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Biodiversity lost

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Biodiversity lost: The phylogenetic relationships of a complete mitochondrial DNA genome sequenced from the extinct wolf population of Sicily

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Abstract

Using next-generation sequencing, we obtained for the first time a complete mitochondrial DNA genome from a museum specimen of the extinct wolf (*Canis lupus*) population of the island of Sicily (Italy). Phylogenetic analyses indicated that this genome, which was aligned with a number of historical and extant wolf and dog mitogenomes sampled worldwide, was closely related to an Italian wolf mtDNA genome (the observed proportion of nucleotide sites at which the two sequences are different was p = 0.0012), five to seven times shorter than divergence among Sicilian and any other known wolf mtDNA genomes (p range = 0.0050 – 0.0070). Sicilian and Italian mitogenomes joined a basal clade belonging to the mtDNA haplogroup-2 of ancient western European wolf populations (Pilot et al., 2010). Bayesian calibration of divergence times indicated that this clade coalesced at MRCA = 13.400 years (with 95% HPD = 4000 – 21.230 years). These findings suggest that wolves probably colonized Sicily from southern Italy towards the end of the last Pleistocene glacial maximum when the Strait of Messina was almost totally dry. Additional mtDNA and genomic data will further clarify the origin and population dynamics before the extinction of wolves in Sicily.

Key words: Sicilian wolf; *Canis lupus*; complete mtDNA genome; next-generation sequencing; island extinctions.

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Introduction

In the last centuries, the extension, abundance and genetic diversity of many animal populations have been negatively influenced by humans (Li et al., 2016). In particular, populations of large vertebrates and top predators have decreased due to anthropogenic environmental changes such as deforestation, decline of natural prey and direct persecution (Chapron et al., 2014). The wolf (*Canis lupus*) is one of the few large predators that survived the Pleistocene faunal turnover (Loog et al., 2018), but its populations have fluctuated widely throughout the Holocene and in recent centuries as a result of the excessive hunting of wild ungulates and direct shootings (Leonard et al., 2005; Randi, 2011). During those periods, the wolves disappeared from the southern and central regions of North America and from most of the countries of western and central Europe. Although some wolf populations are currently expanding, re-colonizing parts of their historical territories, aided by legal protection, controlled hunting and active conservation, demographic declines have led to the local extinction of several small and isolated populations (Linnell et al., 2008). For example, the last wolf Honsu (*C. l. hodophilax*), a dwarf subspecies endemic to the main islands of Japan, was killed in 1905 (Ishiguro et al., 2009). The wolf Ezo (*C. l. hattai*), found only in Hokkaido, Japan, was uprooted from the island in the late 1800s (Ishiguro et al., 2010). Furthermore, the small isolated wolf population of Sierra Morena, in central Spain, has recently been confirmed extinct (Gómez-Sánchez et al., 2018).

Fossil remains indicate that wolves have been present in Europe since at least the end of the middle Pleistocene at about 0.5-0.3 million years ago (Sotnikova and Rook 2010). However, those ancestral populations, which exhibited a suite of distinct ecologic and morphologic traits, have been completely substituted by contemporary wolves that have spread throughout Europe *c*. 25,000 - 20,000 years ago (Pilot et al., 2014; Loog et al., 2018). The extant wolves in the Italian peninsula (a distinct subspecies *Canis lupus italicus* Altobello, 1921) are genetically divergent from all other wolf populations in Europe, due to their long-lasting isolation south of the Alps, and to recent natural and anthropogenic bottlenecks (Lucchini et al . 2004, Pilot et al 2014). The wolves of peninsular Italy barely survived in the second half of the twentieth century, when they were limited to the southernmost parts of the Apennines and numbered less than 100 individuals (Boitani 1984). During this long period of demographic decline and range contraction, wolves in the Alps and the Apennines lost almost all of their mtDNA diversity, which in the past was much

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larger than the current one (Dufresnes et al., 2018). Since the middle of the last century, the recovery of Italian wolves has been spectacular, but the fate of Sicilian wolves has been less positive and the population has become extinct (Angelici et al., 2019).

The historical records of Sicilian wolves are scarce. Their phylogeographic origins, historical distribution and abundance on the island are largely unknown (but see: Angelici et al., 2019). Most probably the wolves were already rare in the first half of the 1800s, probably due to the loss of habitat and of natural preys (Minà Palumbo 1868). Direct hunting was one of the main causes of the decline of the Sicilian wolf population and final extinction (Pratesi 1978). Although some anecdotal reports mentioned the presence of wolves until 1959 (Pasa A., 1959). An mtDNA control-region sequence showed that the skin of an alleged wolf shot in 1924 in Bellolampo (Palermo; preserved in the Regional Museum of Terrasini), belongs either to a domestic dog (Angelici et al. 2019), morphologically confused with a wolf, or to a hybrid showing maternal mtDNA introgression. Although morphological traits and molecular identifications of the few specimens still preserved in museums have recently been described (Angelici et al. 2019), the origin and phylogenetic relations of the extinct Sicilian wolf population still need further investigation.

Sicily has been repeatedly isolated and connected to mainland peninsular Italy as a result of the eustatic fluctuations of the Mediterranean (Anzidei et al., 2012). Paleogeographic reconstructions and paleontological data document periods of intense African-Sicilian faunal interchange across the Strait of Sicily during the Messinian bridge, c. 5.3 million years ago (Stock et al., 2008). More recently, at the glacial peaks of the Pleistocene, the north-eastern coasts of Sicily have been repeatedly connected to Calabria, the tip of the south-western Italian peninsula (Antonioli et al., 2014). The Strait of Messina, currently 3.2 km wide and 80-120 m deep, has been drained at glacial peaks (Antonioli et al., 2014), and temporary terrestrial bridges have been used to colonize the island from a number of animal populations (Palombo 2018), including several amphibians and reptiles (Frits et al., 2004 et al., 2016), a galliform bird (*Alectoris graeca whitakeri*, Randi et al., 2003), and European wild cats (*Felis silvestris*, Mattucci et al., 2013).

In this study, we hypothesize that wolves in Sicily have been effectively isolated from mainland Italian populations at least since the flooding of the last Pleistocene land-bridge across the Strait of Messina. We sequenced for the first time a complete mitogenome of the Sicilian wolf, obtained from a museum-preserved specimen. With these data, we aim to evaluate the phylogenetic relationships of this individual's mtDNA with extant and historical wolves from Italy

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and worldwide, and to estimate the Sicilian wolf divergence times based on a completed mitogenome and not limited to very short mtDNA control-region sequences. Moreover, assuming that in the case of hybridization, morphologic identifications can be misleading, we aimed to identify the origin of the sequenced mitogenome, either if from a wolf or a dog ancestral maternal population via hybridization. We hope that these results will contribute to reconstructing a plausible scenario of wolf colonization of the island of Sicily.

Materials and Methods

DNA extraction

A tissue sample was collected from a stuffed wolf specimen (Fig. 1) preserved at the Civic Museum "Baldassare Romano", Termini Imerese (Palermo; Italy). The origin of this specimen is not documented, although Angelici and Rossi (2018) suggest that the wolf was probably killed on Monte San Calogero near Termini Imerese (Palermo). We sampled the distal portion of the second finger of the front leg, including the bone of the phalanx and the nail. The DNA was extracted only from the inner parts of the phalanx and the nail, which are somehow protected by exogenous contaminations and degradation. Before extraction, the sample surfaces were exposed to UV radiation for 30 minutes. We split the sample into two parts, phalanx and nail, which were processed independently. We collected the inner dry tissue remains by drilling the nail and grinding the phalanx. Thus we obtained two lots of powder, which weighted 324 mg and 252 mg, respectively. The tissue powder was stored into two sterile UV-decontaminated test tubes. We used these aliquots to independently extract two DNA samples using a ChargeSwitch® Forensic DNA Purification Kit (Thermo Fisher), following the producer's procedure. We chose a silica bonding method which includes a few extraction steps and therefore should minimize DNA losses. The samples were lysed overnight in agitation at 50°C; the DNA bound to magnetic beads was cleaned by three washing step. We eluted twice the extractions by incubation for 10 minutes at room temperature with 25 ml of elution buffer obtaining a final volume of 50 ml of DNA solution. Since historical DNA is already fragmented, incubation at 50°C during the night should not affect fragmentation, thus maximizing DNA recovery. We repeated the elution once again, but we did not

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find any residual trace of DNA, showing the efficiency of the dilution step. Finally, the two DNA extractions were pooled to obtain a single DNA sample. To minimize the risk of contaminations by exogenous DNA, the samples were processed in a environmental DNA lab where decontaminating procedures have been previously applied to ensure DNA-free zones. Nothing else was processed in the lab those days. We used a separate area of the lab dedicated to special applications, easy to decontaminate and that was never employed for *Canis* spp DNA. All bench-tops and equipment were flamed or cleaned with bleach and ethanol. We used a 1% solution of sodium hypochlorite, sprayed on the lab surfaces and left for 5 min before drying and wiped with 70% ethanol to produce levels of gaseous chlorine at the recommended exposure limits (Ballantyne et al., 2015). All the equipment were exposed to UV overnight in a sterile laminar-flow hood. We used pipette tips with sterile aerosol filters.

NGS library preparation and DNA sequencing

The quality and concentration of the pooled DNA was checked and quantified by spectrophotometer analysis using Nanodrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA), Qubit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and TapeStation (Agilent Technologies Inc. Santa Clara, California) equipments. An empty extraction sample containing all the chemicals in the kit, but without DNA, was used as a negative control. No DNA was detected in the blank sample by spectrophotometric analysis of the meat station. Due to the absence of DNA, we did not sequence the negative samples. The composition of fragment size and concentration for each size range of the input DNA was analyzed in the Tapestation. The volume of the input DNA solution was determined by evaluating the concentration of the most frequent fragment range, between 200 and 400 bp. The smaller fragments were removed by purification following the Agencourt (Bechman) beads protocol.

We used the Illumina Truseq DNA nano kit (Illumina Inc., San Diego, CA, USA) for library preparation according to the manufacturer instructions with some modifications as indicated below. We chose not to apply the fragmentation steps because the DNA was already fragmented as shown by the spectrophotometric analysis. The extracted DNA resulted already in small fragment useful for the sequencing steps. In the second phase, the DNA fragments were blunted using the repair mixture 2 at 30 ° C for 30 minutes. The shortest DNA fragments were removed with 250 µl of undiluted Sample Purification Beads reagent (SPB). A single adenine nucleotide was added to the 3’

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ends of the blunt fragments to prevent self-ligation and provide complementary bases for adapters. Adapter ligation was performed using LIGation Mix2; adapter dimers were removed by SPB cleaning. The library was PCR-amplified with the PCR Primer Cocktail at 95°C for 3 minutes, 8 cycles of: 98°C for 20 seconds, 60°C for 15 seconds, 72°C for 30 seconds, 72°C for 5 minutes, hold at 4°C. A library composed by a fragment distribution centered at the 295 bp was obtained; It was normalized at the concentration of 4nM and then diluted at 12.5 pM. PhiX Control library (v2; Illumina) was added and the library was sequenced on a MiSeq platform (Illumina Inc., San Diego, CA, USA), using a SBS MiSeq Reagent Kit v2 with a 150 Paired End run.

Bioinformatic analyses and mtDNA genome assemblage

Image analysis, base calling and data quality assessment were performed on the MiSeq that generated the BCL files. Casava 1.8 embedded into Illumina Base space wrapper (Illumina Inc., San Diego, CA, USA) was used for de-multiplexing and for the conversion of the BCL files to compressed Fastq files. Adapter sequences were removed with AdapterRemoval 2.3.0 (Schubert et al. 2016); reads were trimmed for low quality bases and overlapping reads for at least 11 nucleotides were merged using the following parameters: trimns, minlength 30, trimqualities, minquality 35, collapse. The processed reads were mapped against the complete mtDNA reference genome of an Italian wolf (GenBank accession number KF661048) by means of the BWA-MEM 0.7.17 aligner algorithm (Li and Durbin, 2010). We extracted 2130 reads, which mapped onto the reference mitogenome. Mapped reads were filtered using MapDamage 2.0 (Jónsson et al., 2013) and quality scores of C->T or G->A transitions, potentially due to post-mortem DNA damage, were adjusted according to the position in reads and damage patterns. Filtered reads mapped onto mtDNA (6.618 paired end reads) were extracted from the BAM file using SAMTools 1.4 (Li et al., 2009), reconverted to Fastq format and then assembled into a complete consensus sequence with Spades 3.11 (Bankevich et al. 2012) for subsequent analysis of concatenated mtDNA genome . The quality of assembled mtDNA genome was evaluated using Quast tool (Gurevich et al., 2013). DNA SNPs (Table 2) were validated by manual inspection using IGV 2.4.6 (Robinson et al., 2011) . BWA-MEM was used to map specific reads of sex-determining Y gene (SRY) region present within our FASTQ files against dog SRY sequence (AF107021.1) as reference. To confirm the presence of SRY sequences within our sample, we performed a search for mapped reads with blast algorithm as implemented in NCBI website (Altschul et al. 1990) with the parameters: *expect threshold: 10, word size: 28, match score: 1,*

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mismatch score: -2, gap costs: linear; filtered for low complexity, the more significant match was with Canis lupus familiaris chromosome Y genomic sequence, accession number KP081776.1, with a 100% of identity and a e-value of $5e^{-49}$

The processed reads were mapped against the complete mtDNA reference genome of an Italian wolf (GenBank accession number KF661048) by means of BWA-MEM 0.7.17 aligner algorithm (Li and Durbin, 2010). The new Sicilian wolf mitogenome was then realigned with the sequences of the three Italian wolf mtDNA genomes in GenBank: KF661048.1 (Thalmann et al. 2013); KU696389.1 and KU644662.1 (Koblmuller et al. 2016), and with a sample of the many complete *Canis lupus* mitogenomes in the GenBank using ClustalW (Higgins et al., 1994) in MEGAX (Kumar et al. 2018) with default parameters. Correct translations were taken into account. Short overlapping DNA segments, eventual incomplete stop codons and mutations at the three codon positions were identified using MEGAX*.* RNA sequences were identical to homologous *Canis lupus* sequences in GenBank, so we did not reconstruct secondary structures. Missing and ambiguous bases were excluded and not used in phylogenetic analyses.

Phylogenetic analyses and estimates of divergence times

We analysed two different datasets that were generated aligning the new Sicilian and other *Canis lupus* mitogenomes downloaded from GenBank (Table 1). Set#1 includes 51 complete modern wolf mitogenomes used by Koblmuller et al. (2016), three historical wolf samples, 14 dogs (*C. l. familiaris*) and five Himalayan wolves (named *C. l. laniger* or *C. l. chanco*) that were used as outgroups. Set#2 includes 26 modern wolf mitogenomes used by Thalmann et al. (2013), two historical Ezo wolf (*C. l. hattai*) and three historical Honsu wolf (*C. l. hodophilax*; Matsumura et al. 2014), four Himalayan wolves and four coyote mitogenomes that were used as outgroups. In these two alignments, we excluded the control-regions (CR), which were only partially sequenced in the historical and in some of the modern samples. The alignments were manually checked in MEGAX and adjusted. Moreover we blasted the new Sicilian wolf mtDNA CR in GenBank to search for eventual matching with other wolf or dog CRs. In particular we were aiming to assess if the new Sicilian wolf CR was shared with the available CRs sequenced from the historical wolves which lived in the Alps and Italian peninsula (Dufresnes et al. 2018) or in Sicily (Angelici et al. 2018).

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Table 1. List of complete *Canis* mitogenomes aligned and used in phylogenetic analyses (n = number of mitogenomes).

Alignment	Mitogenomes (n)	References
Set#1	Sicilian wolf (1)	This study (GenBank MH891616.1)
	Italian wolves (1)	Thalmann et al. 2013 (KF661048.1)
	Modern wolves (50)	Koblmuller et al. 2016
	Historical wolves (3)	Koblmuller et al. 2016
	Dogs (14)	Koblmuller et al. 2016
	Himalayan wolves (5)	Koblmuller et al. 2016
Set#2	Sicilian wolf (1)	This study (MH891616.1)
	Italian wolves (3)	Thalmann et al. 2013 (KF661048.1) Koblmuller et al. 2016 (KU696389.1; KU644662.1)
	Modern wolves (23)	Thalmann et al. 2013
	Historical Ezo wolves (2) Historical Honsu wolves (3)	Matsumura et al. (2014)
	Himalayan wolves (4)	Thalmann et al. 2013
	Coyotes (4)	Thalmann et al. 2013

We performed a preliminary DNA substitution model selection of the mitogenome alignments in Set#1 and Set#2 using IQtree 1.6.8. (Nguyen et al. 2014) with the Model Finder+Tree reconstruction procedure. We used IQtree to compute maximum-likelihood (ML) trees with the best-fit model (TN+F+R6; TN = TN93; Tamura & Nei, 1993; F = empirical base frequencies; R6 = generalized free-rate Gamma-distribution with 6 categories; Yang 1995). The trees were rooted using the five Himalayan wolf (*C. l. laniger* and *C. l. chanco*; Set#1) or the four coyote (DQ480509, DQ480510 and DQ480511, Björnerfeldt et al. 2006; KF661096.1, Thalmann et al. 2013; Set#2) mitogenomes as outgroups. For comparative purposes, we analysed the Set#1 and Set2# alignments in MEGAX using the FindBest DNA Model maximum-likelihood (ML) procedure. The best-fit substitution model was TN93+G, followed by HKY+G (G = G-distribution with 6 categories, corresponding to R6 in IQtree). We used the Neighbor-joining procedure (NJ; Saitou and Nei 1987) with these substitution models and heterogeneous lineages evolution. Support to the phylogenetic

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tree internodes were determined by 1000 interior-branch length test of minimum evolution trees (ME; Nei and Kumar 2000) and by 1000 bootstrap samplings of NJ trees (Tamura et al. 2013). We identified and visualized transitions, transversions, variant sites and repeat motifs copy numbers in MEGAX. All positions containing missing data were excluded.

We used the Set#2 alignment to compute a Bayesian majority-rule phylogenetic tree using BEAST 2.5.1 (Drummond et al. 2012), with the HKY+G model. Markov chain Monte Carlo (MCMC) samples were drawn every 1000 generations from a total of 1,000,000 generations, following a discarded burn-in, based on Tracer 1.7.1 (Drummond and Rambaut 2007) outputs, of 100,000 generations. BEAST was used to infer the age of the most recent common ancestor (MRCA) of a clade joining the Italian and Sicilian wolf mtDNA, which was calibrated using the divergence times among Japanese wolves (MRCA = 46,800; 95% highest probability density HPD = 37,500–58,000 years) as estimated by Matsumura et al. (2014). Both the uncorrelated log-linear model (Drummond et al. 2006) and the strict clock model were tested for the molecular clock, and both the Bayesian skygrid model (Gill et al. 2013) and a constant model for the population size. The convergence and performance of different models were assessed using Tracer 1.7.1. The consensus tree was visualized with FigTree 1.4.4 (A. Rambaut et al. 2018) or with TreeAnnotator in BEAST. The configuration files for BEAST were done using BEAUti (in the BEAST package).

Results

The quantity and quality of the DNA extracted from the inner nail and distal bone phalange of an historical wolf from the now extinct Sicilian population was good enough to obtain reliable mtDNA sequences by next-generation procedure. Good DNA quality was indicated by a value of the ratio 260/280 nm = 1.9, which suggested a low presence of inhibitors. The total double-strand DNA concentration was = 1.6 ng/µl. DNA fragments were distributed normally with size ranging from 40 bp to 1040 bp, centered at 250 bp, with a concentration of 0.6 ng/µl. Based on the results of these quality-controls we omitted the initial DNA fragmentation step for library preparation. The DNA was directly PCR-enriched for fragments spanning from 165 bp to 655 bp, centered at 295 bp, (Fig. 2). We obtained a library of about 12 GB for the paired-end readings (5.8 GB for the fastq R1 and 5.9 GB for the fastq R2 files). We recovered about 3,150 paired-end readings (about 6,300 readings in total; DNA concentration = 2.49 ng/µl) with an average length of 100 bp. Taking into account the

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presence of overlapping and duplicate readings and that the mitogenome of the wolf is about 16.500 bp, the coverage for the mtDNA was *c*. 20X.

The reads were assembled into a complete Sicilian wolf mtDNA, which was 16,678 bp long (GenBank accession number: MH891616.1). Direct inspection (in MEGAX) of the alignment with reference dog and Italian wolf homologous sequences, showed that all the expected mtDNA tRNA, rRNA and protein-coding genes were correctly identified and mapped; these sequences did not show any anomalous stop codon and translated into the expected RNAs or proteins. Thus, we assumed that this mtDNA genome was authentic. In comparison with the known Italian wolf mtDNA genomes, the Sicilian wolf exhibited 14 silent transition substitutions and only one first-position G-A mutation that changed a V into an M aminoacid residue at codon 21 of the ATP6 gene in the Sicilian wolf (Table 2). The mtDNA CR of the Sicilian wolf was 1,219 bp, that is 50 bp shorter than the corresponding sequence of the Italian wolf CR due to 10 missing copies of a CGGTACACGT repeat (Kim et al. 1998).

*Table 2. List of mutation differences between the Italian wolf reference (*KF661048.1; Thalmann et al. 2013) *and the new Sicilian wolf (*MH891616.1) *mitogenomes. The variant sites were identified and visualized in MEGAX.*

mtDNA gene	Position	Mutation
ND1	3458	C-T
ND1	3497	C-T
ND2	4025	A-G
ND2	4352	T-C
COX1	6072	T-C
COX1	6111	T-C
ATP6	8025	G-A [V-M]
ND3	9637	T-C
ND3	9763	C-T

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ND4	11326	T-C
ND4	11515	G-A
ND4	11838	G-A
ND5	13575	T-C
ND6	13863	T-C
ND6	14937	T-C
CR	15615	C-T
CR	15629	C-T
CR	15804	C-T
CR	16018	C-T

The observed proportion of nucleotide sites at which the Sicilian and Italian wolf mitogenomes are different (*p-distance*) was p = 0.0012 (identical to the estimated TN93 distance), that is five to seven times shorter than genetic distances (D) among the Sicilian and any other wolf mitogenomes (D = 0.0050 – 0.0070). The p-distance between Sicilian and Italian wolves CR was D = 0.0181; hence, as expected (Stoneking 2000), the CR mutated faster than the RNA and protein-coding mtDNA sequences.

The best-fit model of nucleotide substitution in Set#1 and Set#2 was TN+F+R6 (BIC = 67166.7818); the next one was the HKY+F+R6 (Hasegawa et al. 1985), with BIC = 67181.2424. The maximum-likelihood (ML) trees computed by IQtree with the best-fit model are shown in Fig. 3 (Set#1) and Fig. 4 (Set#2). In both trees the mitogenome of the Sicilian wolf joined a basal phylogenetic clade that included the Italian wolf and two mtDNA genomes respectively sequenced from a wolf sampled in Poland (KF661045.1) and in Belarus (KU696390.1). This clade (hereafter named the Italian clade) was basal to all the other modern wolf and dog genomes, excluding the two Pleistocenic wolves included in set#1: KF661088.1 (estimated age 28,000 years, from Alaska) and KF661081.1 (18,000 years from Russia). However, the internodes connecting the main wolf clades were weakly supported by bootstrap analyses (Fig. 3 and Fig. 4). The Sicilian wolf mitogenome in the Italian clade was basal to the peninsular Italian wolf mitogenome. Bootstrap and interior-branch length tests showed that the Italian clade was 99% - 100% supported. The same position of the Sicilian wolf mitogenome in the Italian clade and the same topology of the trees were obtained in MEGAX with the best-fit TN93+G substitution model and the NJ procedure.

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The Bayesian majority-rule consensus phylogenetic trees (Fig. 5) obtained analysing the Set#3 mtDNA genomes fully supported the IQtree and MEGAX results. The Japanese and Italian wolf clades were basal to all the other modern wolf and dog genomes. The average coalescence time of the Italian clade, as estimated in BEAST following Matsumura et al. (2014), was MRCA = 13,400 years (with 95% highest probability density HPD = 4000 – 21,230; Fig. 6). The divergence times of the Italian wolf and Japanese wolf from the other wolf clades were similar (*c*. 100,000 years), highlighting the ancient origins of wolves in peninsular Italy and Sicily in comparison to other wolves and dogs worldwide. The Italian and Sicilian wolf mitogenomes belong to the wolf haplogroup-2, that includes all the ancient wolves sampled in western Europe dating from between 44,000 and 1,200 years ago (Pilot et al. 2010).

The mtDNA CR of the Sicilian wolf was blasted in GenBank to search for eventual best match matching with domestic dog CRs. The CR of the Sicilian wolf did not match with any of the dog sequences known so far, thus supporting its origin in a wild wolf populations. The sequence was identical to a partial mtDNA CR sequence found by Dufresnes et al. (2018) in a different wolf sample from Sicily (their haplotype H3 from sample AN855; Museo di Zoologia P. Doderlein, Palermo). The sequence was also identical to two partial mtDNA CR sequences (MK129178.1 and MK129179.1) found by Angelici et al. (2018). Thus, this mtDNA genomes was apparently unique for the extinct wolf population of Sicily.

We analyzed the stored genomic DNA reads to search for specific sex markers. We identified SRY sequences matching with the homologous *Canis lupus* chromosome Y genomic sequences present in GenBank (AF107021.1), thus indicating unequivocally that the studied specimen was a male.

Discussion

In this study we obtained for the first time a complete mitogenome of a wolf from the now extinct Sicilian population. This wolf was likely killed on Mt. San Calogero, near the city of Termini Imerese, probably in the last years of the nineteenth century, very near to the extinction of the island population, for the last documented wolf was killed in 1935. The DNA extracted from this sample was of quality good enough to obtain reliable mtDNA coverage and sequences using next-generation procedures. Due to the well known high copies number of the mtDNA genome per cell,

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depending on the type of tissue (Bogenhagen et al., 1974), mtDNA coverage can easily exceed the coverage of genomic DNA sequences (Picardi et al., 2012), thus facilitating reliable library construction. These results could also be explained by the relatively good quality of the DNA, which was extracted from the inner part of a nail and distal phalange bone, in fact a protected environment from exogenous contaminations and from DNA degradation. The tissue sample was collected from a wolf killed about one century ago, which should be considered historical and not truly ancient.

The control-region of the new Sicilian wolf mitogenome is identical to a partial CR sequenced from a different Sicilian wolf sample (Dufresnes et al. 2018), and to two partial CR sequences obtained from another Sicilian wolf specimen, as mentioned by Angelici et al. (2018). These results, pending further evidence from molecular studies of additional Sicilian wolf samples, suggest that, during the last few decades before the extinction, the wolf population of Sicily showed a unique mtDNA haplotype and low genetic diversity. Phylogenetic analyses also indicate that the mitogenome of the Sicilian wolf is closely related to the predominant mitogenome of the past and extant wolf population in peninsular Italy (Dufresnes et al. 2018; Randi et al. 2014; Montana et al. 2017). The Sicilian and Italian wolf mitogenomes join in a strongly supported clade (the Italian clade) which includes also two mtDNAs sequenced from two wolves sampled in Poland and in Belarus, respectively.

This clade is basal to all the other modern wolf and dog haplogroups sequenced so far, with the exception of most of the ancestral sequences obtained from Pleistocenic wolves (Thalmann et al. 2016; Koblmuller et al. 2016), and from the now extinct Japanese wolves (*C. l. hodophilax*; Matsumura et al. 2014). The origin and fate of the Japanese wolves has been described Matsumura et al. (2014). Both the Japanese and Italian wolf clades, which apparently split *c*. 130,000 – 100,000 years from all the other modern wolf haplogroups worldwide, belong to the mtDNA haplogroup-2 (Pilot et al. 2010). This haplogroup has been detected in the ancient western European wolf population that were largely substituted by the recent spread of modern wolves, which showed the more recent mtDNA haplogroup-1 (Pilot et al. 2010). However, these mitogenomes clearly indicate that both extant wolves in Italy and extinct wolves in Sicily are by far more recent than Himalayan wolves, formerly considered a subspecies of *C. lupus* and named *C. l. laniger* or *C. l. chanco*, but now ranked as distinct species *C. himalayensis* (Aggarwal et al. 2007). They diverged *c*. 550,000 (95% HDP = 495,100–605,600) years ago (Matsumura et al. 2014), and predated the evolutionary radiation of Eurasian and New World *C. lupus*.

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In this study we used Matsumura et al. (2014) estimates of Japanese wolf mtDNA divergence time to compute a MRCA = 13,400 years (95% HPD = 4000 – 21,230) of the Italian wolf clade. The mtDNA genome of the Sicilian wolf is basal to the Italian wolf clade, thus a MRCA = 13,400 years can be considered as an approximate estimate of island-mainland wolf mtDNA divergence. Although phylogenetic relationships and divergence time estimates obtained from complete mtDNA genomes should, in principle, outperform estimates obtained using only shorter sequences, they should anyway be used with caution. First, the mtDNA is a maternal haploid genome informative only on single-gene relationships and not on population-species phylogeny. Then, the sample size used in our and other studies (e.g., Angelici et al. 2019) are by far too small to exclude uncertainty. The wolf population in Sicily is extinct, the available museum specimens are few and perhaps not always suitable for genomic studies, thus the sample size of the target population could not be much expanded. However, we believe that the addition of complete mitogenomes from other haplogroup-2 wolf populations could improve the phylogenetic structure and connections of the Italian wolf clade, allowing more reliable estimates of divergence times. Moreover, sequences form chromosomal genes could contribute to better describe the phylogeographic history of the wolf population in Sicily.

The mtDNA CRs of Sicilian wolves are distinct from homologous CR sequences of historical Italian wolves obtained by Dufresnes et al. (2018). Wolves in peninsular Italy were certainly abundant a few centuries ago, but the museum specimens suitable to DNA sequencing are too few to conclude that the Sicilian haplotypes were absent in the historical mainland population. Hence, we cannot exclude that the Sicilian mtDNA haplotypes evolved in peninsular Italy. However, based on the available data, the most parsimonious hypothesis is that those haplotypes evolved in Sicily following wolf colonization of the island. The divergence time of 13,400 years (95% HPD = 4000 – 21,230) of the Sicilian wolf mtDNA is compatible with the age of the last land bridge between the island and the south-western tip of Italy, which, according to Antonioli et al. (2012) lasted for at least 500 year from 21,500 to 20,000 years ago. A late Pleistocene colonization of peninsular wolves before the Messina strait was definitely flooded less than 20,000 years ago does not exclude earlier colonization waves, which, seems, nevertheless undocumented by the available Sicilian wolf specimens. These estimates of mitogenome divergence time and landbridge connections should, anyway, be considered as preliminary due to the uncertainty (and large HPD) inevitably consequent to both paleogeographic and molecular dating.

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Conclusions

During late Holocene a number of species, and in particular ungulates (red deer, fallow deer, roe deer, wildboar), the natural prey of wolves, went extinct in Sicily like due to anthropogenic pressures (La Mantia and Cannella 2008). The concomitant consequences of habitat transformations, ungulate decline and overhunting most probably pushed the wolf population of Sicily to decline and finally disappearing. The few available stuffed specimens evidence smaller body size and paler coat colours of the last wolves in Sicily in comparison to the Italian wolves. Moreover wolves in Sicily did not show the darker fur strip on the front legs, a morphological trait typical, but not unique of the peninsular Italian wolf population (Altobello 1921; Ciucci and Boitani 2003). Dwarfism and local phenotypic adaptations are typical of some island vertebrate populations. Moreover, we cannot exclude that during the final population bottleneck wolves in Sicily crossbred and hybridized with free-ranging dogs, perhaps accelerating the speed of the extinction vortex (see: Gómez-Sánchez et al. 2018). Future genomic data and analyses could perhaps shed more light on the extent of homozygosity and eventual domestic dog introgression in the lost population of wolves in Sicily.

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Figure Legends

Figure 1. A picture of the sampled and genotyped Sicilian wolf from the Civic Museum "Baldassare Romano", Termini Imerese (Palermo; Italy).

Figure.2: Capillary DNA analysis on Tape station electrophoretic systems demonstrating the size bands distribution for the obtained library

Figure 2. Maximum-likelihood tree of wolf mitogenomes used by Koblmuller et al. (2016), including three historical wolf samples, 14 dog (*C. l. familiaris*) genomes and the new Sicilian wolf mitogenome. Five Himalayan wolf (here named *C. l. laniger* or *C. l. chanco*) mtDNAs were used as outgroups. The Italian clade is indicated. Bootstrap values at the internodes.

Figure 3. Neighbor-joining tree of modern wolf mtDNA genomes (control-region excluded) used by Thalmann et al. (2013), and the new Sicilian wolf mtDNA genome. Four coyote (*C. latrans*) mtDNAs were used as outgroups. The Italian clade is indicated. Bootstrap values at the internodes.

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Figure 4. Consensus Bayesian phylogenetic tree computed by BEAST 2.5.1 (Drummond et al. 2012) with the HKY+G model (Hasegawa et al. 1985). We used a subset of wolf mtDNA genomes (control-region excluded) published by Matsumura et al. (2014), including two historical Ezo wolf (*C. l. hattai*), five historical Honsu wolf (*C. l. hodophilax*) and the new Sicilian wolf mtDNA genome. Four coyote mtDNAs were used as outgroups. The Markov chain Monte Carlo samples were drawn every 1000 generations from a total of 1.000.000 generations, following a discarded burn-in of 100.000 generations.

Figure 5. Age estimates (indicated by bar lengths) of the nodes of the consensus phylogenetic trees computed by BEAST 2.5.1 with the HKY+G nucleotide substitution model.

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Figure 1.

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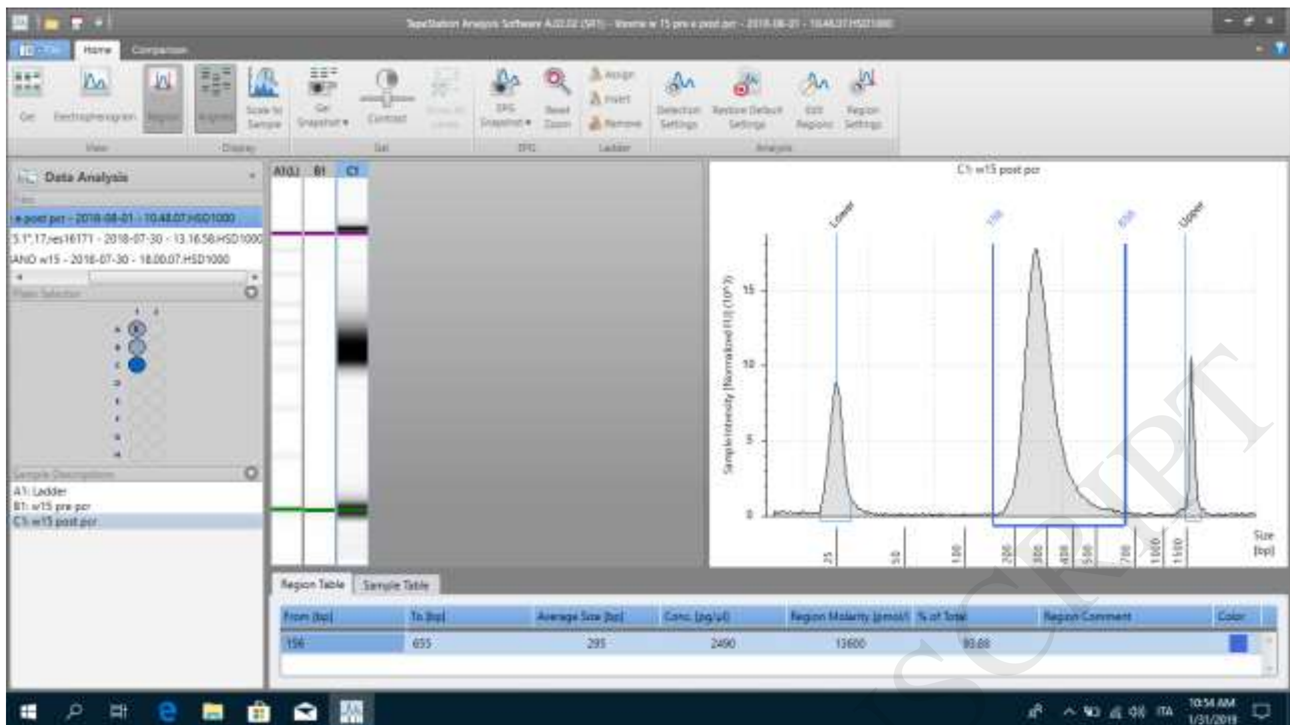

Figure 2

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Figure 3.

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Figure 4.

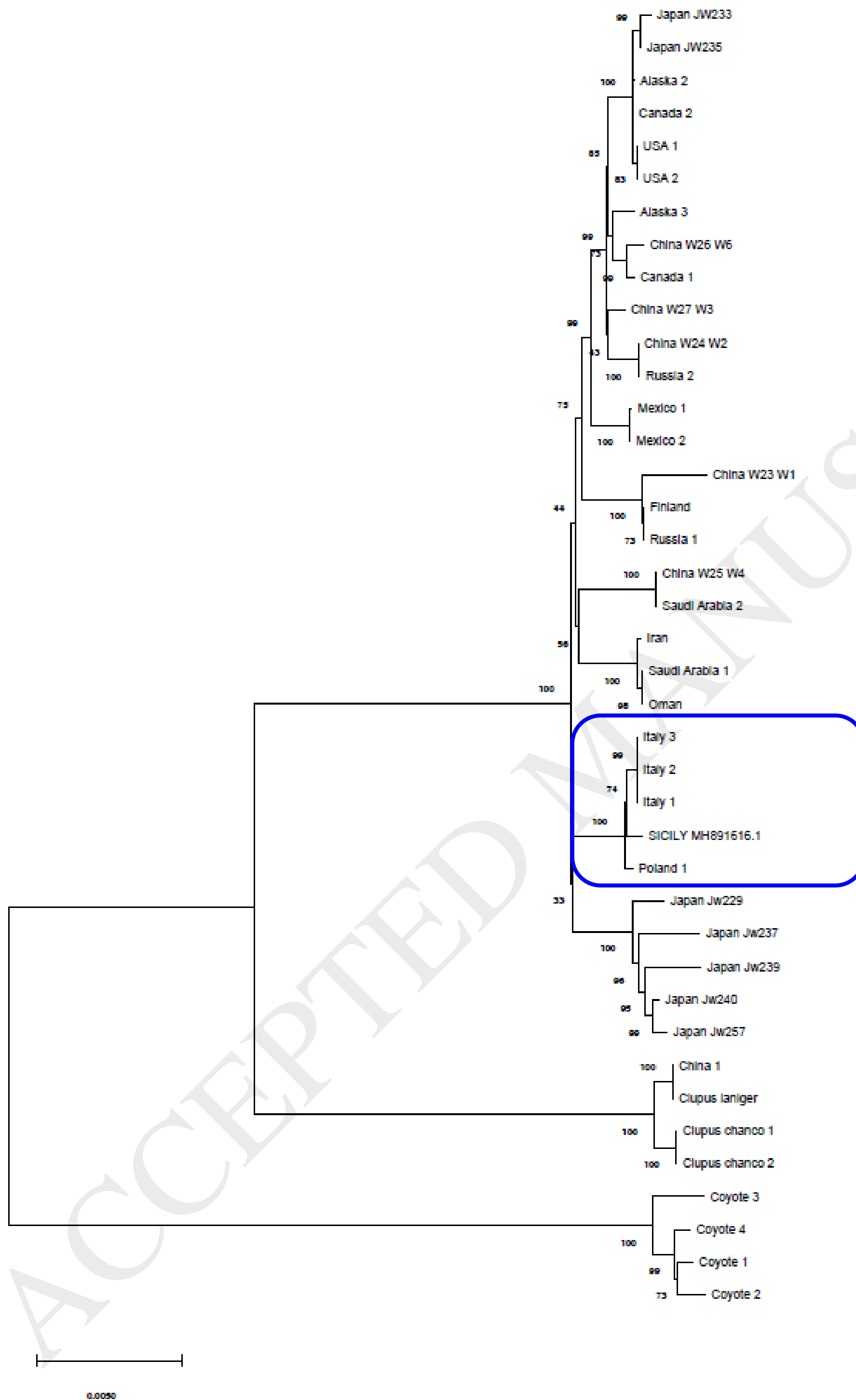

Figure 5.

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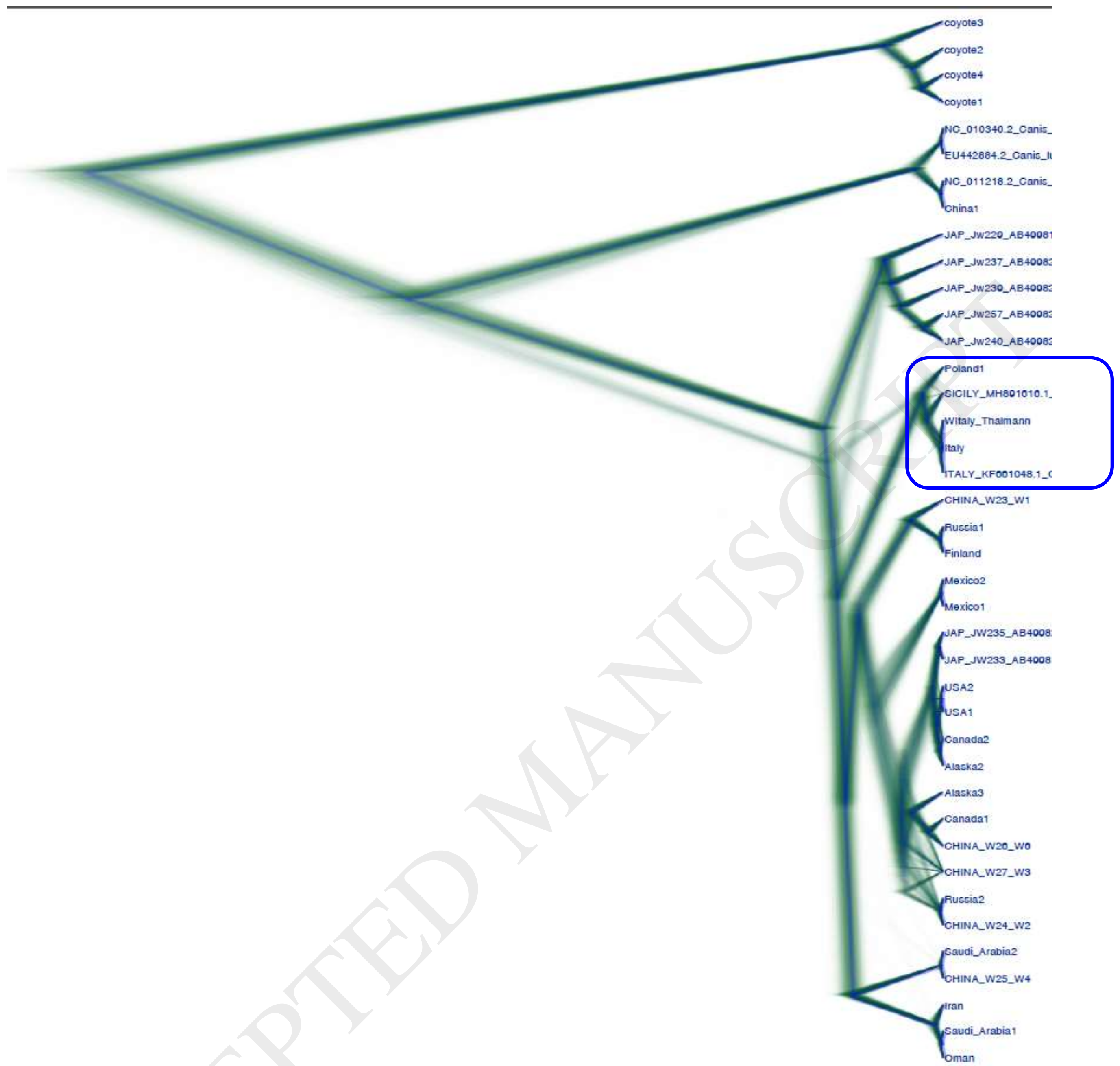

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Figure 6.