The Sicilian wolf: genetic identity of a recently extinct insular population

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Abstract:

During historical times many local populations of grey wolf *(Canis lupus)*, once the most diffused mammal in the world, became extinct. Among these the Sicilian population, the biggest island of the Mediterranean Sea, was eradicated by human persecution in the early decades of the XX century.

In order to reconstruct the genetic identity of the Sicilian wolf, we used ancient DNA techniques to analyse the mitochondrial DNA of the six known specimens actually stored in museums.

We have successfully extracted and amplified a mtDNA fragment of the control region (CR) from four samples. Our analyses show that two samples have the same haplotype, that differs by two substitutions from the most diffused Italian haplotype (W14) and one substitution from the second Italian rare haplotype (W16). One of the others two samples shows a new wolf haplotype never described before and the fourth a haplotype common in dogs.

Furthermore, all the detected wolf haplotypes in this study belonged to the mitochondrial haplogroup to which the Pleistocene European wolves and several current southern Europe haplotypes belong.

Unfortunately, this endemic population of Mediterranean wolf, was definitively lost before it has been possible to understand its uniqueness and importance regards conservation.

Key words: *Canis lupus*, grey wolf, extinction, Sicily, haplotypes, mtDNA, aDNA

Introduction

The extinction of animal species is a biological phenomenon that can have ecological and social repercussions (Stuart Chapin III et al., 2000; Sodhi et al., 2009). A remarkable case is represented by the extinction of mammal island populations, which is a rather recurrent event (eg Alcover et al, 1998; Hanna & Cardillo 2013) that can be due to a number of different causes, including climate changes (Eldredge, 1999) and the direct or indirect human actions like persecutions, habitat fragmentation or introductions of invasive allochthonous species (Clavero & Garcia-Betthou 2005).

In the Mediterranean islands, many species or populations of endemic mammals have disappeared in relatively recent times (Alcover et al., 1999; Marra 2005; Bover & Alcover 2008). Sicily, the largest island in the Mediterranean Sea, located south of the Italian Peninsula (about 37°45'0 N; 14°15'0 E), in historic times has seen the disappearance of many species, such as the red deer (*Cervus elaphus*), the roe deer (*Capreolus capreolus*), the fallow deer (*Dama dama*), the wild boar (*Sus scrofa*, then recently reintroduced), the Eurasian otter (*Lutra lutra*), and even the grey wolf (*Canis lupus*), the only large autochthonous predator of the island (La Mantia & Cannella, 2008).

In particular, the Sicilian wolf represented the only insular population of grey wolf in the Mediterranean area, and one of the few historic insular populations in the world, together with the Japanese grey wolf (*Canis lupus hodophilax*), and the Hokkaido grey wolf or Ezo wolf (*Canis lupus hattai*), both extinct between the end of 1800s and the beginning of 1900s (Walker, 2005). The Sicilian wolf disappeared from the Island in the early decades of the twentieth century because of human persecutions consequently to livestock damages (eg. Minà Palumbo, 1858; Chicoli, 1870) though there is no unanimity on the exact extinction timing with the last official capture referred to a specimen shot down in Bellolampo, Palermo, in 1924. However, many other sightings and records of individuals killed in subsequent years have been reported, until at least the 1935 (Giardina, 1977; La Mantia & Cannella, 2008), or even possibly the 1950s and 1960s (Toschi, 1959; Cagnolaro et al., 1974; Angelici et al., 2016). To date, historical museum specimens attributed to the last grey wolf individuals lived in Sicily are extremely scarce: no more than 7 samples, constituted by skins, stuffed specimens, one skull, and skeletal remains are stored in few Italian museums (Lo Valvo, 1999; Angelici and Rossi, 2018). Compared to the Apennine grey wolf (*Canis lupus italicus* Altobello, 1921), namely the subspecies widespread along the Italian peninsula (Nowak and Federoff 2002; Montana et al., 2017a), the few complete Sicilian wolf specimens show peculiar distinctive characteristics, including a smaller size and a paler coat colour (see Angelici and Rossi, 2018).

However, despite these morphological differences and its ancient isolation from the mainland populations, with the last continental bridge between Italy and Sicily estimated to have occurred from about 25,000 to 17,000 years ago (Antonioli et al., 2014), the first studies specifically focused on the genetic identity of the Sicilian wolf started only in recent years (see Angelici et al., 2015).

A possible reason relies on the fact that the first scientific investigations on the Italian grey wolf started in 1970s (Cagnolaro et al., 1974; Zimen & Boitani, 1975), when the population was reduced to less than 100 individuals in isolated areas in the Central and Southern Apennines (Boitani, 2003), and when the Sicilian population was already extinct. Another possible reason might be attributable to the fact that insular and continental populations were generally considered to be the same (e.g. Sarà, 1999), and even in the most accurate studies published on the wolf in Italy (e.g. Ciucci and Boitani, 2003), questions about the identity of the Sicilian wolf have never been faced. A recent morphological and morphometric analysis on museum samples has suggested that the Sicilian wolf could be considered a valid subspecies, for which the name *Canis lupus cristaldii* has been proposed (Angelici & Rossi 2018).

A possible contribution to clarify these taxonomic uncertainties can be provided by the analysis of the mitochondrial DNA (mtDNA), often applied for systematic, phylogeographic and phylogenetic studies (e.g. Avise et al., 1987; Jaarola & Searle, 2002; Gaubert et al., 2012; Montana et al., 2017). Indeed, many insular mammal populations show specific or even unique mtDNA haplotypes which can distinguish them from the continental populations from which they originated (e.g. Koh et al., 2014), paralleling above mentioned morphological differences, including insular dwarfism (eg McFadden & Meiri, 2013). Additional insight can be provided by the analyses carried out on ancient DNA (aDNA) preserved in fossil or museum specimens, which allow to obtain information directly from the time frame investigated (Rizzi et al., 2012). Recent methodological improvements in the field of ancient DNA analysis, such as the identification of the bone elements which better preserve DNA (Allentoft et al., 2015; Pinhasi et al., 2015), the growing access to aDNA through extraction methods tailored to ultra-degraded molecules (Dabney et al., 2013a) and also the advances in sequencing technologies, allow a better comprehension of fundamental processes such as patterns of evolution, population genetics and palaeoecological changes (Rizzi et al., 2012; Hofreiter et al., 2015; Leonardi et al., 2017). Furthermore, aDNA analysis can provide also information about spatial and temporal dynamics of extinct and extirpated species and in this such as the Japanese wolf (*Canis lupus hodophilax*) extinct in historical times as the Sicilian wolf (Ishiguro et al., 2009, 2016; Matsumura et al., 2014). In particular, the study of ancient mtDNA allows to considerably expand the phylogeographic knowledge on many animal species (Leonard et

al. 2000; Barnes et al., 2007; Cieslak et al., 2010; Casas-Marce et al., 2017; Paijmans et al., 2017; Palkopoulou et al., 2018).

Therefore, the aim of this work is to clarify the genetic identity and the origin of the last specimens of *C. lupus* from Sicily through the study of the ancient mtDNA, providing additional information on its ancient population dynamics.

Materials and methods

Sample details

After an accurate research on the few available samples, we collected teeth, fur, skins or claws, depending on the availability of the remains and the preservation of the materials (Table 1), from 6 museum specimens dated between the end of the XIX century to the early decades of the XX century, representing some of the last individuals of the Sicilian *Canis lupus* lived on the island before its human-driven extinction (Table 1). The first sample (Sic1) was collected from a skull (tooth), and from a stuffed specimen, both preserved at the Museum of Natural History, Section of Zoology “La Specola”, University of Florence. The second (Sic2) and the third (Sic3) samples were collected respectively from the skull of an immature individual and from a stuffed adult wolf, both preserved at the Museum of Zoology "P. Doderlein", University of Palermo. The fourth (Sic4, with anomalous features thus possibly attributable to a feral dog or a hybrid) and the fifth (Sic5) samples were collected from an adult skin, and from a mounted specimen, respectively, both preserved at the Regional Museum of Terrasini, Palermo. The last sample (Sic6) was collected from an immature individual, preserved at the Museum of Zoology "P. Doderlein", University of Palermo. For more details about specimens see Supplementary information.

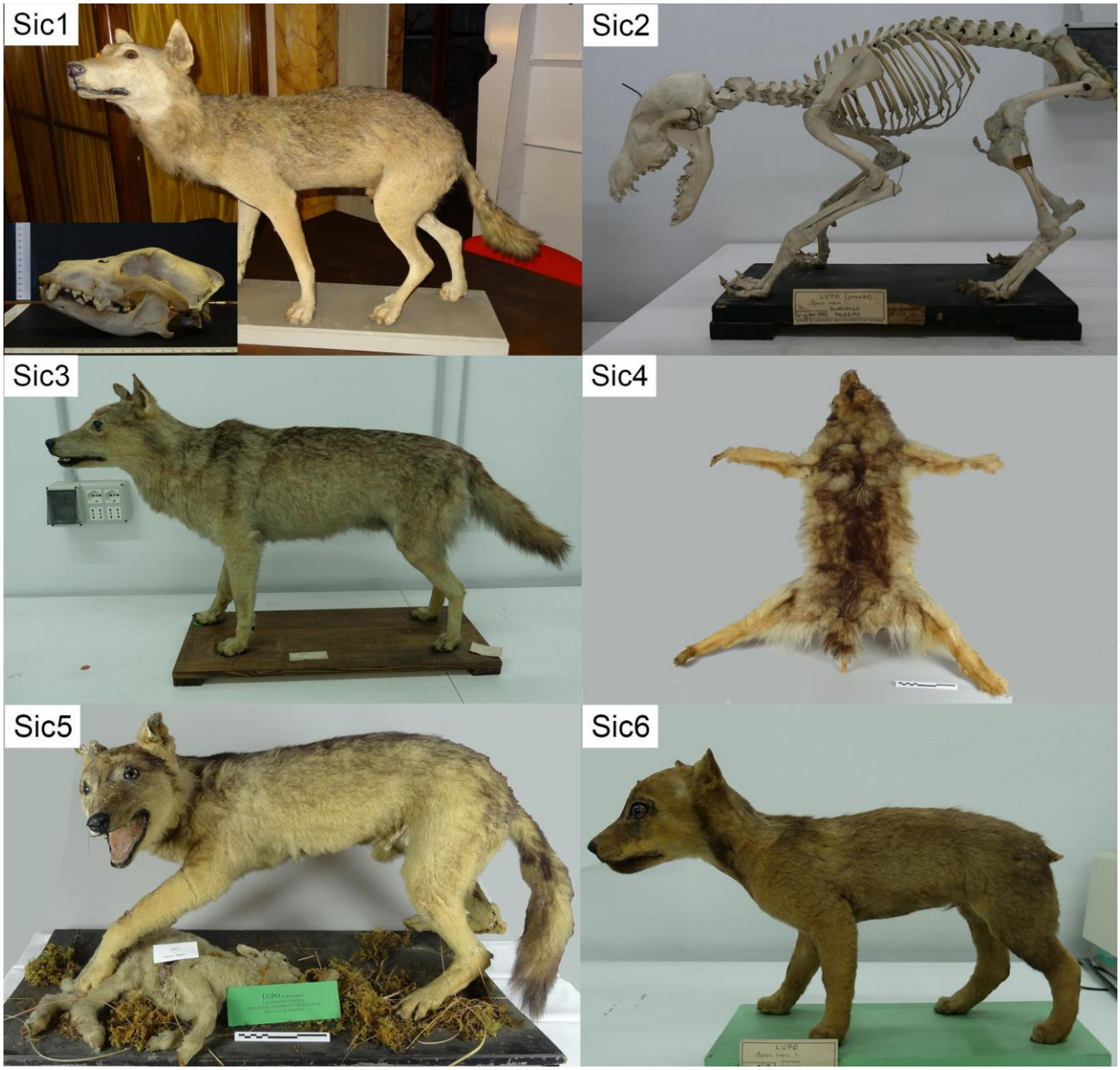

Figure 1: In this figure all the samples analysed in this study with the relative laboratory codes are represented.

Lab ID	Museum ID	Museum	Types of samples	Date
Sic1	C11875	Museo di Storia Naturale 'La Specola', Firenze, Italy	1.Tooth 2.Fur	1883
Sic2	AN/855	Museo di Zoologia 'Pietro Doderlein', Palermo, Italy	Tooth	~1880-1920
Sic3	M/18	Museo di Zoologia 'Pietro Doderlein', Palermo, Italy	Fur	~1880-1920
Sic4	9	Museo 'Regionale Interdisciplinare di Terrasini', Terrasini (PA), Italy	1.Leather with fur 2.Claw	1924
Sic5	9263	Museo 'Regionale Interdisciplinare di Terrasini',	Leather with fur	~1880-1920

		Terrasini (PA), Italy		
Sic6	M/17	Museo di Zoologia 'Pietro Doderlein', Palermo, Italy	Fur	~1880-1920

Table 1: List of the analysed samples in this study. The lab and museum ID are listed above together with the museums, types of material from with we extracted DNA and the approximate ages. For more information about specimens see Supplementary Information.

Ancient DNA procedures

All the collected samples were processed at the Ancient DNA Laboratory of the University of Bologna, Ravenna Campus, in a physically isolated area, reserved to the manipulation of ancient or historical specimens (Fulton et al., 2012; Knapp et al., 2012). All the phases of the experiments were performed following strict ancient DNA standards, selected for this study to avoid contaminations by exogenous DNA and to make the results reliable (Cooper & Poinar, 2000; Gilbert et al., 2005; Knapp et al., 2015). Decontamination and preparation of samples, DNA extraction and amplification set up were performed in different rooms, located in the pre-PCR facility, where the researchers worn suitable disposable clothing constituted by coverall suits, boots, arm covers, double pair of gloves, face masks and face shields, and follow established workflow and procedures. All the surfaces, benchtops and the instruments were regularly cleaned after each experiment with bleach and ethanol or DNA Exitus plus (AppliChem GmbH, Darmstadt, Germany) (Esser et al., 2006) and UV irradiated for 60 minutes. Tips with aerosol-resistant filter were utilized with reserved pipettes and reagents (DNA, DNAse and RNAse free) were stored in small aliquots and irradiated with UVc for 60 minutes before the use (except those that cannot undergo to ultraviolet irradiation). Extraction blanks and amplification controls were processed along the samples. No modern DNA or modern specimens have been ever introduced in the aDNA laboratory. Amplification and downstream analyses were performed in a post-amplification facility, physically distant from the pre-amplification area.

DNA extraction

Samples were decontaminated by UVc irradiation for 40 minutes for each side and then processed in small groups (2-3 samples) along with an extraction blank for each batch. Whenever available, in

order to make results more reliable, for each specimen we analysed multiple samples from different types of tissues (e.g. tooth and skin), otherwise multiple independent DNA extractions were carried out on different samples from the same tissue type (Tab. 1). DNA from teeth samples (10-50 mg), was extracted by means of a silica-based protocol, modified from Dabney et al. (2013a) and Allentoft et al. (2015). Instead, DNA from fur, skin or claws (5-20 mg) was extracted with a protocol specific for samples containing keratin (Campos and Gilbert, 2012) (see Supplementary materials for detailed explanations about the procedures and the protocols). DNA and extraction blanks were quantified by Qubit® dsDNA HS (High Sensitivity) Assay Kit (Invitrogen™Life Technologies - Carlsbad, CA, USA).

Mitochondrial DNA amplification and sequencing

The mitochondrial HVR1 region was amplified by 4 primer pairs to overcome the challenges related to the diagenesis of ancient DNA, that are predominantly manifested as breakage of genetic material in small fragments, poor DNA content and also nucleotide substitutions (e.g. deaminations; Dabney et al., 2013b). DNA samples were amplified for several HVR1 mitochondrial fragments: i) a 57 bp fragment (99 bp with primers) spanning nucleotide positions 15591-15690 (Stiller et al., 2006), referenced herein as “short fragment”; ii) a 361 bp (404 bp with primers) stretch by means of three overlapping fragments (Leonard et al. 2005, Ersmark et al., 2016), spanning nucleotide positions 15411 - 15814 and referenced herein as “long fragment” (see Supplementary Information for details). All the amplifications were performed twice for each sample in order to confirm the results. PCR products were checked on agarose gel and the positive amplifications were purified by MinElute PCR purification kit (Qiagen), following manufacturer instructions. Sequencing reactions were performed on the purified amplicons on both directions, for the forward and the reverse primer, using the BigDye Terminator kit ver.1.1 (Thermo Scientific). Sequencing was performed on an ABI 310 Genetic Analyzer at the laboratories of Pharmacogenetics and Pharmacogenomics of the Department of Pharmacy and Biotechnology (University of Bologna).

Data analysis

Sequences were firstly visualized on FinchTV (Geospiza) in order to check the chromatograms and their quality. Then, they were edited and aligned by Bioedit v7.2.5 (Hall, 1999) and MEGA7 (Kumar et al., 2016). For each specimen, a consensus sequence was obtained from all the

mitochondrial fragments and all the replicated amplifications. Identical haplotypes were collapsed in DNASP v.5 (Librado and Rozas, 2009) and matched in BLAST (Altschul et al., 1990) to determine possible correspondences with modern and ancient canid haplotypes already published in GenBank.

To provide a first overview of the geographic and temporal relationships of our newly analysed samples, we assembled an extended dataset consisting of sequences from modern and ancient wolves and dogs. Moreover, due to the different fragments size obtained for the Sicilian samples analysed (57 bp and 361 bp), we built several alignments.

For the short fragment, two alignments were constructed, one aimed at comparing our specimens to all the ancient sequences available and also to the current Italian haplotypes (shorter alignment 1A, n = 125), and the other to set all the sequences here obtained in the landscape of modern dogs and wolf populations (shorter alignment 1B, n=146). They were used only in network based methods to infer the phylogenetic relationships among mtDNA haplotypes of the ancient and modern wolves and dogs, due to the very short sequence data. We thus constructed a Median-Joining network using the software PopArt (Leigh et al., 2012) for the alignments 1A and 1B.

For the long fragment here amplified, we created a database that contains 124 worldwide modern wolf and dog sequences and also ancient wolf sequences from literature. However, the majority of the sequences downloaded do not cover the first bases of the longer fragment here obtained and thus we cut the sequences of the database at 330 bp and we created the alignment 2 which contains modern and ancient wolf sequences (See Supplemental material for details about alignments). JModeltest2 (Darriba et al. 2012) and the Akaike Information Criterion (AIC) were used to identify the best nucleotide substitution model for the alignment 2 concerning the longer fragment. The best evolutionary model was applied to build a Bayesian tree using MrBayes on the Alignment 2. The Bayesian analysis was run for $20x10^6$ generations, with a 10% burn-in and a sampling frequency each 1000 iterations and rooted using a coyote homologue sequence (*Canis latrans*, GenBank access number DQ480509) as an outgroup. Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/) was used to check the convergence of parameters result from the two runs and the final tree was visualised with FIGTREE v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Authenticity of the data

Following the strict criteria and procedures developed for ancient DNA analysis (see Materials and methods) we found no contamination in the extraction blanks and PCR controls and results were confirmed by the double extractions and multiple amplifications of the same mtDNA fragment. Moreover, sequences were considered authentic since the data were confirmed in the overlapping portions of each adjacent fragment of the three primer pairs or in the overlapping portions of different stretches from independent amplifications.

DNA extraction and amplification

We successfully amplified 57 bp of the mtDNA control region in 4 out of the 6 analysed samples (Sic1, Sic2, Sic3 and Sic4). However, as expected from skeletal remains (bones and teeth) which usually contain the highest quantity of endogenous DNA among museum samples (Mulligan, 2005; Wandeler et al., 2007; Green & Speller, 2017), for the two tooth samples (Sic1 and Sic2) we were able to successfully amplify also the longer region (361 bp).

Haplotypes and haplogroups

The longer regions (361 bp) obtained from Sic1 and Sic2 resulted to be identical and largely matched (290 bp) with a historical wolf sample from Hungary dated 1899 (Dufresnes et al., 2018). The haplotype showed by these samples differs in only one substitution from a Bulgarian (ID: KU696388) and from a Polish (ID: KF661045) wolf haplotype. Moreover, this longer fragment differs for two substitutions from the most diffused control region haplotype of the Italian wolf population, named W14, and one substitution from the second Italian rare haplotype, W16 (Randi et al. 2000; Boggiano et al., 2013; Montana et al., 2017b).

Considering the short fragment (57 bp) the haplotype obtained from Sic3 resulted to be never described before in published papers or online database. Instead, the haplotype obtained from sample Sic4 matched with a number of dog sequences, confirming the anomalous morphological characteristics of this sample that, probably, corresponds to a feral dog (Table 2).

Interestingly all the detected haplotypes belong to the wolf mitochondrial haplogroup 2 proposed by Pilot and colleagues (2010), to which belong all the European Pleistocene's wolves and several haplotypes from Southern Europe.

Phylogenetic Analyses

The Median-joining network based on the alignment 1A showed that the haplotype shared by Sic1 and Sic2 was close to the modern European and Italian haplotype W16 and also to an ancient wolf haplotype widespread from North-East Asia to Europe (Figure 2a).
The Median-joining network, by comparing the 4 haplotypes detected in this study to modern dogs and wolves (Figure 2b) showed that samples Sic1, Sic2 and Sic3 grouped very close to modern Italian wolf haplotypes and to other European individuals that belong to the haplogroup 2 described by Pilot and colleagues (2010) - both prehistoric and modern - and a few mutational steps far from some wolf and dog haplotypes (Figure 2b).

Sample Sic3, which showed a unique haplotype found only in this specimen, was placed one mutation away from Sic1 and Sic2, and it did not match with any other ancient or modern specimen (Figure 1a). When compared to current haplotypes this sample kept showing its uniqueness, although it clearly belongs to the same isolated cluster of modern Italian haplotypes and other wolves of the Late European Pleistocene (Hg2) among dog haplotypes (Figure 1b) . Sample Sic4 appeared very distant to the other ancient Sicilian specimens analysed in this study, grouping with three ancient specimens, retrieved in Italy (Verginelli et al. 2005), Perù (Leonard et al., 2002) and New Zealand (Frantz et al., 2016) which resulted more recent than 3,200 years ago and assigned either morphologically, genetically or both to modern domestic dogs.

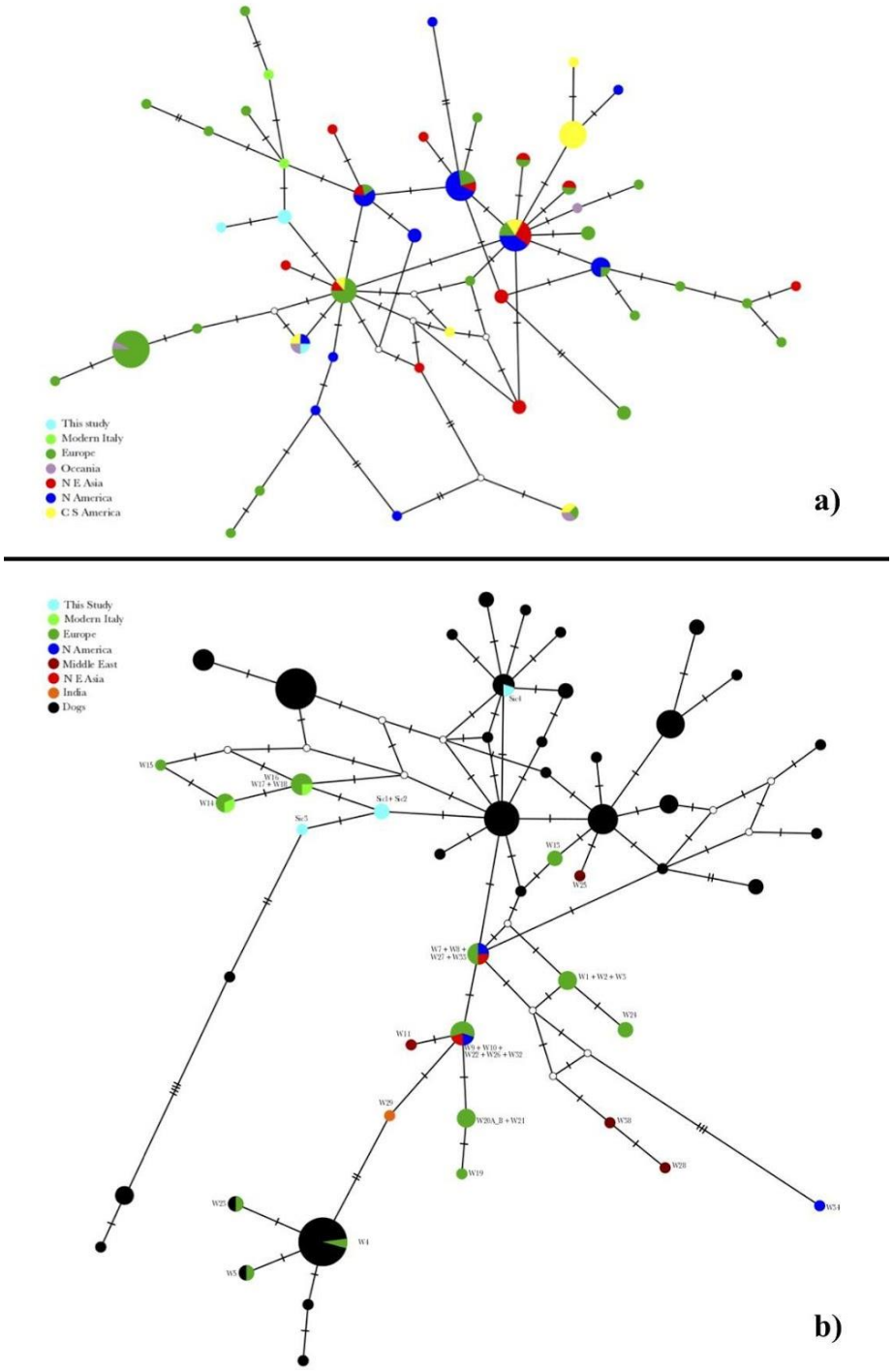

Fig. 2: Median-joining network of mtDNA haplotypes based on a) the alignment 1A (57 bp), comprehensive of ancient sequences downloaded from Genbank, and b) the alignment 1B (57bp) in which the samples analysed in this study were compared to modern wolves and dog breeds. White dots represent median vectors while a single bar-line represents a nucleotide mutation in the alignment.

The best fit evolutionary model for the alignment 2 including the longer fragments was the HKY + I + G with I= 0.5840; G category= 4, G shape = 0.4450 and Kappa = 62.19.

The Bayesian tree obtained from MRBAYES (Fig.3) using the above mentioned substitution model showed a very clear topology where the extant wolf haplotypes might be split into two clades roughly corresponding to the two main wolf haplogroups (namely 1 and 2) described by Pilot and colleagues (2010) and Montana and colleagues (2017).
Interestingly, although the limited length of the sequence alignment did not allow to obtain robust supports for most of the internodes, the split between the two haplogroups was supported.

Furthermore, all the ancient haplotypes were included in the haplogroup 2. The Sicilian haplotype grouped in a monophyletic lineage, inside the haplogroup 2, which included a Swiss 14,500-years-old wolf haplotype (Thalmann et al., 2013), wolf modern haplotypes from Italy (W14 and W16), Balkans (W15) and East Europe (W17) (Montana et al., 2017b). Moreover, the Sicilian haplotype, and the ones mentioned above, plotted in a subclade very close to a Siberian 42,000-year-old and a Russian 33,500-year-old specimens (Thalmann et al., 2013; Ersmark et al., 2016), making together a monophyletic group.

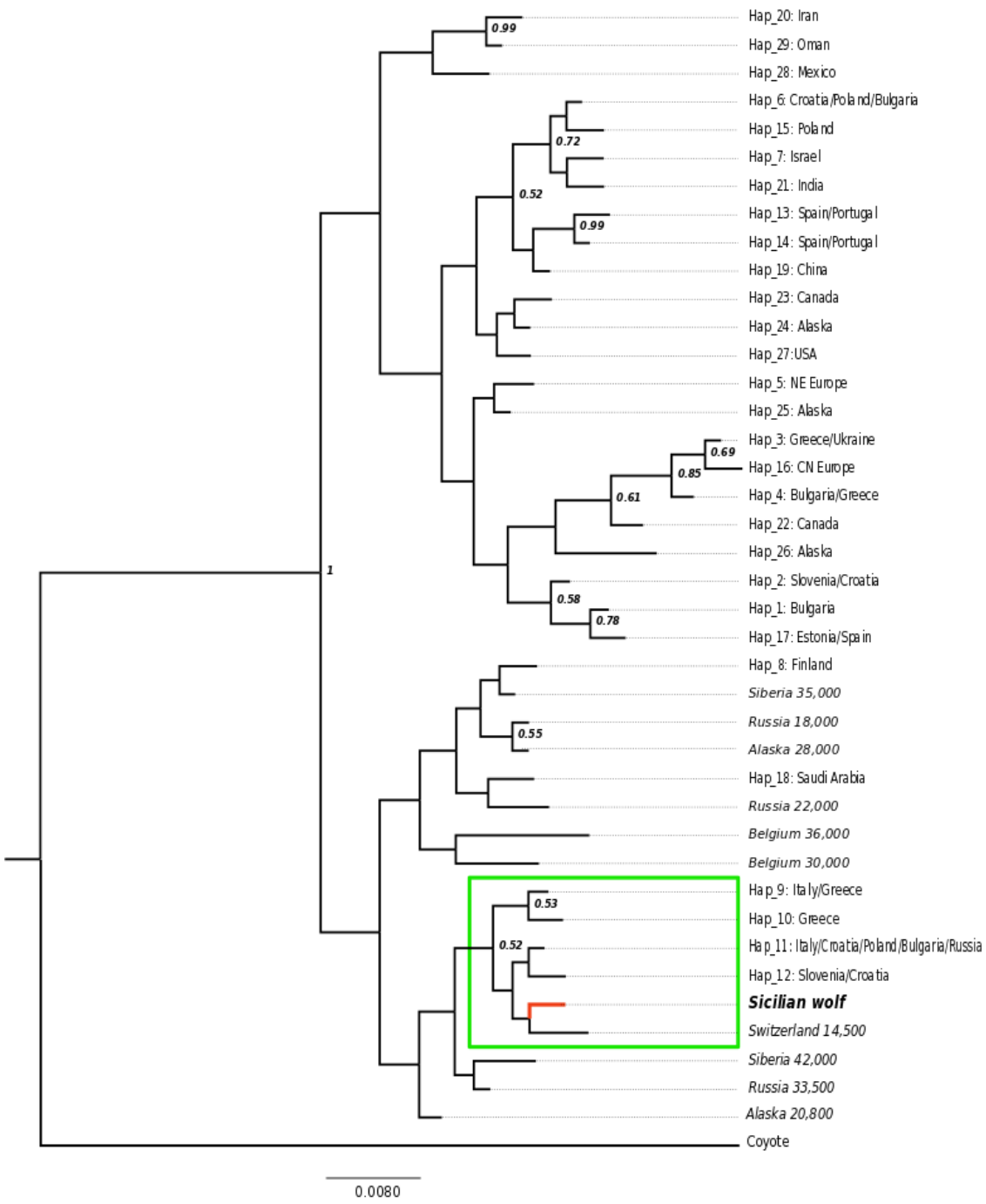

Fig 3: Bayesian tree obtained by using MrBayes software. The tree was generated using the Alignment 2 (330bp) and the coyote sequence as an outgroup.

Discussion

In this study we detected three wolf haplotypes belonging to four historical canid samples from Sicily. The sample named Sic3, shows an undescribed 57 bp wolf haplotype, possibly due to an *in*

situ mutation fixed by the long-term isolation in Sicily since the landbridge with the mainland vanished.

The samples Sic1 and Sic2 returned a 361bp long sequences which share the same haplotype, that overlaps a published 290 bp haplotype from a sample recovered in 1899 in Hungary (Dufresnes et al., 2018). It was not possible to carry out a complete comparison on the whole sequence here obtained but we could suppose, although we cannot prove because of the mismatch in the length of the sequences amplified in the two studies, that the sequence of the Sicilian wolves could differ in some diagnostic and highly variable nucleotides not typed from Dufresnes and colleagues (2018). The network in Fig. 2a shows that the Sicilian wolves, together with the two modern Italian haplotypes, were part of the genetic pool of the Pleistocene wolves, placed a bit on a side with other European samples. Furthermore, as highlighted from the tree based on the longer fragments (Fig. 3) the Sicilian haplotype keep showing a close proximity to the current southern and eastern European haplotypes.

When Sicily separated itself from continental Italy we do not know how large the wolf island population was, as we do not know if after the separation there have still been contacts between the two populations, i.e. if there have been cases of natural introduction, or even human introduction. It must be said that both the possibilities are events difficult to hypothesize. Considering the great depth, and the strong currents in the Strait of Messina (eg Bignami and Salusti 1990) the spontaneous colonization from the Italian peninsula to Sicily appears possible only in the early stages of the sea-level rise. As regards the second hypothesis, it seems unjustified to introduce a predator culturally seen as a competitor and considered harmful to man and to his activities. On the contrary, the introduction of dogs already domesticated, coming from the European continent, as well as from North Africa and Middle East, seems more likely.

The results could be compatible with the presence in the past of a greater genetic diversity not only throughout Europe but also in the Italian peninsula, probably due to contacts with populations of Southern Europe (see Dufresnes et al. 2018). So, starting from a common pool with different haplotypes, in peninsular Italy and Sicily, due to geographic isolation, alternative haplotypes would have stabilized. In other words, the haplotypes found in Sicily may have been present also on the peninsula or even outside, but later disappeared as a result of the loss of diversity that all southern European populations experienced in the last centuries. In the same way, the same situation may have occurred in Sicily during the last 20,000 years where the haplotypes that are found in Italy and southern Europe today may have existed in the island until the human driven eradication process.

An interesting result is the identification of Sic4 as a domestic dog, at the time probably mistaken for a wolf due to some phenotypic affinity, or even for its being stray or feral. There is only very scarce available information about the history of this specimen and we do not know if it has been shot due predation on livestock. It should also stress that this specimen was killed in 1924 and may have been identified as a wolf because at that time the species was yet on the verge of extinction and the surviving wolves, due to the lack of conspecifics and also due to the large number of free-ranging dogs, may have generated Wolf x Dog hybrids. Those free-ranging hybrids, most likely similar to wolves, could have been considered dangerous by the population and, eventually, this species had become a fearsome animal to which most people were no longer familiar with.

The present study shows how investigations on overlooked old specimens, especially if related to insular population, may lead to results worthy of note from a biogeographical and systematic point of view (eg. Turvey at al., 2017). In this case it also should be stressed how a population gone extinct before it has been possible to understand its uniqueness and importance as regards conservation.

According to its long time isolation, morphologic differentiation and genetic identity, we think that the Sicilian wolf may be considered a valid subspecies, as recent genetic studies tend to confirm for other insular wolf populations (eg. Matsumura et al., 2014; Wechworth et al., 2015).

Despite the results, this research represents a preliminary approach about the study of the genetic identity of the Sicilian wolf, targeted only to a portion of the control region of the mtDNA. In the future, it will be undoubtedly interesting to carry out the study of the whole genome of the same samples, to evaluate the contribution of paternal and maternal DNA and to better understand the evolutionary dynamics of the wolf population in this island.

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