

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/312599642>

# A new mitochondrial haplotype confirms the distinctiveness of the Italian wolf (*Canis lupus*) population

Article in *Mammalian Biology* · May 2017

DOI: 10.1016/j.mambio.2017.01.005

CITATIONS

19

5 authors, including:



**Luca Montana**

13 PUBLICATIONS 198 CITATIONS

SEE PROFILE



**Marco Galaverni**

WWF Italy

114 PUBLICATIONS 2,453 CITATIONS

SEE PROFILE

READS

1,183



**Romolo Caniglia**

Institute for Environmental Protection and Research (ISPRA)

154 PUBLICATIONS 2,419 CITATIONS

SEE PROFILE



**Elena Fabbri**

Institute for Environmental Protection and Research (ISPRA)

201 PUBLICATIONS 2,821 CITATIONS

SEE PROFILE



## Short communication

A new mitochondrial haplotype confirms the distinctiveness of the Italian wolf (*Canis lupus*) populationLuca Montana<sup>a,b,\*</sup>, Romolo Caniglia<sup>a,1</sup>, Marco Galaverni<sup>a,1</sup>, Elena Fabbri<sup>a</sup>, Ettore Randi<sup>a,c</sup><sup>a</sup> Laboratorio di Genetica, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), Via Ca' Fornacetta 9, 40064 Ozzano dell'Emilia, Bologna, Italy<sup>b</sup> Département de Biologie, Université de Sherbrooke, 2500, Boul. de l'Université, Sherbrooke, QC, J1K 2R1, Canada<sup>c</sup> Department 18/Section of Environmental Engineering, Aalborg University, Sohngårdsholmsvej 57, 9000 Aalborg, Denmark

## ARTICLE INFO

## Article history:

Received 29 October 2016

Accepted 18 January 2017

Handled by Frank E. Zachos

Available online 22 January 2017

## Keywords:

*Canis lupus italicus*

Bottleneck

Conservation genetics

Isolation

Mitochondrial DNA

## ABSTRACT

In the past century the Italian wolf has been repeatedly indicated as a distinct subspecies, *Canis lupus italicus*, due to its unique morphology and its distinctive mtDNA control region (CR) monomorphism. However, recent studies on wolf × dog hybridization in Italy documented the presence of a second mtDNA CR haplotype (W16), previously found only in wolves from Eastern Europe, casting doubts on the genetic uniqueness of the Italian wolves. To test whether this second haplotype belongs to the Italian wolf population, we genotyped 92 wolf DNA samples from Italy, Slovenia, Greece and Bulgaria at four mtDNA regions (control-region, ATP6, COIII and ND4 genes) and at 39 autosomal microsatellites. Results confirm the presence of two mtDNA multi-fragment haplotypes (WH14 and WH19) in the Italian wolves, distinct from all the other European wolves. Network analyses of the multi-fragment mtDNA haplotypes identified two strongly differentiated clades, with the Italian wolf WH14 and WH19 multi-fragment haplotypes rooted together. Finally, Bayesian clustering clearly assigned all the wolves sampled in Italy to the Italian population, regardless of the two different multi-fragment haplotypes. These results demonstrate that the W16 CR haplotype is part of the genetic pool of the Italian wolf population, reconfirming its distinctiveness from other European wolves. Overall, considering the presence of unique mtDNA and Y-linked haplotypes, the sharply different frequencies of genome-wide autosomal alleles and the distinct morphological features of Italian wolves, we believe that this population should be considered a distinct subspecies.

© 2017 Deutsche Gesellschaft für Säugetierkunde. Published by Elsevier GmbH. All rights reserved.

During recent centuries the wolf (*Canis lupus*) was progressively eradicated from most European countries through human persecution, habitat loss and decline of its natural prey (Breitenmoser, 1998). In the Italian peninsula wolves were drastically reduced in number and distribution after the Second World War. Zimen and Boitani (1975) estimated that in the 1970s only approximately 100 individuals survived, split into two fragmented populations in the Central and Southern Apennines. Nonetheless in just 40 years the Italian wolf population recovered and recolonized most of its historical range in the Apennines and in the Western Alps, currently including about 1500 (±300) wolves (Galaverni et al., 2016). Nowadays, the population is still expanding and recolonizing the

Central-Eastern Alps, with a recently described episode of admixture with Dinaric wolves (Fabbri et al., 2014; Ražen et al., 2016).

The Italian wolf population represents an interesting case study for its uniqueness worldwide. At the beginning of the 20th century Altobello (1921) described the Italian wolf as a distinct subspecies, *C. l. italicus*, based on its peculiar morphological traits: relatively small average size (110–148 cm body length, plus the tail), dark bands along the back and forelimbs, black tail tip, and distinct craniometrical features, recently confirmed by Nowak and Federoff (2002). This uniqueness was later supported by genetic studies describing (i) only one private mitochondrial DNA (mtDNA) control region (CR) haplotype (named W14; Randi et al., 2000), (ii) two unique Y-chromosome microsatellite haplotypes (YH17 and YH26; Randi et al., 2014), (iii) microsatellite allele frequencies sharply different from any other wolf population or dog breed (Randi et al., 2000; Randi and Lucchini, 2002), and (iv) very high genome-wide  $F_{ST}$  distance (0.15–0.29) from other wolf populations from Europe (67k single nucleotide polymorphisms – SNPs –, Stronen et al.,

\* Corresponding author at: Département de Biologie, Université de Sherbrooke, 2500, Boul. de l'Université, Sherbrooke, QC, J1K 2R1, Canada.

E-mail address: [Luca.Montana@USherbrooke.ca](mailto:Luca.Montana@USherbrooke.ca) (L. Montana).

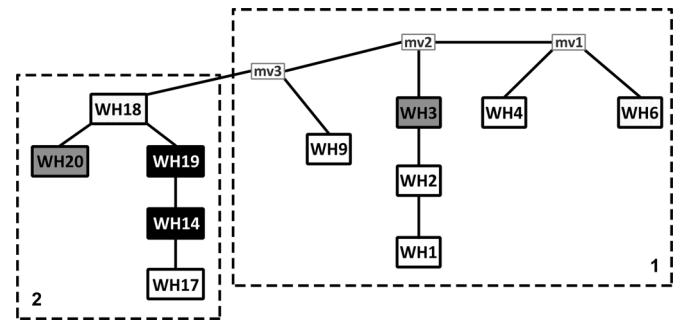
<sup>1</sup> These authors contributed equally to this work.

2013; 78k SNPs, Pilot et al., 2014) and North America (48k SNPs, VonHoldt et al., 2011). Coherently, the Italian wolf population is one of the most differentiated worldwide also according to Principal Component Analyses (Pilot et al., 2014; Stronen et al., 2013) or Bayesian clustering analyses (Stronen et al., 2013; VonHoldt et al., 2011). This high degree of differentiation could result from multiple bottlenecks during a period of isolation south of the Alps, possibly dating back to the last glaciation when the Italian peninsula constituted one of the main southern European refugia (Lucchini et al., 2004).

Despite the monomorphism observed for decades at the mtDNA CR (Randi et al., 2000), Boggiano et al. (2013) and Randi et al. (2014), working on wolf x dog hybridization in Italy, recently documented six unrelated canids carrying the mtDNA CR haplotype named W16 by Randi et al. (2000), which was previously reported in wild wolves from Bulgaria (Randi et al., 2000; H6 in Pilot et al., 2010), Croatia (WCRO3 in Gomerčič et al., 2010), North Carpathians (Pilot et al., 2010), and France (W2 in Valière et al., 2003). The presence of this haplotype in the Italian wolf population could be the consequence of: (1) a spontaneous mutation in the mtDNA CR after the population split, (2) immigration from neighbouring populations where haplotype W16 has been detected (i.e. Croatia; Gomerčič et al., 2010), or (3) hybridization between Italian wolves and dogs.

To test these hypotheses and clarify the origin of the W16 haplotype found in the Italian wolf population, we analyzed DNA samples extracted from 92 wild wolves from three genetically distinct populations: Italian (WIT,  $n=45$ ; including the three previous-sampled canids carrying the W16 haplotype described by Randi et al. (2014)), Dinaric (Slovenia – WSLO,  $n=20$ ) and Balkan (Greece – WGRI,  $n=10$ ; Bulgaria – WBUL,  $n=17$ ) populations. All samples were sequenced at part of the mtDNA CR and at three additional mtDNA coding genes: ATPase 6 and ND4, chosen for their high variability, similar to that of the mtDNA CR (Elson et al., 2004; Rutledge et al., 2010), and COX III, conversely selected because it is one of the most conserved genes in the mtDNA genome (Björnerfeldt et al., 2006). Total DNA from each individual was extracted using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN® Inc., Hilden, Germany). A 498-bp fragment of the left peripheral and central domain of the mtDNA CR was amplified using primers WDLOPL and H519 (Fabbri et al., 2014). In addition, we used four primer pairs (For8049-Rev8501; For8255-Rev8891; For10104-Rev10647; For11093-Rev11741; Björnerfeldt et al., 2006) to amplify 588 bp of ATPase 6, the first 414 bp and last 433 bp (total 847 bp) of ND4 gene and 231 bp of COX III. All the amplifications were performed by polymerase chain reactions (PCR) in a 10  $\mu$ L solution with 2.0  $\mu$ L of 20–40 ng/ $\mu$ L DNA, 10X PCR Buffer with 2.5 mM  $Mg^{2+}$ , 0.3  $\mu$ M of primer mix (forward and reverse), and 0.25 units of Taq Polymerase (5 PRIME Inc., Gaithersburg, USA). PCRs were run with an initial denaturation step at 94 °C for 2 min, followed by 45 cycles of 94 °C denaturation for 15 s, 55 °C annealing for 15 s, and 72 °C extension for 30 s, with a final extension at 72 °C for 10 min. Negative (no DNA in PCR) and positive (samples with known genetic profile) controls were used at every laboratory step. PCR products were purified using ExoSAP-IT (Affimetrix, Inc., Cleveland, Ohio, USA) and sequenced in both directions using an Applied Biosystems 3130XL Genetic Analyzer automatic sequencer. Sequences were visualized and corrected in SeqScape v.2.5 (Life Technology) and aligned in Geneious v.7.1. (2005–2014 Biomatters Limited). Geneious was also used to manually inspect and correct alignment ambiguities, which mostly affect the placement of *indels*.

Wolf CR haplotypes were compared to the haplotypes described and deposited in GenBank using BLAST. CR haplotypes were named following Randi et al. (2000), since they had the same length and were longer than those from similar mtDNA CR studies on wolves (Fabbri et al., 2014; Pilot et al., 2010; Vilà et al., 1999). Haplotypes from the genic regions were also given unique codes (e.g. A1 for



**Fig. 1.** Median-Joining network of the 11 wolf haplotypes. The network was built using complete multi-fragment haplotypes (2164 bp) and accounting for gaps in the alignment. The two dashed-line rectangles denote the haplotypes corresponding to haplogroups 1 (right side) and 2 (left side). Haplotypes are connected through three median vectors (mv1, mv2, mv3) and different colors represent haplotypes belonging to different populations (Italian wolves: black; Dinaric wolves: grey; Balkan wolves: white).

ATPase6, C1 for COX III and N1 for ND4). The four mtDNA fragments were then concatenated in a multi-fragment (MF) alignment of 2164 bp. DnaSP v.5.10.01 (Librado and Rozas, 2009) was first used to search for identical sequences and then to estimate nucleotide diversity,  $\pi$  (Nei and Li, 1979), its standard deviation (SD) and haplotype diversity ( $H_d$ ) for each of the four mtDNA regions and for the multi-fragment sequences. Haplotype diversity was calculated for the whole sample set and for each geographic population. We finally built a Median-Joining network in Network v. 5.0 (Bandelt et al., 1994; www.fluxus-engineering.com) using the MF haplotype sequences to explore their relationships.

We found a total of 10 CR haplotypes (Table 1), all previously described (Gomerčič et al., 2010; Pilot et al., 2010; Randi et al., 2000). All the Italian samples carried the W14 CR haplotype, except five canids that carried the W16 CR haplotype (including the three analyzed in Randi et al. (2014)).

The highest haplotype diversity was observed at the CR ( $H_{dALL} = 0.7578$ ), whereas the lowest value among the three coding mtDNA genes was found at COX III ( $H_{dALL} = 0.4933$ ; Table 2). The CR also showed the highest nucleotide diversity ( $\pi = 0.0156$ ,  $SD = 0.0015$ ; Table 2), whereas the lowest  $\pi$  value was observed at ND4 ( $\pi = 0.0031$ ,  $SD = 0.0004$ ; Table 2). Interestingly, all the mutations on the genic regions were synonymous, except for two SNPs (592C>T and 781A>G) on the ND4 fragment. Those SNPs produced the amino acidic variations Leu198Phe and Thr261Ala, and were only found in the N5 haplotype, detected in wolves from Italy and Greece (Table 1). Finally, the concatenated sequences showed a total of 11 haplotypes (Table 1). SNPs at ATPase 6 and COX III were diagnostic enough to differentiate haplotypes belonging to haplogroup 1 (from Iberia to Eastern Europe, absent in Italy) and haplogroup 2 (Italian peninsula and Eastern Europe), where it overlaps geographically with haplogroup 1 described by Pilot et al. (2010), confirming their phylogeny. Moreover, all Italian wolves shared the same ND4 haplotype (N5), carrying either the W14 or the W16 CR haplotype, combined into the WH14 and WH19 MF haplotypes, respectively. Other European wolves carrying the W16 CR haplotype presented a different ND4 haplotype (N4), combined into the WH18 MF haplotype (Table 1). The N5 haplotype was also found in two wolves from Greece, but linked to the W15 CR haplotype, never found in Italy (Table 1).

The MF haplotype network (Fig. 1) highlighted the presence of two strongly differentiated clades, consistent with Pilot et al. (2010), with haplotypes WH14–20 belonging to haplogroup 2 and WH1–9 to haplogroup 1. Interestingly, the relations within the network suggest that WH14 most likely originated from WH19 and not vice versa.

**Table 1**  
Distribution of mtDNA multi-fragment haplotypes found in our wolf samples. CR haplotypes were named following Randi et al. (2000). Haplotypes W2, W3, W4, W5, W9, W14, W15, and W16 respectively correspond to haplotypes named H16, H23, H4, H13, H10, H22, H27, and H6 by Pilot et al. (2010) whereas haplotype W17 corresponds to haplotype named WCRO6 by Gomerčič et al. (2010).

MF <sup>a</sup>	ATP6 <sup>b</sup>	COIII <sup>c</sup>	ND4 <sup>d</sup>	CR <sup>e</sup>	BG <sup>f</sup>	GR <sup>f</sup>	SLO <sup>f</sup>	ITA <sup>f</sup>	Total
WH1	A1	C1	N1	W1	1	–	–	–	1
WH2	A1	C1	N1	W2	1	–	–	–	1
WH3	A1	C1	N1	W3	–	–	16	–	16
WH4	A1	C2	N2	W4	–	3	–	–	3
WH6	A2	C1	N2	W5	5	3	–	–	8
WH9	A1	C1	N3	W9	5	–	–	–	5
WH14	A3	C3	N5	W14	–	–	–	40	40
WH17	A3	C3	N4	W15	–	2	–	–	2
WH18	A3	C3	N4	W16	5	2	–	–	7
WH19	A3	C3	N5	W16	–	–	–	5	5
WH20	A3	C3	N4	W17	–	–	4	–	4
Total samples					17	10	20	45	92
Total haplotypes					5	4	2	2	–
Private MF haplotypes					3	2	2	2	–

<sup>a</sup> MF = Multi-Fragment haplotypes. The name of the haplotypes follows the nomenclature present in Montana et al. (under review).

<sup>b</sup> ATP6 = ATPase 6 haplotypes.

<sup>c</sup> COIII = COX III haplotypes.

<sup>d</sup> ND4 = NADH 4 haplotypes.

<sup>e</sup> CR = Control region haplotypes.

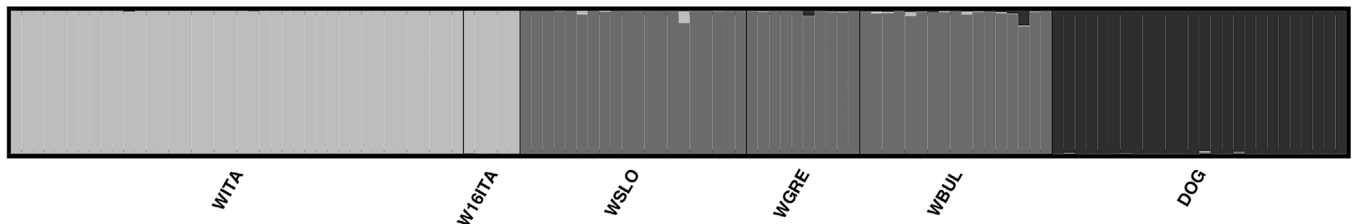
<sup>f</sup> BG = Bulgaria; GR = Greece; SLO = Slovenia; ITA = Italy.

**Table 2**  
Haplotype numbers (n. H), nucleotide diversity ( $\pi$ ), its standard deviation (SD), and the haplotype diversity (Hd) for each of the four single mtDNA regions and for the multi-fragment (MF) sequences.

Region	n. H	$\pi$	SD ( $\pi$ )	Hd <sub>ALL</sub> <sup>a</sup>	Hd <sub>BG</sub> <sup>b</sup>	Hd <sub>GR</sub> <sup>b</sup>	Hd <sub>SLO</sub> <sup>b</sup>	Hd <sub>ITA</sub> <sup>b</sup>
ATP6	3	0.0050	0.0006	0.5208	0.6985	0.7333	0.3368	0.0000
COIII	3	0.0102	0.0013	0.4933	0.4412	0.7333	0.3368	0.0000
ND4	5	0.0031	0.0004	0.6928	0.7721	0.5333	0.3368	0.0000
CR	10	0.0156	0.0015	0.7578	0.7794	0.8222	0.3368	0.2020
MF	11	0.0072	0.0007	0.7661	0.7794	0.8222	0.3368	0.2020

<sup>a</sup> ALL = all 92 samples.

<sup>b</sup> BG = Bulgaria; GR = Greece; SLO = Slovenia; ITA = Italy.



**Fig. 2.** Bayesian clustering of wolf samples produced by STRUCTURE. Each individual is represented by a vertical bar fragmented into  $K$  sections, according to its proportional membership to the inferred genetic clusters. Sampling regions are indicated in the labels (ITA = Italy, SLO = Slovenia, GRE = Greece, BUL = Bulgaria). At the optimal number of  $K = 3$  clusters, Italian wolves, European wolves and dogs are clearly separated. The five wolves sampled in Italy carrying the WH19 MF haplotype (W16ITA) fully cluster with the Italian wolves carrying the WH14 MF haplotype.

To further clarify the origin of the W16 CR haplotype in Italy, the 92 wild wolves previously sequenced at the four mtDNA fragments, plus 26 village dogs sampled in the Northern and Central Apennines, were also genotyped at 39 canine autosomal STRs that were used in a recent study on wolf population genetics and hybridization in Europe (Randi et al., 2014). Multilocus genotypes were used in a Bayesian clustering analysis implemented in Structure 2.3.4 (Falush et al., 2003), run with four independent iterations for each value of  $K$  ( $K = 1-8$ ), 500 000 Markov Chain Monte Carlo (MCMC, discarding the first 50 000 as burn-in), the admixture and independent allele frequency models and no prior information (option “usepopinfo” not activated). The  $\Delta K$  statistics (Evanno et al., 2005) implemented in Clumpak (Kopelman et al., 2015) revealed that the optimal number of genetic clusters was three. At  $K = 3$  all dog genotypes were assigned to one cluster with average  $Q_{\text{DOG}} = 1.00$  (90% CI: 0.99–1.00), all European wolf genotypes to a second cluster with  $Q_{\text{WEU}} > 0.985$  (90% CI: 0.98–0.99) and all Italian wolves

to a third cluster with  $Q_{\text{WIT}} = 1.00$  (90% CI: 0.99–1.00). Individual  $q_i$  values ranged between  $0.99 < q_{\text{DOG}} < 1.00$ ,  $0.89 < q_{\text{WEU}} < 1.00$  and  $0.99 < q_{\text{WIT}} < 1.00$ . The five canids carrying the W16 CR haplotype were unambiguously assigned to the Italian wolf population with  $q_i$  values ranging between 0.995 and 0.998 (Fig. 2).

These findings indicate that wolves carrying the W16 CR haplotype belong to the Italian wolf population, and confirm the distinction of the Italian wolf population from all other European populations. Consequently, we reject the hypotheses of an introgression of this haplotype into the Italian population as a result of immigrations from neighbouring wolf populations. Moreover, as the W16 haplotype was never found in dogs (Gundry et al., 2007), an ancient introgression of this haplotype via hybridization with free-ranging dogs seems unlikely. Consequently, our results suggest the presence of two, not one, CR haplotypes in the Italian wolf population (W14 and W16; Randi et al., 2000) corresponding to two private MF haplotypes (WH14 and WH19; Montana et al.



under review), revealing more diversity than previously thought for this population (Lucchini et al., 2004; Randi et al., 2000; Randi and Lucchini, 2002). Therefore, we hypothesize that during the past century wolves with W14 and W16 haplotypes survived in Italy, but the strong demographic decline left a vast majority of individuals carrying the W14 CR haplotype. Conversely, the W16 CR haplotype was probably too rare to be detected before the recent activation of large-scale monitoring projects based on the analyses of carcasses, live-trapped wolves and non-invasively collected samples from the entire Italian territory (Caniglia et al., 2014; Fabbri et al., 2014; Randi et al., 2014).

Interestingly, the presence of the ND4 haplotype N5 only in wolves from Southern European countries (Italy and Greece) might suggest a shared physiological adaptation, and selection studies could aid to explain this variation.

Overall, our results reject the hypothesis that Italian wolves share any mtDNA haplotype with other wolf populations and reaffirm their mitochondrial distinctiveness (confirming data from whole mtDNA sequences; Koblmüller et al., 2016). Such uniqueness parallels what has been described also for sex chromosomes (Y-linked haplotypes, Randi et al., 2014; and X-chromosome haplotypes, Pilot et al., 2014). These findings, together with the sharply distinct microsatellite allele frequencies of Italian wolves compared to any other wolf population and dog breed, show that they represent a well-differentiated cluster (Randi et al., 2014; Randi and Lucchini, 2002), strongly supported also by results from genome-wide SNPs (Pilot et al., 2014; Stronen et al., 2013; VonHoldt et al., 2011) and even whole-genome sequences (Fan et al., 2016).

At the beginning of the 20th century, the distinctive phenotypic traits of the Italian wolves led Altobello (1921) to describe a distinct subspecies, *C. l. italicus*. More recently, Nowak and Federoff (2002), after a careful statistical comparison of morphometric traits with other Eurasian wolves and dogs, reinforced the evidence of such morphological uniqueness.

Therefore, taken together, we believe that this genetic and phenotypical differentiation is pronounced enough to consider the Italian wolf population as a distinct subspecies, *C. l. italicus*, as recently proposed also for other highly-differentiated wolf populations, e.g. the Mexican wolf (*C. l. baileyi*; Fredrickson et al., 2015) and the Alexander Archipelago wolf (*C. l. lagoni*; Weckworth et al., 2015). A similar subspecies designation has already been widely accepted for other Italian mammal species, such as the Mesola red deer (*Cervus elaphus italicus*; Zachos et al., 2014) and the Italian roe deer (*Capreolus capreolus italicus*; Randi et al., 2004). Such recognition for the Italian wolf could support more appropriate conservation efforts in a context of population expansion threatened by the persistently low acceptance by local communities (Chapron et al., 2014).

## Acknowledgements

We warmly thank everybody that contributed to obtain the wolf and dog samples used in this study, in particular P. Genov and T. Skrbinšek for Slovenian, Bulgarian and Greek samples. We also thank D. Palumbo and M. Festa-Bianchet for useful discussions, the Associate Editor and two anonymous reviewers for their helpful comments on the manuscript. This project was supported by ISPRA, by the Italian Ministry of Environment, Direction of Nature Protection.

## References

- Altobello, G., 1921. Fauna d'Abruzzo e Molise. In: Mammiferi IV., pp. 38–45.  
 Bandelt, H.-J., Forster, P., Röhl, A., 1994. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16, 37–48.

- Björnerfeldt, S., Webster, M.T., Vilà, C., 2006. Relaxation of selective constraint on dog mitochondrial DNA following domestication. *Genome Res.* 16, 990–994, <http://dx.doi.org/10.1101/gr.5117706>.  
 Boggiano, F., Ciofi, C., Boitani, L., Formia, A., Grotto, L., Natali, C., Ciucci, P., 2013. Detection of an East European wolf haplotype puzzles mitochondrial DNA monomorphism of the Italian wolf population. *Mamm. Biol.—Z. Säugetierkd.* 78, 374–378, <http://dx.doi.org/10.1016/j.mambio.2013.06.001>.  
 Breitenmoser, U., 1998. Large predators in the Alps: the fall and rise of man's competitors. *Biol. Conserv.* 83, 279–289.  
 Caniglia, R., Fabbri, E., Galaverni, M., Milanese, P., Randi, E., 2014. Noninvasive sampling and genetic variability, pack structure, and dynamics in an expanding wolf population. *J. Mammal.* 95, 41–59, <http://dx.doi.org/10.1644/13-MAMM-A-039>.  
 Chapron, G., Kaczensky, P., Linnell, J.D.C., von Arx, M., Huber, D., Andrén, H., López-Bao, J.V., Adamec, M., Álvares, F., Anders, O., Balčiauskas, L., Balys, V., Bedř, P., Bego, F., Blanco, J.C., Breitenmoser, U., Brøseth, H., Bufka, L., Bunikyte, R., Ciucci, P., Dutsov, A., Engleder, T., Fuxjäger, C., Groff, C., Holmala, J., Jerina, K., Kluth, G., Knauer, F., Kojola, I., Kos, I., Krofel, M., Kubala, J., Kunovac, S., Kusak, J., Kutal, M., Liberg, O., Majić, A., Männil, P., Manz, R., Marboutin, E., Marucco, F., Melovski, D., Mersini, K., Mertzani, Y., Mysłajek, R.W., Nowak, S., Odden, J., Ozolins, J., Palomero, G., Pannoni, M., Persson, J., Potočnik, H., Quenette, P.-Y., Rauer, G., Reinhardt, I., Rigg, R., Ryser, A., Salvatori, V., Skrbinšek, T., Stojanov, A., Swenson, J.E., Szemethy, L., Trajçe, A., Tsingarska-Sedefcheva, E., Vaňa, M., Veeroja, R., Wabakken, P., Wölfl, M., Wölfl, S., Zimmermann, F., Zlatanov, D., Boitani, L., 2014. Recovery of large carnivores in Europe's modern human-dominated landscapes. *Science* 346, 1517–1519, <http://dx.doi.org/10.1126/science.1257553>.  
 Elson, J.L., Turnbull, D.M., Howell, N., 2004. Comparative genomics and the evolution of human mitochondrial DNA: assessing the effects of selection. *Am. J. Hum. Genet.* 74, 229–238.  
 Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* 14, 2611–2620, <http://dx.doi.org/10.1111/j.1365-294X.2005.02553.x>.  
 Fabbri, E., Caniglia, R., Kusak, J., Galov, A., Gomerčić, T., Arbanasić, H., Huber, D., Randi, E., 2014. Genetic structure of expanding wolf (*Canis lupus*) populations in Italy and Croatia, and the early steps of the recolonization of the Eastern Alps. *Mamm. Biol.—Z. Säugetierkd.* 79, 138–148, <http://dx.doi.org/10.1016/j.mambio.2013.10.002>.  
 Falush, D., Stephens, M., Pritchard, J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* 164, 1567–1587.  
 Fan, Z., Silva, P., Gronau, I., Wang, S., Armero, A.S., Schweizer, R.M., Ramirez, O., Pollinger, J., Galaverni, M., Ortega Del-Vechyo, D., Du, L., Zhang, W., Zhang, Z., Xing, J., Vilà, C., Marques-Bonet, T., Godinho, R., Yue, B., Wayne, R.K., 2016. Worldwide patterns of genomic variation and admixture in gray wolves. *Genome Res.* 26, 1–11, <http://dx.doi.org/10.1101/gr.197517.115>.  
 Fredrickson, R.J., Hedrick, P.W., Wayne, R.K., VonHoldt, B.M., Phillips, M.K., 2015. Mexican wolves are a valid subspecies and an appropriate conservation target. *J. Hered.* 106, 415–416, <http://dx.doi.org/10.1093/jhered/esv028>.  
 Galaverni, M., Caniglia, R., Fabbri, E., Milanese, P., Randi, E., 2016. One, no one, or one hundred thousand: how many wolves are there currently in Italy? *Mamm. Res.* 61, 13–24, <http://dx.doi.org/10.1007/s13364-015-0247-8>.  
 Gomerčić, T., Sindičić, M., Galov, A., Arbanasić, H., Kusak, J., Kocijan, I., Gomerčić, M.D., Huber, D., 2010. High genetic variability of the grey wolf (*Canis lupus* L.) population from Croatia as revealed by mitochondrial DNA control region sequences. *Zool. Stud.* 49, 816–823.  
 Gundry, R.L., Allard, M.W., Moretti, T.R., Honeycutt, R.L., Wilson, M.R., Monson, K.L., Foran, D.R., 2007. Mitochondrial DNA analysis of the domestic dog: control region variation within and among breeds. *J. Forensic Sci.* 52, 562–572, <http://dx.doi.org/10.1111/j.1556-4029.2007.00425.x>.  
 Koblmüller, S., Vilà, C., Lorente-Galdos, B., Dabad, M., Ramirez, O., Marques-Bonet, T., Wayne, R.K., Leonard, J.A., 2016. Whole mitochondrial genomes illuminate ancient intercontinental dispersals of grey wolves (*Canis lupus*). *J. Biogeogr.* 1–11, <http://dx.doi.org/10.1111/jbi.12765>.  
 Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A., 2015. CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Mol. Ecol. Resour.* 15, 1179–1191, <http://dx.doi.org/10.1111/1755-0998.12387>.  
 Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452, <http://dx.doi.org/10.1093/bioinformatics/btp187>.  
 Lucchini, V., Galov, A., Randi, E., 2004. Evidence of genetic distinction and long-term population decline in wolves (*Canis lupus*) in the Italian Apennines. *Mol. Ecol.* 13, 523–536, <http://dx.doi.org/10.1046/j.1365-294X.2004.02077.x>.  
 Nei, M., Li, W.-H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U. S. A.* 76, 5269–5273.  
 Nowak, R.M., Federoff, N.E., 2002. The systematic status of the Italian wolf *Canis lupus*. *Acta Theriol.* 47, 333–338.  
 Pilot, M., Branicki, W., Jędrzejewski, W., Goszczyński, J., Jędrzejewska, B., Dykyy, I., Shkvrya, M., Tsingarska, E., 2010. Phylogeographic history of grey wolves in Europe. *BMC Evol. Biol.* 10, <http://dx.doi.org/10.1186/1471-2148-10-104>.  
 Pilot, M., Dąbrowski, M.J., Hayrapetyan, V., Yavruyan, E.G., Kopalani, N., Tsingarska, E., Bujalska, B., Kaminiski, S., Bogdanowicz, W., 2014. Genetic variability of the grey wolf *Canis lupus* in the caucasus in comparison with Europe and the Middle East: distinct or intermediary population? *PLoS One* 9, <http://dx.doi.org/10.1371/journal.pone.0093828>.

- Ražen, N., Brugnoli, A., Castagna, C., Groff, C., Kaczensky, P., Kljun, F., Knauer, F., Kos, I., Krofel, M., Luštrik, R., Majič, A., Rauer, G., Righetti, D., Potočnik, H., 2016. Long-distance dispersal connects Dinaric-Balkan and Alpine grey wolf (*Canis lupus*) populations. *Eur. J. Wildl. Res.*, 137–142, <http://dx.doi.org/10.1007/s10344-015-0971-z>.
- Randi, E., Lucchini, V., 2002. Detecting rare introgression of domestic dog genes into wild wolf (*Canis lupus*) populations by Bayesian admixture analyses of microsatellite variation. *Conserv. Genet.* 3, 31–45, <http://dx.doi.org/10.1016/j.earscirev.2006.09.002>.
- Randi, E., Lucchini, V., Christensen, M.F., Mucci, N., Funk, S.M., Dolf, G., Loeschcke, V., 2000. Mitochondrial DNA variability in Italian and east European wolves: detecting the consequences of small population size and hybridization. *Conserv. Biol.* 14, 464–473, <http://dx.doi.org/10.1046/j.1523-1739.2000.98280.x>.
- Randi, E., Alves, P.C., Carranza, J., Milosevic-Zlatanovi, S., Sfougaris, A., Mucci, N., 2004. Phylogeography of roe deer (*Capreolus capreolus*) populations: the effects of historical genetic subdivisions and recent nonequilibrium dynamics. *Mol. Ecol.* 13, 3071–3083, <http://dx.doi.org/10.1111/j.1365-294X.2004.02279.x>.
- Randi, E., Hulva, P., Fabbri, E., Galaverni, M., Galov, A., Kusak, J., Bigi, D., Bolíková, B.Č., Smetanová, M., Caniglia, R., 2014. Multilocus detection of wolf x dog hybridization in Italy, and guidelines for marker selection. *PLoS One* 9, <http://dx.doi.org/10.1371/journal.pone.0086409>.
- Rutledge, L.Y., Patterson, B.R., White, B.N., 2010. Analysis of *Canis* mitochondrial DNA demonstrates high concordance between the control region and ATPase genes. *BMC Evol. Biol.* 10, 1–12.
- Stronen, A.V., Jedrzejewska, B., Pertoldi, C., Demontis, D., Randi, E., Niedziałkowska, M., Pilot, M., Sidorovich, V.E., Dykyy, I., Kusak, J., Tsingarska, E., Kojola, I., Karamanlidis, A.A., Ornicans, A., Lobkov, V.A., Dumenko, V., Czarnomska, S.D., 2013. North-south differentiation and a region of high diversity in european wolves (*Canis lupus*). *PLoS One* 8, 1–9, <http://dx.doi.org/10.1371/journal.pone.0076454>.
- Valière, N., Fumagalli, L., Gielly, L., Miquel, C., Lequette, B., Poulle, M., Weber, J., Arlettaz, R., Taberlet, P., 2003. Long-distance wolf recolonization of France and Switzerland inferred from non-invasive genetic sampling over a period of 10 years. *Anim. Conserv.* 6, 83–92, <http://dx.doi.org/10.1017/S1367943003003111>.
- Vilà, C., Amorim, I.R., Leonard, J.A., Posada, D., Castroviejo, J., Petrucci-Fonseca, F., Crandall, K.A., Ellegren, H., Wayne, R.K., 1999. Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*. *Mol. Ecol.* 8, 2089–2103.
- VonHoldt, B.M., Pollinger, J.P., Earl, D.A., Knowles, J.C., Boyko, A.R., Parker, H.G., Geffen, E., Pilot, M., Jedrzejewski, W., Jedrzejewska, B., Sidorovich, V.E., Greco, C., Randi, E., Musiani, M., Kays, R., Bustamante, C.D., Ostrander, E.A., Novembre, J., Wayne, R.K., 2011. A genome-wide perspective on the evolutionary history of enigmatic wolf-like canids. *Genome Res.* 21, <http://dx.doi.org/10.1101/gr.116301.110>.
- Weckworth, B.V., Dawson, N.G., Talbot, S.L., Cook, J.A., et al., 2015. Genetic distinctiveness of Alexander Archipelago Wolves (*Canis lupus ligoni*): reply to Cronin et al. (2015). *J. Hered.*, 1–3, <http://dx.doi.org/10.1093/jhered/esv026>.
- Zachos, F.E., Mattioli, S., Ferretti, F., Lorenzini, R., 2014. The unique Mesola red deer of Italy: taxonomic recognition (*Cervus elaphus italicus* nova ssp., Cervidae) would endorse conservation. *Ital. J. Zool.* 81, 136–143, <http://dx.doi.org/10.1080/11250003.2014.895060>.
- Zimen, E., Boitani, L., 1975. Number and distribution of wolves in Italy. *Mamm. Biol.—Z. Säugetierkd.* 40, 102–112.