue samples collected across the Italian wolf range from 1984 to 1999 identified a unique mitochondrial DNA (mtDNA) control region haplotype (Randi et al. 2000; Randi and Lucchini 2002; Lucchini et al. 2004), confirming previous studies based on smaller sample sizes (Wayne et al. 1992; Vilà et al. 1997). This haplotype (W14 in Randi et al. 2000; W4 in Vilà et al. 1997), possibly a result of random genetic drift and persistent low levels of effective population sizes, was not found in any other wolf population worldwide and therefore represents a highly diagnostic marker for Italian wolves (Randi et al. 2000; Wayne and Vilà 2003; Pilot et al. 2010). Moreover, mtDNA analyses have so far failed to detect maternally inherited introgression of dog haplotypes in Italian wolves, and the W14 sequence was not described in any of the 30 dog breeds and nine feral dogs analyzed from the central Apennines (Randi et al. 2000; Lucchini et al. 2004). Monomorphism for the W14 haplotype was also confirmed in the re-colonizing wolf population in the Alps (Lucchini et al. 2002; Valière et al. 2003). However, more extensive genetic analyses did not involve mitochondrial haplotyping (Verardi et al. 2006; Fabbri et al. 2007; Iacolina et al. 2010; Caniglia et al. 2011), and no intensive sampling was ever conducted in the historical source populations from central and southern Italy.

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In this study, we performed a non-invasive genetic survey based on mtDNA and microsatellite analysis as part of a broader ecological study of wolves from the Abruzzo Lazio and Molise National Park (PNALM) and adjacent areas, central Italy. This was one of the historical refuges where Italian wolves persisted when the risk of extinction was highest elsewhere in Italy (Zimen and Boitani 1975). We analyzed the mtDNA control region of faecal samples collected in winter 2006 along snow trajectories and assessed genetic identity of individual samples by multilocus microsatellite analysis and molecular sex determination.

One-hundred and seven fresh ($6 \leq h \leq 48$) wolf faecal samples were collected and stored in 99% ethanol during intensive snow-tracking surveys of resident wolf packs, whose territory was identified by means of snow-tracking and radio-telemetry. DNA was extracted from approximately 200 mg of each sample using a QIAamp DNA Stool Mini Kit (QIAGEN). One blood and three muscle samples were also collected from wolves found dead during the study, and an additional blood sample was obtained from a live-trapped wolf. DNA was purified from muscle and blood using standard phenol/chloroform protocols.

A 630 bp fragment of the mitochondrial DNA control region was amplified using the primer L-Pro (Douzery and Randi 1997 and references therein), which binds to position 15,432 of the Eurasian wolf tRNA Proline gene (Arnason et al. 2007), and the control region internal primer H576 (Randi et al. 2000). A shorter, overlapping fragment of 405 bp was also amplified for all tissues and a subset of feacal samples using L-Pro and the control region internal primer H350 (Randi et al. 2000) to check for sequencing accuracy. Polymerase chain reaction (PCR) amplification was conducted in a total volume of 10 μl with 100 ng of total DNA, 1 × PCR buffer, 2.5 μg bovine serum albumin, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer and 0.5 units of *Taq* DNA polymerase (Invitrogen). Thermal profiles consisted of an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 15 s at 52 °C and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. PCR products were cycle-sequenced using BigDye Terminator v3.1 chemistry (Applied Biosystems). Cycle sequencing reactions were purified by isopropanol precipitation and resolved on an ABI 3100 automated capillary sequencer. Raw sequence chromatographs from both strands were edited and aligned against GenBank *Canis lupus lupus* mtDNA genome (Accession number: NC009686.1) using CodonCode Aligner 3.7.1 (CodonCode Corporation). Alignment was done using 576 bp and 350 bp sequences obtained from PCRs with primers L-Pro/H576 and L-Pro/H350, respectively.

We assessed allelic variation at 12 microsatellite loci (CPH2, CPH3, CPH5, CPH6, CPH7, CPH8, CPH22, C09.250, FH2010, FH2088, FH2096, vWF.X) (Ostrander et al. 1993; Shibuya et al. 1994; Fredholm and Winterø 1995; Francisco et al. 1996; Dolf et al. 2000). Four multiplex PCRs were performed each for three loci using forward primers labelled with FAM, HEX, and NED fluorescent dyes (Applied Biosystems) in 10 μl total volume containing 10 ng of DNA, 0.5X Multiple PCR Master Mix (QIAGEN) and 0.2 μM of each primer. Thermal profiles followed the manufacturer's protocol with annealing at 55 °C. Each multiplex PCR was loaded separately on an Applied Biosystems 3100 genetic analyzer and allele sizes scored against a GeneScan500 ROX size standard using GENEMAPPER 4.0 (Applied Biosystems). Alleles were scored using a multiple-tubes approach (Taberlet et al. 1996). Heterozygotes were defined if alleles appeared at least twice after three PCR replicates. Samples were instead scored as homozygotes after eight positive and consistent amplifications of the same allele. Several samples required up to 20 PCR replicates in order to obtain a sufficient number of positive amplifications. Genotyping errors due to false alleles, allelic dropout or locus-specific errors were assessed using the bimodal test and the difference in capture history (DCH) test implemented in DROPOUT (McKelvey and Schwartz 2005). Errors due to allelic dropout, null alleles and stuttering were also tested using MICROCHECKER (Van Oosterhout et al. 2004). We used GENALEX 6.4 (Peakall and Smouse 2006) and GENECAP (Wilberg and Dreher 2004) to derive consensus genotypes from different PCRs of the same sample, to estimate probability of identity, locate identical genotypes and assess levels of heterozygosity. Multilocus genotypes differing by one allele only were assigned to the same individual (Paetkau 2003).

Molecular sexing was conducted by multiplex PCR amplification of two fragments from the wolf DBX intron 6 and DBY intron 7 of 249 bp and 118 bp, respectively, using four PCR primers as described in Seddon (2005). PCR products were checked on 2% agarose gels. When tested with wolf tissue samples of known sex, the amplification consistently gave two fragments in males and one fragment in females.

Relatedness between each pair of PNALM wolves was assessed by the Lynch and Ritland (1999) estimator r_{xy} using IDENTIX (Belkhir et al. 2002). The estimator r_{xy} is the probability that a random allele from an individual x is identical by descent with a random allele taken from individual y in a population (e.g. $r_{xy} = 0.5$ for full-sibs, $r_{xy} = 0.25$ for half-sibs and $r_{xy} = 0$ for unrelated individuals). We used the Bayesian clustering approach with admixture implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) to investigate evidence of hybridization between wolves and dogs. A reference set of multilocus genotypes of Italian dogs ($N = 95$) and wolves from Italy ($N = 107$), Croatia ($N = 27$), Bulgaria ($N = 39$) and northeastern Europe ($N = 47$) was used to estimate the number of clusters with the highest posterior probability (Randi and Lucchini 2002; Lucchini et al. 2004). We run 100,000 Markov Chain Monte Carlo steps without prior population information for a number of populations K ranging from one to seven (number of putative groups plus two) using a burn-in period of 30,000 iterations. We calculated the mean likelihood $L(K)$ over 10 runs for each K. We assessed the mean difference between successive likelihood values of K, $L'(K)$, and the absolute value of the difference between successive values of $L'(K)$. We assessed the most likely value of K following the ΔK method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). The average estimated membership to the K inferred clusters was calculated for each individual across the 10 runs using CLUMPP (Jakobsson and Rosenberg 2007). Reference individuals were then assigned to one of the K clusters and this information used as prior in a new simulation (using $K = 3$) aimed at assessing the proportion of admixture of each PNALM wolf. Signals of introgression were checked in the past two generations using the GENSBACK option.

We successfully amplified all 5 tissue samples and 90 out of the 107 faecal samples for both mitochondrial and nuclear DNA markers. All sequenced samples exhibited the same haplotype W14, with the exception of three faecal samples collected in the same wolf pack territory on three different sampling occasions between February and March 2006. These samples differed by a single C–T transition at position 171 from the W14 control region haplotype. A nucleotide BLAST search using the blastn algorithm confirmed a 100% match of the W16 haplotype with mtDNA control region sequences reported for wolves from Bulgaria (Randi et al. 2000; W6 in Pilot et al. 2006), Croatia (WCR03 in Gomerčić et al. 2010), Slovakia and the North Carpathians (W6 in Pilot et al. 2006) and two captive-released individuals captured in France (W2 in Valière et al. 2003). To dismiss the possibility of sequencing, capillary electrophoresis or scoring errors, we re-extracted DNA from these samples and confirmed the occurrence of the W16 haplotype by running three additional PCR amplifications and sequencing reactions for each sample.

Initial microsatellite analysis produced 84 unique genotypes. Average number of alleles per locus was 5.3 ± 0.36SE (range from 3 to 9 alleles). Observed and expected heterozygosities were 0.71

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and 0.60, respectively. The DCH test revealed that no locus had significantly more errors than any other locus, thus we did not drop any loci from further analyses. The bimodality test identified a mode for samples differing at three loci and a relatively large proportion of samples differing by only one or two loci. We re-amplified all samples with genotypes derived from a consensus of positive PCR replicates but showing a low frequency of at least one alternative genotype. We also re-amplified samples that initially did not amplify at particular loci and re-scored the entire data set. We conducted four additional iterations of error identification and reanalysis of data for samples differing by ≤3 loci to assess that no new individuals were generated by genotyping errors. The final data set showed a minimal separation distribution with a unimodal mode for samples differing at four loci and identified a total of 69 distinct wolf genotypes (25 males, 42 females, two unsexed). The probability that two unrelated individuals, drawn at random from a population, had the same multilocus genotype was $P_{ID} = 5.07 \times 10^{-6}$, while the same probability for full siblings was $P_{SIB} = 2.08 \times 10^{-4}$. Our results reiterate that scoring of multilocus genotypes obtained using conventional multiple-tubes approaches from non-invasive genetic samples can be prone to DNA amplification errors, which can eventually affect numerical estimates of individual wolves. Probability of genotyping errors can be significantly reduced by integrating detection of false alleles, allelic dropouts and database errors via computer techniques and subsequent laboratory reanalysis of problematic samples for those loci identified by error checking procedures (McKelvey and Schwartz 2005). The three wolf samples showing a W16 haplotype belonged to two males and one female, respectively. They had different multilocus genotypes at more than three loci. Mean error rate per locus for these three samples ranged from 0.10 (vWF.X) to 0.34 (CPH5) and mean yield per locus was 64.4%. The statistic based on the rate of change in the log probability of the data between successive K values estimated using the Bayesian clustering analysis resulted in a modal value of $\Delta K = 707.8$ for $K = 3$. The assignment analysis using population information and the admixture model showed that one of the male and the female genotypes had significantly high posterior probability ($q^i = 0.993$ and $q^i = 0.992$, respectively) of belonging to the cluster containing the major proportion of the Italian wolf genomes. A relatively low coefficient of relatedness was recorded between these two samples ($r_{xy} = 0.140$; PNALM wolf samples range: $-0.305 < r_{xy} < 0.485$). On the other hand, the second male multilocus genotype had a relatively lower posterior probability of belonging to the Italian wolf cluster ($q^i = 0.516$) and a posterior probability of $q^i = 0.346$ and $q^i = 0.122$ of sharing a parent and a grandparent, respectively, in the cluster characterized by wolf genotypes from Bulgaria, Croatia and northeastern Europe (Table 1). Coefficients of relatedness with the other two W16 wolves were 0.005 and −0.005, respectively, indicating no parental relationships. A negligible proportion of genotypes of the three W16 samples ($q^i < 0.01$) was allocated to the dog cluster.

The occurrence of the W16 mtDNA control region haplotype in wolves from central Italy is unexpected based on previous genetic surveys of Italian wolves, and raises questions of conservation concern about their origin. A similar case was reported in France, near Montpellier, where two wolves, sharing a W2 haplotype (W16 in Randi et al. 2000) and believed to be captive-released individuals due to their unusual confidence in humans, were subsequently captured in 1999 (Valière et al. 2003). However, no additional genetic markers were used by the authors to further investigate the identity of the W2 wolves from France. On the other hand, we compared the three W16 samples from the PNALM to dogs and wolves of other European populations in order to ascertain evidence of hybridization and introgression. The assignment test did not support the hypothesis of a hybrid origin with dogs of the three W16 samples. We therefore envision three alternative hypotheses

Table 1
Average values of the estimated membership coefficients for individual wolves from Italy, eastern and northeastern Europe, the three PNALM wolves showing the W16 haplotype, and dogs in each of the three clusters defined by the Bayesian clustering analysis and the rate of change in the log probability of data between successive K values. For Italian PNALM wolves sharing the W16 haplotype, we also reported the probability of having a parent (q_1^i) and/or a grandparent (q_2^i) from either of the two alternative clusters.

Samples	Cluster		
	I	II	III
Bulgarian wolves	1.000	0.000	0.000
Croatian wolves	0.997	0.001	0.001
NE European wolves	0.949	0.000	0.051
Italian wolves	0.004	0.992	0.004
Dogs	0.000	0.001	0.999
PNALM 107 (♂)	0.000	0.993	0.000
q_1^{107}	0.000		0.000
q_2^{107}	0.000		0.001
PNALM 085 (♂)	0.002	0.516	0.000
q_1^{85}	0.346		0.001
q_2^{85}	0.122		0.001
PNALM 034 (♀)	0.000	0.992	0.000
q_1^{34}	0.001		0.000
q_2^{34}	0.014		0.006

to explain the occurrence of this haplotype in the Italian wolf population. First, during the last glaciation, frequencies of mtDNA haplotypes of wolves in the Italian Apennines were most probably affected by random drift and low effective population size, which presumably led to the fixation of the most frequent haplotype (Randi et al. 2000; Lucchini et al. 2004). According to this scenario, the W16 haplotype may have originated from a spontaneous mutation in the non-coding, control region of the mtDNA wolf genome during the approximately 10,000 years that followed the end of the last glacial period. The W16 haplotype could have therefore been left undescribed in previous population genetic studies, which did not consider comprehensive sampling in central and southern Italy where wolf populations survived historical contraction. By increasing sampling intensity, a higher number of mtDNA wolf haplotypes were indeed detected in several East European countries than previously thought, challenging the idea that the majority of Eurasian wolf populations have unique haplotypes (Pilot et al. 2006). Remnant populations of the historical wolf range may therefore still host a W16 haplotype yet undetected due to its very low frequency and insufficient genetic sampling (Koblmüller et al. 2009; Schwartz and McKelvey 2009). According to this first hypothesis, the population decline and bottleneck experienced by Italian wolves may have left more variation than previously thought. Allelic diversity and heterozygosity in the PNALM wolf population was, in fact, comparable to or higher than the overall Italian wolf population diversity found in previous studies (Lucchini et al. 2004; Fabbri et al. 2007). An alternative explanation could be immigration from neighbouring countries where the W16 haplotype has been detected (i.e., Croatia; Gomerčić et al. 2010). This is however unlikely due to the long distance and the extensive cultivated areas in the Po valley, which make effective barriers to wolf dispersal between the eastern Alps and the Apennines (Lucchini et al. 2004). Moreover, no other W16 haplotype was ever reported for wolves from the northern Apennines (Randi et al. 2000; Randi and Lucchini 2002; Lucchini et al. 2004). The results of our assignment test suggest a third and more likely explanation which involves anthropogenic introgression of Italian wolves with captive-released wolves or commercialized wolf × dog hybrids (e.g. Czechoslovakian wolf-dogs), a timely conservation issue in Italy (Boitani and Ciucci 1993; Verardi et al. 2006; Randi 2008). Introgression with dogs was confirmed by microsatellite analyses (Randi 2008), and a non-Italian mtDNA haplotype, different from W16,

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was previously detected among captive wolves owned illegally and confiscated by the Italian Department of Forestry (Wu479 in Randi and Lucchini 2002). All microsatellite alleles characterized in this study for the three W16 individuals were also found in the PNALM wolves carrying the W14 haplotype. However, whereas a high proportion of two of the W16 samples multilocus genotypes were assigned to a cluster of Italian wolf genomes, the third W16 sample showed high probability of admixed ancestry with East European wolves. According to the q^i values, this last sample most likely corresponds to an F1 hybrid with East European wolves, whereas the other two could be backcrosses of later generations diluted into the parental Italian wolf population. Moreover, coefficients of relatedness of the putative F1 hybrid male with the other two wolves were close to zero, whereas a value of relatedness higher than an expected third-order relationship (0.125) was recorded between the female and the other male. According to our third hypothesis, these results would therefore suggest recurrent, temporally distinct but localized hybridization events. Although introgression from wolf × dog commercialized hybrids (e.g., Czechoslovakian wolf-dogs) cannot be ruled out, hybridization likely involved one or few individuals from an eastern European lineage, possibly originated from illegally released captive wolves. It is noteworthy that a privately owned zoo, hosting non Italian wolves, is located in the immediate surroundings of the PNALM area and that wolves were known to have repeatedly escaped from this facility (L. Sammarone, Italian Department of Forestry, pers. comm.).

Further genetic investigation of the PNALM wolf population, as well as of others from central and southern Italy may be necessary to better understand the origin of this haplotype and assess the conservation implications of our findings. Meanwhile, management efforts are needed to enforce confiscation of illegally owned captive wolves and control the number of free-ranging and vagrant dogs in order to prevent further spreading of anthropogenic introgression, especially in National Parks and other protected areas. We also urge National and European administrations to strictly regulate commercialization, ownership and breeding of wolf × dog hybrids (e.g., Czechoslovakian wolf-dogs), especially in areas where these could interbreed with wild wolves.

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