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Genomic characterization of the Italian wolf (*Canis lupus*)**:** the genes involved in black coat colour determination and application of microarray technique for SNPs detection.

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TABLE OF CONTENTS

I - ABSTRACT

This study provides a comprehensive genetic overview on the endangered Italian wolf population. In particular, it concentrates on two research lines.

On one hand, we focused on melanism in Italian wolves in order to isolate a mutation related with black coat colour in canids. With several reported black individuals (an exception at European level), the Italian wolf population constituted a challenging research field posing many unanswered questions. As found in North American wolf, we reported that melanism in the Italian population is caused by a recently discovered melanocortin pathway component, the K locus, in which a beta-defensin protein acts as an alternative ligand for the Mc1r. This research project was conducted in collaboration with Prof. Gregory Barsh, Department of Genetics and Paediatrics, Stanford University.

On the other hand, by means of a customized Canine microarray we performed analysis on a significant number of SNPs (Single Nucleotide Polymorphisms) useful to integrate or substitute existing microsatellite markers for individual genotyping and wolf-dog hybrids identification. Thanks to DNA microchip technology, we obtained an impressive amount of genetic data which provides a solid base for future functional genomic studies. This study was undertaken in collaboration with Prof. Robert K. Wayne, Department of Ecology and Evolutionary Biology, University of California, Los Angeles (UCLA). I spent a three-months period at UCLA as visiting student supported by a Marco Polo grant, in order to process the Italian samples.

KEYWORDS: melanism, black phenotype, coat colour, K locus, SNPs, microarray, Italian wolf.

II - INTRODUCTION

The increased amount of black or nearly black pigmentation (**melanism**) is a common characteristic in mammals. Melanism occurs frequently in wolf phenotype but it is not equally widespread within all the diverse populations across the world.

In North America, dark wolves' frequency is quite consistent, reaching almost 50% in some areas, such as Yellowstone National Park. On the contrary, melanism is extremely rare or absent in the majority of the European populations.

Melanistic individuals have been occasionally reported in the Italian wolf population. With no proved evidence of black wolves' existence before 1982, melanism is not considered a typical characteristic of the Italian wolf phenotype.

Nevertheless over the last twenty years, black wolves have been detected in the Apennines near Arezzo and, more recently, in other areas of the Tuscan-Emilian Apennines. Moreover, dark individuals sighting and carcasses discoveries are increasing all over the Northern Apennines.

One explanation to the black coat occurrence in Italy relies on the hypothesis of a mutation fixation in a local population - plausibly in the Romagna Apennines -, which may have expanded northward along the Apennines.

Another hypothesis suggests that melanism in Italian wolf depends on hybridization with a black domestic dog. Nonetheless, there is no genetic evidence of dog introgression in the Italian wolf population supporting this theory and only few hybridisation events were described. It follows that the dog gene introgression - if it ever happened - did not occurred recently.

In order to find the solution to such a dilemma, the detection of the DNA mutations which causes melanism is the only practical way to comprehend the origin of melanistic phenomenon.

The second research line focuses on the identification thanks to the microarray technique of thousands of single nucleotides polymorphisms characterizing the Italian wolf population.

Italian wolf population is an ecotype of the European wolf from which remained separated for around 150 - 200 years. Because of its long-lasting historical isolation and peculiar morphological characteristics, the Italian wolf represents an extremely interesting case study. Furthermore, the Italian population is characterized by a reduced genetic variability and the fixation of a single mitochondrial haplotype.

The genetic characterization so far utilized bases on eighteen diagnostic microsatellites loci which identify species, individuals, sex, and kinship.

A previous study characterized around only fifty SNPs within the population.

In our research we were able to obtain a remarkably higher number of genetic markers (127,000 SNPs), thanks to the development of a new DNA microarray technique. In 2004, it was created a Custom Canine SNP microarray which allows the simultaneous analysis of thousands of SNPs. Such technique was applied by UCLA on a wide project. In this research samples of numerous canine breeds, North American and Canadian wolves, coyotes, jackals, red wolves, Ethiopian wolves, European wolves and Italian wolves were collected and analyzed.

This thesis consists of six chapters.

Chapter 1 reviews the current status of knowledge on wolf (distribution and legal protection) at European and Italian level, on domestication process, on dog genetics and on pigmentation. It also highlights the peculiarity of the Italian wolf population with particular focus on black individuals and their genetic characterization.

Chapter 2 identifies the main purposes of this work.

Chapter 3 discusses materials and methods used in this project to collect samples and analyze data.

Chapter 4 reports results on both the research lines on melanism and SNPs determination.

Chapter 5 discusses and critically analyzes the results providing a critical assessment of the work in comparison with relevant research lines.

Chapter 6 draws the final conclusions and suggests future research perspectives.

1 – BACKGROUND

1.1 Status of knowledge of wolf and dog

1.1.1 Wolf

The wolf (*Canis lupus*, Linnaeus 1758) is a large carnivore, member of the family *Canidae*[1]. Second only to brown bear (*Ursus arctos*, Linnaeus 1758), wolf is the largest predator in Europe.

The species is geographically widespread and occupies a high variety of habitats. In fact despite its territorial nature, the wolf is capable of dispersing several hundred kilometres from home territory. Dispersal is the main behavioural trait which leads to the colonization of new areas and thus the gene flow enhancement of genetic diversity which increases the wolf phenotypic variation (Mech 1970; Boitani 1995; 2000). Considering such remarkable morphological variability the identification of several separated subspecies of *Canis lupus* in the Eurasian area is controversial.

There is much taxonomic debate over the placement of the Eurasian wolf subspecies. For example Sokolov & Rosolino (1985) reported nine living Eurasian subspecies.

1.1.2 Historical and current worldwide wolf distribution

Historically, the wolf was the world's most widely distributed mammal. This canid lived throughout the northern hemisphere from North Pole to India, spreading over three continents (Fig. 1).

As a result of wolf extirpation from much of its former range, its distribution critically declined in the last century. Insofar, wolf population is nearly extinct throughout most of Western Europe (Boitani 1995), Mexico, and USA (Mech 1970). Consequently, the International Union for Conservation of Nature (IUCN) lists the wolf among the species of **Least Concern**[2] (LC).

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[1] The Canidae family includes dogs, wolves, coyotes, foxes, and jackals.

[2] IUCN 2001. IUCN Red List Categories and Criteria: version 3.1. IUCN Species Survival Commission. IUCN, Gland, Switzerland and Cambridge, UK. ii 30 pp.

Wolf worldwide distribution occurs primarily in wilderness and remote areas, namely in Canada, Alaska, northern USA, Europe, and Asia spanning from about latitude 75°N to 12°N (Sillero-Zubiri *et al.* 2004). Nowadays the major populations live in Canada, Alaska and Montana with thousands of individuals which are recolonizing the original distribution areas.

In North America, the extirpation of the wolf began with the arrival of the European pilgrims and it was carried out so methodically that in 1930s the species resulted extinct all over the continent with the exception of Alaska and Minnesota (Ciucci and Boitani 1998).

In Europe wolves are distributed from sea level up to over 2000 meters a.s.l. Nevertheless, in central and southern areas, human persecution has pressed on wolves to settle in mountainous areas above 600 meters a.s.l. (Sulkava and Pulliainen 1999).

Thanks to domestication and artificial selection (See chapter 1.1.6), nowadays it is disseminated all over the world, as the domestic dog (*Canis familiaris*, Linnaeus 1758).

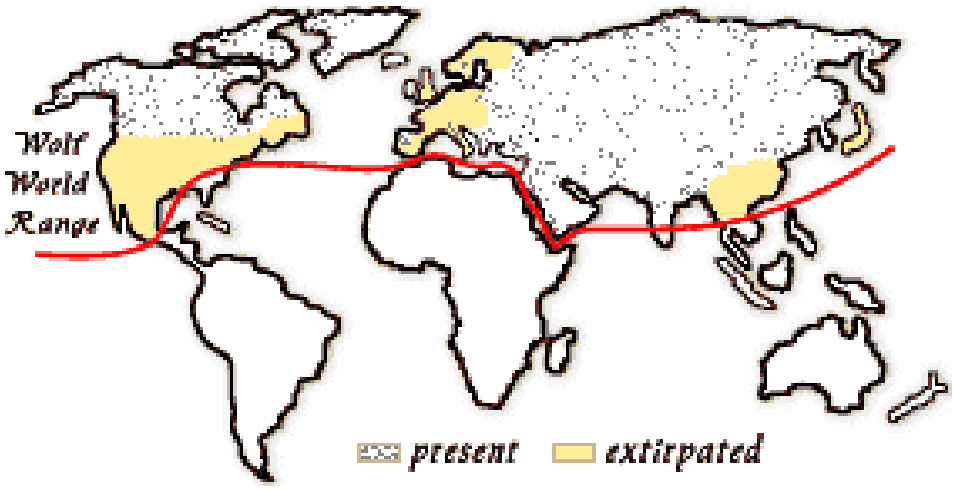

Figure 1 World wide wolf distribution.

1.1.3 Population status in Europe

As said, until the end of the 19th century, wolf inhabited all continental Europe. Yet, after less than a century and a half of persecution, it was nearly extinct in northern and central Europe. Only few populations outlived in the mountainous areas of Portugal, Spain, Italy, Greece, Yugoslavia, and Scandinavia (Breitenmoser 1998).

The height of contraction the European wolf population was reached in 1960s and 1970s due to prey species depredation and loss of suitable habitat availability.

At present, wolf population is extensively increasing in number while expanding the distribution range. The estimated number of wolves in geographic Europe is likely to exceed 10,000 individuals. Nonetheless, European wolf presents a metapopulation structure constituted by partially isolated local fragments (Fig. 2).

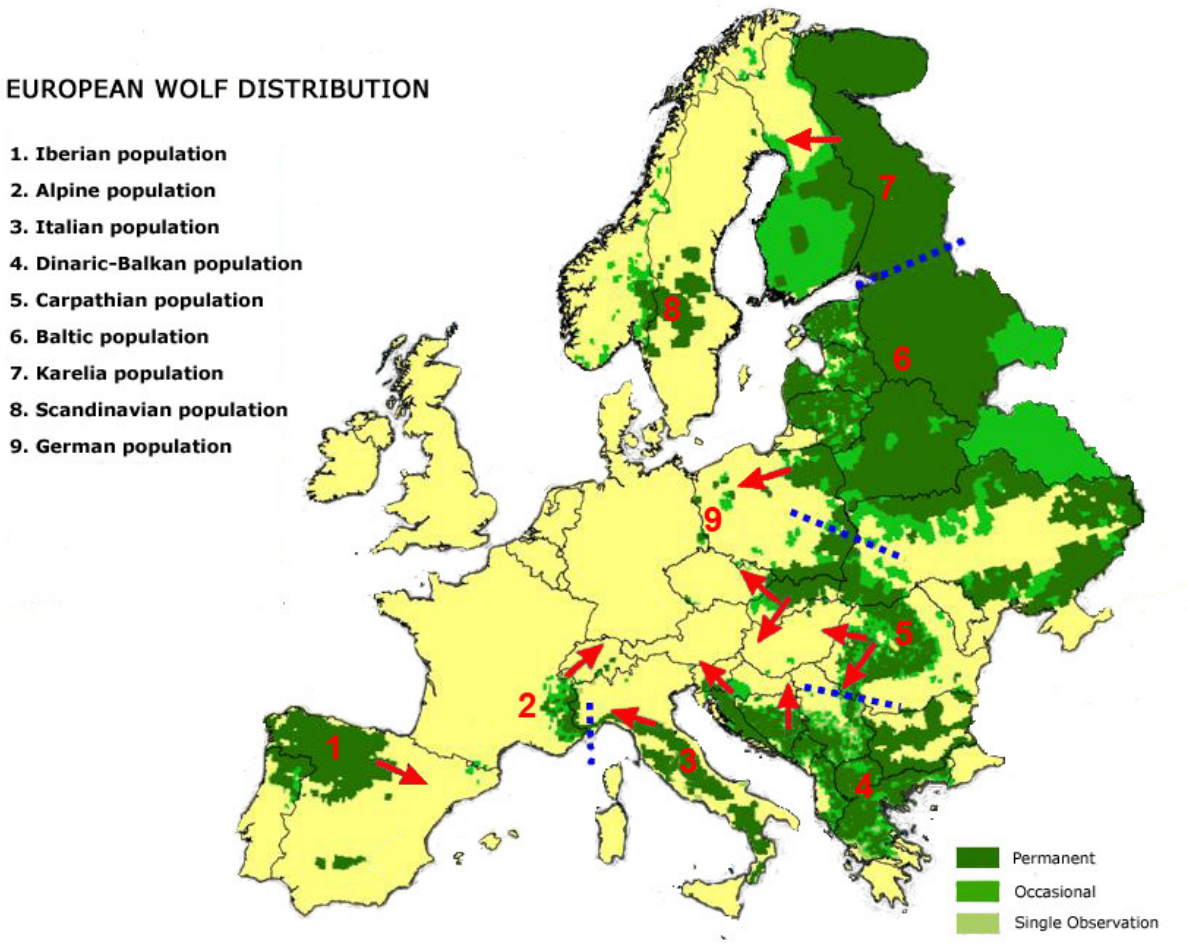

Figure 2 Current wolf distribution in Europe, indicated by the green areas. In evidence the nine population (red number) expansions tendencies (red arrow) and in blue the limit between that populations (modified from LCIE website).

The main conservative traits of such metapopulation have been outlined in the framework of the Large Carnivore Initiative for Europe[3] (LCIE 2007) as follows:

1. The **Iberian population** is increasing (approaching around 2,500 individuals) and spreading toward south and east despite the widespread illegal killing (poison baits, shooting, etc.). Most of the population is established in the north-west area including the western Basque country. In fact due to the inadequacy of coherent management and over-hunting, wolf is classified as **Near Threatened**[4] **(**NT). Nevertheless, the very small sub-population isolated in Sierra Morena (around 50 individuals) should be classified as **Critically Endangered**[5] (CR).

It is disputed whether the Iberian wolf (*Canis lupus signatus*, Cabrera 1907) may be classified as a distinct sub-species.

2. The **Alpine population** occupies an area that includes most of the Western Alps in France and Italy south of Valle d'Aosta. Even though isolated animals occasionally trespass the borders, until now there is no evidence of permanent establishment in Switzerland (Boitani 2003, Marucco *et al.* 2005). The native population was exterminated in the 1920s. The present population is the recent outgrowth (starting from 1992) of the Italian wolf with which, in fact, shares the same Italian haplotype (Fabbri *et al.* 2007). At present, the Alpine population has limited genetic and demographic contacts with the wolves living on the Apennines[6]. This population is still numerically exiguous (around 100-120 individuals) although it is increasing fast (on average 10% per year). Its small size justifies the assessment in category **Endangered**[7] (EN) and thus it is fully protect under both national and international law.

Mortality causes are mainly car or train accidents and poaching or illegal killings.

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[3] LCIE is a working group of the species survival commission of the International Union for Conservation of Nature (hereinafter IUCN).

[4] See above note 2.

[5] See above note 2.

[6] It is estimated that less than one migrant individual per year is successful. It ensues that the Alpine population is classified as a subpopulation under IUCN Red List guidelines

[7] See above note 2.

3. The **Italian population** was widespread across the Italian territories (Cagnolaro *et al*. 1974) until the end of the 19th century with the exception of Sardinia Island. It was exterminated in the Alps in the 1920s (Brunetti 1984) and in Sicily in the 1940s (Cagnolaro *et al*. 1974). Wolves occur in the whole Apennines range from Liguria to Calabria (Aspromonte) and extending into northern Lazio and central western Tuscany (provinces of Siena, Grosseto and Pisa) (Ciucci & Boitani 1998, Corsi *et al.* 1999, Boitani 2003). Nowadays, the Italian wolf population is estimated to be of at least 500-800 individuals distributed along the Apennines while in the 1970s the number was drastically reduced to 70-100 individuals. Both the positive growth trend and the expansion process toward north and east make possible the gradual re-colonisation of previously inhabited areas. The current distribution range extends along the Apennines and the western Alps but, as said, the Italian population has limited exchanges with the Alpine population. Furthermore, recent genetic studies demonstrate that gene flow is unidirectional (from Apennines toward the Alps).

The Italian wolf population is classified as **Vulnerable**[8] (VU) because of human extermination (poisoning, poaching, car accidents). The stochastic nature of such events suggests maintaining a cautionary assessment.

4. This **Dinaric-Balkan** population is estimated around 5,000 individuals. The population range extends from Slovenia to north-central Greece covering the whole Dinaric mountain area through Croatia, Bosnia-Herzegovina, western Serbia, Kosovo, Montenegro, Macedonia, Albania, western and southern Bulgaria. Being limitedly managed, the population appears to be in favourable conservation status (**Least Concern**[9] (LC)). Rare dispersal individuals may trespass the borders to and from Italy thus enabling occasional gene flow between the neighbouring populations.

Hunting killings both legal and illegal, poisoning, shortage of wild preys, and the habitat fragmentation (e.g. highways construction) are common and widespread. However, ad-hoc management actions should be implemented in the marginal parts of wolf habitat mainly in

[8] See above note 2.

[9] See above note 2.

Slovenia, Croatia and southern Greece where wolves are fully protected but human pressure has a significantly negative impact.

5. The **Carpathian** population (c. 5,000 individuals) is classified as **Least Concern**[10] (LC).

The population extends across several countries, from Northern Bulgaria to Eastern Serbia, Romania, south-western Ukraine, Slovakia and southern Poland. Most of the population is established in the central Carpathian Mountains. Wolves' packs in Romania and Ukraine are particularly abundant and prosperous. On the contrary, southern Poland and Slovakia are critical areas which may urge for ad-hoc conservation measures. This is the consequence of the incoherent management regimes applied in the neighbouring countries (i.e. Ukraine, Poland and Slovakia). Widespread usage of poison baits and illegal killings remain the main threats. Moreover, in Ukraine wolf is often considered as a pest species while in Slovakia and Romania wolves are game species.

The adoptions of consistent decisions at international level are thus necessary to ensure viability particularly of the marginal parts of the Carpathian population.

6. The **Baltic** population sizes around 3,600 individuals and spread across eastern Poland, Lithuania, Latvia, Estonia, Belarus, northern Ukraine, and part of Russia.

During the 20th century, the standard management policy implied open harvest which seriously reduced wolves' presence. Despite the numeric fluctuation during and after the War World, at the present the Baltic wolves appear to be relatively consistent in number. Nonetheless, small portions of the population in Poland (currently protected) and some of the Baltic States may require conservation measures to preserve their long term existence.

The Baltic population is listed as **Least Concern**[11] (LC).

7. The **Karelia** population lives in Finland (mainly in the south) and Russian Karelia. Almost no data is currently available on the population amount but it is considered not to exceed the total of

[10] See above note 2.

[11] See above note 2.

10,000 individuals. The population, listed as **Least Concern** (LC), may be declining due to persecution and it may thus be assessed as **Vulnerable** (VU).

8. The **Scandinavian** population is estimated to be about 250 mature individuals. The population originated from a pair migrated from Finland and firstly reproduced in 1983. Scandinavian wolves persist isolated since the last (and sole) known genetic exchange in 1991 with a Finnish wolf. Due to the high inbreeding average, the population presents a limited genetic variability and is classified as **Endangered** (EN). The population is distributed in central Sweden and in south-eastern Norway spreading toward southern and northern Sweden and into southern Norway. Both Countries fully protect the species and provide full damage compensation.

9. The **German** population consists of scattered packs living in eastern Germany (Saxony) and western Poland. The species amounts to less than 50 individuals and thus listed as **Critically Endangered** (CR). Such a tiny, fragmented and isolated group was exterminated in Germany in the 19th century. The main threats to its existence are the exiguous number, the high fragmentation rate, and the reduced likelihood of gene flow occurrence.

Box 1 - Distribution Status in Europe

In many European country the wolf is completely extinct: Austria; Belgium; Denmark; France; Germany; Hungary; Ireland; Luxembourg; Netherlands; Switzerland and United Kingdom.

Native - Presence confirmed Austria; Belarus; Belgium; Bosnia and Herzegovina; Bulgaria; Croatia; Czech Republic; Denmark; Estonia; Finland; France; Germany; Greece; Hungary; Ireland; Italy; Latvia; Lithuania; Luxembourg; Macedonia, the former Yugoslav Republic of; Moldova; Netherlands; Norway; Poland; Portugal; Romania; Russian Federation; Serbia and Montenegro; Slovakia; Slovenia; Spain; Sweden; Switzerland; Turkey; Ukraine; United Kingdom.

1.1.4 Conservation status and legal protection

In 1973, the International Union for Conservation of Nature (IUCN) approved the Manifesto Declaration of Principles for Wolf Conservation focusing on human dimension and management of the species. The same year, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) included the wolf as one of the *potentially endangered* species (CITES Appendix II), with the exception of the populations of Bhutan, Pakistan, India and Nepal where wolf was listed as a species in *danger of extinction* (CITES Appendix I).

The IUCN listed the European wolf as of **Least Concern** (LC) and the species is considered of 'community interest'. Since the 70s, several both national and international laws regarding wildlife and environment ensure adequate protection to the endangered European wolf.

Since 1979, the Bern Convention on the Conservation of European Wildlife and Natural Habitats (CCEWNH) listed the wolf as a *strictly protected species* (CCEWNH Appendix II). In 1989, the Committee of CCEWNH adopted an articulate Recommendation on the Protection of the Wolf in Europe (Rec. n. 17/1989). In 1989, the European Parliament approved a Resolution (Doc. A2-0377/88, Ser. A) calling for all the European countries to undertake immediate steps in favour of wolf conservation. Furthermore, the Parliament adopted the IUCN Wolf Manifesto and invited the European Commission to expand and provide financial means to support wolf conservation (Promberger & Schröder 1993). According to the EU Habitats Directive (92/43/EEC 21.5.1992), wolf is a *species in need of habitat conservation* in all European Member States (Appendix II).

In Italy wolf is a strictly protected species. Since 1971, the wolf hunting was prohibited by law and from 1976 the species was fully protected. The Ministry of Environment and the Ministry of Agriculture delegated to the Regional Authorities the proper implementation of laws for wolf conservation. Particularly, Regional Authorities are responsible for compensation of damage caused by wolf on livestock. In 1992, L. n. 157 enlisted the wolf as a strictly protected species. The DPR n 357/1997 incorporated the EU Habitats Directive into national law as a species of European interest, thus requiring a severe protection.

Despite the legal protection framework, human persecution (such as illegal hunting, poaching, etc.) still remains a major threat for the Italian wolf population. The reasons for such illegal killings are to be found, firstly, in the wolf predatory behaviour which often conflicts with human activities such as livestock grazing. On the other hand, authorities power clashes with the violation of the laws. In summary, the main limiting factors for the wolf conservation in Italy are:

- Illegal killing: poaching, shooting and car accident;
- Low habitat suitability and loss of corridors needed for connection and dispersal;
- Low densities and demographic fluctuation;
- Abundance of feral dogs and episodic phenomenon of hybridisation.

1.1.5 Peculiarities and Characteristics of Italian population

In 1921, Altobello classified the Italian wolf population as distinct subspecies *Canis lupus italicus* or Apenninic Wolf in reason of the grey-tawny coat with a grey-black stripe of 10 cm in the middle of the back and the black longitudinal stripe on the frontal legs (Fig. 2a). Yet, these phenotypic characteristics were not enough to identify the Italian wolf as a distinct subspecies.

Figure 2a A young wolf female with the typical coat in spring in Tuscany. (September 2005, Casentinesi Forest National Park, Graziano Capaccioli).

New taxonomic methods based on both morphometrical (Nowak & Federoff 2002) and genetic analyses (Randi *et al*. 2000, Randi & Lucchini 2002) supported Altobello's hypothesis and offered new evidence of such a differentiation from the European wolf.

The reason for the genetic distinction of the Italian population could rely on the existence of geographical barriers which hindered dispersal.

A possible hypothesis may involve the impact of Quaternary glacial cycles which forced species to migrate toward southern refugia (Hewitt 1996, 1999, 2000). Particularly, the Italian geomorphology (Alps Chain and Po River) might have isolated wolves in central-southern Apennines since the Last Glacial Maximum (ca. 18,000 ybp.) reducing habitat expansion and mixing among European wolves.

A second possible scenario may depend on deforestation and direct human persecution - already widespread in the fifteenth century in northern Italy - which might have hampered the connectivity, thus the gene flow, among wolves in the Apennines and the European populations (Lucchini *et al*. 2004).

1.1.6 The debated process of domestication

The canids are an old lineage, separating from the other carnivores about 60 million years ago. Separation of a wolf-like branch, a South American canids branch and a fox-like branch occurred more recently, 7-10 million ybp.

Within the family Canidae, the dog is closely related to wolves, jackals and coyotes, as these canids all share the same number of chromosomes and are all capable of interbreeding to produce fertile offspring even though in nature it is not a typical behaviour.

This makes all of these species potential dog ancestors, and all have been suggested to have played a part in the dog's ancestry. The questions as to where the dog originated, which wild canid gave rise to dogs, and why and how it became domesticated and associated with humans are all still debated.

The origin of domestic dog (*Canis familiaris*) was debated for a long time, because the classification is based on a low number of archaeological samples founded around the world and because the dog presents low genetic differentiation from the other canids.

Wolf and dog are so closely related that in 1993 the American Society of Mammalogists and the Smithsonian Institution's reclassified the dogs from its separate species designation of *Canis familiaris* to *Canis lupus familiaris* as a domestic variant or subspecies of wolf (*Canis lupus*).

However, *Canis familiaris* is still used by most biologists as the scientific name for the dog.

There is no doubt that dogs were associated with humans by the early Neolithic, as a great deal of rock art depicting dogs with humans and clay sculptures of dogs have been found in southwest Asia, Iraq, Turkey and to a lesser extent, Africa, England and Denmark.

A comparison of wolf and dog control region of mitochondrial DNA sequences was considered in light of the average number of changes expected to occur and it was estimated that the earliest dogs may had been domesticated about 135,000 years ago Vila *et al*. (1997). On the contrary the oldest discovered fragment of dog bone is from Germany and dates back to about 14,000 ybp.

Throughout centuries, the dog has evolved to become one of the most variable animal species. Between 300 and 400 dog breeds exist in the world today, and 335 modern dog races are censed from the Fédération cynologique internationale (FCI).

It follows that there is a huge dimensional and phenotypic variability which vary significantly in size and which display an astounding amount of variation in coat type, coat colour, and general morphology. All that diversifications are due to the long process of domestication operated from man.

Much of the diversity found in the domestic dog may be a result of a selection for tameness around people. Selecting an individual for a behavioural trait could alter its development and thus its morphology. Although it is often assumed that the dog is a result of artificial selection, it is possible that early dogs evolved via natural selection. Two different approaches were examined.

The first hypothesis (*monophyletic*) was that domestic dog descended from the wolf. The second hypothesis (*multiple origins*) was that dog evolved from wolf-like ancestors as wolf, jackal, and coyotes.

The Majority of researchers were in favour of the first assumption (*monophyletic*) (Zimen 1981) based on morphological and behavioural characters. More recently, genetic studies showed that the two species have in common a consistent number of microsatellite loci and mitochondrial DNA (Wayne & Vilà 2001). These authors reported that there is only 2% of difference in the sequences of mitochondrial DNA between dog and wolf, while there is 4% of difference between dog and coyote. It is important to underline that same degree of difference (2%) represents the variability observed within the wild wolf populations.

As the dog was the only domesticated species worldwide widespread before the fifteenth century, it was commonly supposed that it evolved from the wolf, a species with a distribution that spanned Asia, North America and Europe. This assumption confounded any efforts to determine where the dog originated, and whether or not New World dogs were either the descendants of Old World dogs or the result of a separate domestication event.

Savolainen *et al*. (2002) while sequencing mtDNA noted that dogs from East Asia possessed significantly more genetic variation than dogs from other parts of the world. This discovery suggested that domestic dog may have originated there.

Morphological evidence also pointed to an East Asian origin of the domestic dog. In fact, a peculiar osteological jaw feature found in domestic dogs was detected in East Asian wolves, while it was absent in all other wolves (Olsen & Olsen 1977).

Another interesting issue related to whether or not there were two different domestication events in the Old World and the New World. Leonard *et al.* (2002) compared ancient Latin American dogs' DNA sequences to fragments from a number of modern dogs and grey wolves. The ancient Latin American dogs did not appear to be as closely related to North American grey wolves as they were to Eurasian grey wolves and dogs.

This suggested not only that North American dogs were the descendants of Eurasian grey wolves, but also that both ancient and modern dogs throughout the world were descendants of Old

World wolves in east Asia. Thus there were no evidence of a separate domestication event occurred in North America.

In conclusion, evidence suggested that the dog most likely has been domesticated more than once (Tsuda *et al.* 1997, Vilà *et al.* 1997). On the other side, there was no proof of separate domestication events in the American and the Eurasian continents.

1.1.7 How does the domestication proceed

As previously mentioned, it is acknowledged that dogs descend from Eurasian grey wolves, and thus that they may have originated in East Asia. Dogs are similar to wolves in terms of both behaviour and morphology. Nevertheless, dogs display a broader spectrum of variability, such as regarding the coat colour and patterns.

Wolves are seasonal breeders (fertile once per year) and may have from two up to six pups per litter. While dogs are non seasonal breeders (female fertility occurs twice per year) and generally have more and bigger litters. Many of the morphological and physiological differences that exist between dogs and wolves may not have been intentionally selected for by humans, and could have been a result of selection for tameness in dogs. Reduction of skull size is also one of the main ways dogs differ from wolves. But, how does selecting animals for a behavioural trait (like tameness) change their overall morphology?

It has already been noted that artificial selection can affect the amount of hormones and neurotransmitters produced by the individuals since behaviour is controlled also by such chemicals. The early development of an animal is also, in part, controlled by these chemicals. It follows that a limited change in the endocrine and neurochemical systems may result in changes to the early development of the animal.

Many researchers consider dogs to be paedeomorphic wolves, meaning that dogs have retained characteristics that are typical of juvenile wolves as adults. For example, the floppy ears, as very young wolf pups. Likewise, the curled sickle tail is also a neotenous trait.

Adult wolves typically have straight tails that are carried at a downward-pointing angle, whereas wolf pups, like many adult domestic dogs, have tails that are carried up above the back. The bark of domestic dogs is another juvenile trait. Adult wolves do barks as an alarm call, but they rarely do. However, wolf pups bark more often than adult wolves. (Coppinger 1983, Coppinger & Coppinger 2001). Adult wolf-sized dogs have head sizes and skull characteristics that are similar to that of a juvenile wolf.

1.1.8 Dog genetics

The Dog is one of the most extensively and worldwide investigated species as for the study of morphology, behaviour, and disease. Consequently, we have a rich literature dealing with several topics concerning the species. One of the most comprehensive study is the Dog Genome Sequencing Project conducted by the Broad Institute (http://www.broad.mit.edu/mammals/dog/) of MIT and Harvard. Based on the whole genome shotgun assembly (CanFam2.0, released in May 2005), researchers sequenced the DNA of Tasha, a female boxer.

The dog genome contains approximately 2,5 billions base pairs. More than 90% of the canine genome is covered, including a total of 1,800 markers. A complete database of 39 chromosomes containing more than 2,5 millions SNPs in the dog genomes have been published (Tab. 1).

The Dog Genome Sequencing Project offered the possibility to find genes, their evolution, and the regulatory mechanisms governing their expression. Furthermore, it has made possible to compare domestic dog and other mammals like rodents and humans. Like domestic dog, grey wolf has a high diploid number (2n = 78) and all acrocentric autosomes. Additionally, the negligible degree of differentiation (2%) between dog and wolf allows the replication of most of the studies on both species.

Chromosome	Number of SNPs
Dog CHR 1:	118963
Dog CHR 2:	94211
Dog CHR 3:	102511
Dog CHR 4:	90810
Dog CHR 5:	83962
Dog CHR 6:	80751
Dog CHR 7:	82525
Dog CHR 8:	69180
Dog CHR 9:	68109
Dog CHR 10:	68942
Dog CHR 11:	80286
Dog CHR 12:	71547
Dog CHR 13:	78958
Dog CHR 14:	62582
Dog CHR 15:	63741
Dog CHR 16:	80891
Dog CHR 17:	75908
Dog CHR 18:	62305
Dog CHR 19:	64248
Dog CHR 20:	55478
Dog CHR 21:	67183
Dog CHR 22:	69622
Dog CHR 23:	67361
Dog CHR 24:	58387
Dog CHR 25:	62481
Dog CHR 26:	59453
Dog CHR 27:	54348
Dog CHR 28:	51436
Dog CHR 29:	49409
Dog CHR 30:	47091
Dog CHR 31:	53804
Dog CHR 32:	48370
Dog CHR 33:	38077
Dog CHR 34:	53725
Dog CHR 35:	40027
Dog CHR 36:	36718
Dog CHR 37:	35738
Dog CHR 38:	33668
Dog CHR X:	61702
TOT	2,544,508

Table 1 List of SNPs for each Dog Chromosome.

1.1.9 Canine coat colour genetics

Pigmentation in most mammals is primarily due to the presence of melanin, which is synthesized in specialized cells called melanocytes. Melanocytes come from the cells of the neural crest, which is located on the dorsal mid-line of the early embryo. Mammals have two forms of melanin in their coats.

The first one is the **eumelanin**, which is dark. It can vary somewhat in colour due to variations in the protein that forms the framework of the pigment granule. The base form of melanin is black. Melanin can also appear brown or blue-grey.

The second pigment, which varies from pale cream through shades of yellow, tan and red to mahogany, is called **phaeomelanin**. In the hairs, melanin is found in minute pigment granules.

In figure 3 is shown the high chromatic variability in wild canids: two completely different morphotype of red fox.

Different gene series determine where eumelanin and phaeomelanin appear on the coat and along the length of the hairs. The genetics of coat colour is largely concerned with the genes that affect the number, shape, arrangement or position of these granules, or the type of melanin they contain.

Figure 3 Two pups of Canadian red fox (*Vulpes vulpes*) by Eddy Bouvier.

Coat colours in domestic dog have many natural phenotypic variants regulated by a wide variety of genes. Some of the genes and alleles involved also cause genetic developmental defects, which are also observed in humans and mice. Furthermore, a gene may give origin to different effects. Each gene encodes the structure of a particular protein, and controls when and where other genes are turned on or off. In the past few years, enhancements have been made in identifying genes involved in pigmentation in dogs. Comparative genomics has both aided and benefited from these findings.

We reported in Table 2 the seven principal genes known to influence canine colour and involved in the complex pathways of coat pigmentation process.

Acronym	CHR	Name and function
MC1R	5	Melanocortin 1 receptor or melanocyte-stimulating hormone receptor
AGEX 1-4	24	Agouti Signal Peptide(ASIP), antagonist of MC1R
COMT	22	Catecolamina O metiltrasferasi
SILVER	10	Silver (formerly PMEL1)
TYRP 1	9	Tyrosinase Related Protein 1
HTR 1	7	Hydroxytriptamine (serotonin) receptor
K locus	16	Beta-defensin 103, alternative ligand for the Mc1r

Table 2 The seven genes involved in dog coat colour determination.

These genes have been identified in dogs as responsible of specific coat colours patterns. Nevertheless, not all alleles have been yet identified at each locus. The identification of these alleles has provided information on interactions in this complex set of genes involved in both pigmentation and neurological development. Some coat colour genes are suspected to be correlated to disease. The alleles found in various breeds have shed light on some potential breed development histories and phylogenetic relationships. The coat colour is such a visible trait that dog breeders have selected for and against specific colours since the late 1800s.

Figure 4 A solid black German Shepherd dog due to human selection.

We studied the genetic bases of the black phenotype (Fig. 4) in dogs to verify the pigmentation mechanisms and to identify genes involved in these complex pathways. All the genes mentioned are involved like inhibitor or antagonist as well as dominant, recessive or incompletely dominant genes.

In several dog breeds, colour has been one of the traits mostly researched for. In fact, it was one of the traits under selection for at least one hundred years. In a few cases, certain colours were selected against because of the belief that these colours may have brought health related problems. Other colours were selected against or for because breeders deemed that such colours may help the breed do better its job. For instance, in the 19th century brown was considered a better camouflage colour and thus selected for the European hunting breeds.

1.1.10 The occurrence of black wolves

Black coated individuals are common in the Class Mammalia. Nonetheless, not all the wolf populations across the world register the same black occurrence. In Northern USA and Canada the occurrence of black phenotypes in wolves is very high reaching over 50% in some areas. On the contrary in Europe melanism in wolf is extremely rare or completely absent in the majority of the populations. The black phenotype is not considered a typical characteristic in Italy as it was never described in the past. Several hundred of wolves' carcasses in the last century were collected by veterinary (Guberti & Francisci 1991; Lovari *et al*. 2007) or stored in museum and none of those were black. Nevertheless, in the last 20-30 years, in the northern Apennines, some black individuals were sighted. In the last few years, dark wolves sighting increased in northern Apennines (Tuscany and Emilia Romagna) and in the Alps.

The occurrence of black-coated individuals in wolf populations is not surprising, but their presence in populations recovering from a severe numerical decline has been considered a possible sign of crossbreeding with the domestic dog. In the northern Apennines, black wolves occur at a non-negligible frequency. Two main studies were conducted on the Italian black wolves:

1. Apollonio *et al.* in 2004 reported that in a 3,300 Km^2 area 22% of the observed wolves and 23% of all the dead wolves found were completely black individuals. A black coated individual belonging to the studied population was analysed using a set of microsattellite loci. The outcomes showed no evidence of hybridization in its ancestry. This result induced to consider that the

occurrence of black phenotype in this area may derived from a natural combination of wolf alleles in coat colour determining genes, and not necessarily from crossbreeding with the domestic dog.

2. Randi and Lucchini in 2002 analyzed a set of 18 microsatellites in order to detect introgression of domestic dog genes into wild wolf population. Premise to the research was the sporadic number of cases of crossbreeding observed in Europe. They deep investigated wolves, feral dogs, known hybrids and wolves presenting unusual phenotype suggesting hybridization in wolf (included two black individuals). Analyses showed that one of the black wolves had mixed ancestry and may have been a hybrid, while the other individual was assigned to the Italian wolf population.

The lack of data on the melanism of the Italian wolf has induced us to search, collect, and arrange all information available. Since 2004, we recorded all black individuals sighting and carcasses found reported by biologists, rangers and photographers, who actively supported the information collection. Thanks to the collaboration of all the people involved, we succeeded in individuating the areas mainly interested by the melanism phenomenon.

1.1.11 Two possible explanations for melanism

As we mentioned before, the introgression of the mutation correlated with black colour could be following a case of hybridisation with a melanic domestic dog. Yet no clever genetic evidence of that hybridization event supports such hypothesis.

The alternative scenario could be that the occurrences of the black coat originated from a natural combination of wolf alleles in coat colour determining genes. Black coated colour could be explained as a fixation of a mutation in a local population, eventually in the northern Apennines, which subsequently was spread to the North with the expansion of the population.

The only way to determine which of the two hypotheses is more likely is that to characterize the mutations involved in the determination of melanism so to detect its origin.

1.2 Fundamentals of Deoxyribonucleic Acid (DNA) Variability

1.2.1 - DNA structure and function

DNA takes the form of double helix formed by four nucleotides: Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). The linear order in which these four nucleotides follow each other in the double helix is called nucleotide sequence. This simple structure is extremely stable and allows the DNA to act as a template for protein synthesis and replication.

Eukaryote organisms have two types of DNA, nuclear (nDNA) inherited from mother and father and mitochondrial (mtDNA) which is inherited only maternally. The nuclear DNA is located in the cellular nucleus. The genes in the nDNA are responsible for external characteristics and for behaviour, but they also have important regulatory functions inside the cells. The mitochondrial DNA is separate and distinct from nDNA and is found in the mitochondria, organelles present in the cell cytoplasm. The coding gene in the mtDNA is strictly regulatory and has little effect on external characteristics or behaviour in comparison to nDNA.

Nuclear DNA is organized in packed units called chromosomes and each cell has two copies of each chromosome (diploid karyotipe). One pair of chromosomes is involved in sex-determination (sex chromosomes, X and Y) and all the other chromosomes are called autosomes. Mammals have only one pair of sex chromosomes, but the number of autosomes varies according to species.

The mechanism of DNA replication is the basis of the hereditary transmission of genetic information. DNA is replicated before each division is completed. Each of the daughter cells receives a new complete set of chromosomes. Each of the two DNA strands (chromatids) is replicated when DNA is denatured and the double helix is opened. The enzyme that catalyzed the replication, the DNA polymerase, binds itself to denatured area and starts to replicate while controlling the insertion of nucleotides. The two new double helixs are identical, each one formed by a parental chromatid and by a complementary chromatid. In this way DNA sequences are faithfully copied and the genetic information coded in the sequences is preserved during cell duplication.

Genes are subunits of DNA located in the chromosomes that encode for particular products, which are mainly proteins. Variations of the same gene, called alleles, lead to alternative form of a trait. Each chromosome pair has two alleles. Some alleles are dominant and tend to be more often expressed, while others are recessive and are only expressed when both alleles of a gene are recessive. The dominant allele masks the effect of the other allele. Genes constitute the functional unit. DNA sequences constituting a gene regulate the transcription. The first part of the gene is made up of a promoter, which is a sequence of a few dozen nucleotides recognised by RNA polymerase. This is followed by coding sequences (exons) that normally alternate with tracts of sequences that are transcribed, but not translated (introns). The gene ends with a termination sequence that interrupts RNA synthesis. Noncoding tandem repeated DNA exists in the genome of every species (repetitive DNA). Tandem repetitive sequences are commonly known as "satellite DNA".

1.2.2 - Genetic mutations polymorphisms and genetic markers

The mayor forces that can cause evolutionary changes are: Natural selection, Genetic Drift, Non-random mating, Migration and spontaneous Mutations. Mutations modify DNA sequences and generate genetic variability both at individual and population levels. The process of replication may present imperfections and some nucleotide mutations may be inserted randomly. A variable gene is defined as polymorphic. Polymorphisms indicate the presence of two or more variants of a DNA sequence. Gene coding polymorphism can generate protein polymorphism. All these elements can be used as markers in the identification and individuation of samples in forensic science. The highly variable non coding DNA sequences, that apparently are not subject to strong pressures from natural selection and therefore evolve rapidly and neutrally, make up the most useful and reliable genetic markers in acquiring evidence in forensic genetics. A genetic marker is a known DNA sequence that can be identified by a simple assay. It can be described as some sort of variation present can arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change

(single nucleotide polymorphism), or a long one, like single strand repeat (STR) also known as microsatellites. Genetic markers have to be easily identifiable, associated with a specific locus, and highly polymorphic, because homozygotes do not provide any information. Detection of the marker can be direct by DNA sequencing, or indirect using allozymes.

1.2.3 Single Nucleotide Polymorphism (SNP)

During the last ten years, the use of molecular markers, revealing polymorphism at the DNA level, has been playing an increasing role in animal genetics studies. Amongst others, the STR DNA marker has been the most widely used, due to its easy use and to the high degree of information provided by its large number of alleles per locus. Despite this, a new marker type, named SNP, discovered in the 80s, is now on the scene and has gained high popularity. SNPs are point mutations that constitute the most common type of genetic variation and are found at a rate 0.5-10 per every 1000 base pairs within the human genome (Collins *et al*. 1997). They can occur both in coding and non coding regions of the genome. The low frequency of single nucleotide substitutions at the origin of SNPs is estimated to be between 1×10^{-9} and 5×10^{-9} per nucleotide and per year at neutral positions in mammals (Li *et al*. 1996, Martinez-Arias *et al*. 2001). A single base changes in a DNA sequence with a usual alternative of two possible nucleotides at a given position (ex substitution Guanine-Adenine on the 57 nucleotide G57A). The probability of two independent base changes occurring at a single position is very low. Another reason is due to a bias in mutations, leading to the prevalence of two SNP types.

Mutation mechanisms result either in transitions: purine-purine (A↔G) or pyrimidine-pyrimidine (C↔T) exchanges, or transversions: purine-pyrimidine or pyrimidine-purine (A↔C, A↔T, G↔C, and G↔T) exchanges. With twice as many possible transversions than transitions, the transitions over transversions ratio should be 0.5 if mutations are random. However, observed data indicate a clear bias towards the transitions. One probable explanation for this bias is the high spontaneous rate of deamination of 5-methyl cytosine (5mC) to thymidine in the CpG

dinucleotides, leading to the generation of higher levels of C↔T SNPs, seen as G↔A SNPs on the reverse strand (Cooper & Krawczak 1989, Wang *et al.* 1998). Some authors consider one base pair indels (insertions or deletions) as SNPs, although these certainly occur by a different mechanism.

In summary this simple biallelic codominant markers are the most prevalent form of genetic variation and hence there is a very high number of loci available (Brumfield *et al*. 2003). Furthermore, the simpler mutational dynamics of SNPs lends the advantage of a lowered rate of homoplasy, and, importantly, there is a capacity for rapid, large scale and cost-effective genotyping (Syvänen 2001, Vignal *et al*. 2002, Chen & Sullivan 2003, Schlötterer 2004).

Miller and Pui -Yan Kwok (2001), describe the life cycle of SNP that it can be summarized in four phases as follow: (i) appearance of new variant allele by nucleotide mutation; (ii) survival against odds of the allele through early generations; (iii) increase to substantial frequency including survival through population fluctuation; (iv) fixation.

The increasing progress made in the molecular techniques used to obtain SNP data, the automation of allele scoring and the development of algorithms for genetic analyses (Abecasis *et al.* 2002) allow to overcome the limitations due to the low heterozygosity of SNPs and to produce an equivalent amount of information as with microsatellites.

The very high density of SNPs in genomes allows analysing several of them at a single *locus* of a few hundred base pairs. Thus represent a more reliable and faster genotyping method since amplifying short sequences and extending single nucleotides, SNPs genotyping should increase PCR success and reduce the allelic dropout and false allele rates. Moreover recent progress of genomics revealed that a relevant part of individual variability is to attributes to single nucleotide polymorphism.

To conclude SNPs are the markers of choice for many applications in medical and evolutionary genetics because they are common widespread and stable and can cause, or be linked to phenotypes of interest.

2 - AIMS

This thesis investigates the genetic diversity of the Italian wolf population applying the newest available genetic analysis techniques. Particularly, it deals with the debated cases of melanism in Italian wolves.

The principal aims of this study are the following:

1) detection of functional genes involved in wolf's morphological changes in consequence of domestication and captive-breeding. Starting from the existing literature on domestic dog, the research focused on the genes involved in the pigmentation process of hairs and black coat-colour determination so to clarify the relationship between anomalous phenotypic patterns and particular mutations;
2) samples collection and development of a database containing phenotypic information and geographical coordinates. Having created a communication network for collecting phenotypic data, this study took into account also the information, both historical and current, about black wolves' carcasses, sighting, documents, and photographs;
3) characterization of the main black phenotypic traits of the Italian wolf, with particular emphasis on coat appearance and hair morphological analysis;
4) analysis of the genetic variability within the Italian wolf population and comparison with the other Eurasian populations;
5) detection of diagnostic markers (SNPs) in wolves, dogs and hybrids in order to determine the potential dog gene introgression within the wolf population;
6) wolf individual identification (genotyping) and parentage determination by means of polymorphic SNPs instead of microsatellite markers;
7) optimization and application of the techniques tested on invasive samples to non-invasive samples analysis.

3 – MATERIALS AND METHODS

3.1 Sampling

In this work genetic analyses were conducted in the conservation genetic laboratory of the ex Italian Wildlife Institute (INFS, currently ISPRA) and at the Conservation Biology Genetic Laboratory of University of California, Los Angeles (UCLA).

We collected different kind of biological samples, as described:

- Tissues from carcasses preserved in absolute ethanol.
- Fresh blood preserved in Longmire's buffer.
- Pelts or hairs stored in absolute ethanol or in a dry envelop.
- Scats samples conserved in absolute ethanol.

In order to investigate the black mutation we analysed more than 200 invasive samples (tissues, blood and pelts) of phenotypically black and wild type wolves and dogs. We focused mostly samples collected throughout Italy so to obtain representative results from all over the country, from south to north. Additionally, we collected hairs from four purebred deep black dogs: one Toy Poodle, one shepherd dog, and two Labrador Retriever. As for the SNPs microarray analysis, we selected 100 invasive samples, and we processed 28 of them.

In 2004, we collected all data available as regards to both invasive and noninvasive Italian canids sampling. Our purpose was that to select all samples with complete information (phenotypic description, geographic coordinates) and create a more detailed database.

Having excluded all samples lacking information, the samples were divided in five different phonotypical categories, described as follow:

1) The Wild Type (WT) samples were 338 characterized by the typical grey- brownish coat colour.
2) The Black Wolves (BW) samples 9 varying from complete black, dark, and black with white spots. These samples were collected but one (from Abruzzi National Park) in the northern

Apennines. Additionally, we recently received 3 invasive samples suspected to be black wolves (1 from Croatia and 2 from Belarus).

3) The Hybrids (H) samples were 41 and presented anomalous phenotypic characteristics (such as spurs, stripes, white nails) or genetically dog-wolf hybrid (13 loci microsatellite).
4) The Dogs (D) were 201 mostly pertaining to free range individuals or mutts collected in kennels.
5) The Black Dogs (BD) were 13: 4 were purebreds black and with the exception of one black all the other 9 were random - breed black and tan individuals.

We firstly processed a small subset of samples representatives for all the five categories. By sequencing that selected subset it was possible to make comparisons for detection and determination of mutations related with phenotypes.

We collected literature on the domestic dog and a mostly focused on genes involved in coat colours determination.

A consistent number of samples were provided with field information, date, place and coordinates. We plotted such data by means of Geographic Informative System software (**ESRI ArcView 3.1**) to determine the geographical distribution which we compared to all available data relating to black wolves Italian distribution.

As second step we processed almost 180 genotypes (13 loci microsatellite) obtained from noninvasive scat sampling. Over more than 400 genotypes were checked for the black mutation. Such samples were collected in the framework of several Noninvasive Genetic Monitoring Projects conducted since 2000 particularly in Emilia Romagna and Tuscany.

With few exceptions, no phenotypic information but geographic was available. Nevertheless, in some cases we obtained personal communications and pictures which suggested the presence of black individuals in restricted areas. Specific thanks to the involvement of biologists, rangers and photographers, sighting data were of utmost importance to individuate the areas mainly interested

by the melanism phenomenon. Collectors provided the phenotypes description. Nevertheless, when possible, either we collected the carcasses or we asked for pictures.

Brownish, dark or completely black phenotypes were, indeed, characteristics difficult to describe. In fact, the very wide pattern of chromatic variance of coat colour frequently originated ambiguous descriptions. It ensues that different observer may define a dark individual either as completely black or darker than normal.

3.2 DNA extraction

There are various methods of extracting DNA which, nonetheless, share common phases. The main steps in the DNA extraction protocol are: lysis, precipitation, wash and resuspension.

The first treatment carried out in extracting DNA is the lyses of the cell membranes and proteins. This treatment disintegrates all the protein structures and releases DNA in solution. Digestion buffer may contain either proteinase K (ProK) or thiocyanate guanidine (GUS) digests proteins. Both produce the chemical disintegration of the protein structure. An anionic detergent supports the activity of ProK or GUS solubilising the cell membranes and denaturing the proteins. DNA is separated from the residues of the proteins and cells digestion in order to obtain a DNA solution free from other biological substances. The last step is elution, DNA is resuspended in a buffer solution, which is based on Tris and EDTA.

We processed all the samples utilizing a robotic automated extraction (see Box 2 for a thorough description).

The automated extraction guaranteed a rapid purification of total DNA thus involving minimal handling. This fast and reliable method enabled up to 96 simultaneous genomic DNA extractions.

Box 2 - The **Robotic Liquid Handling System: MultiPROBE II ex** (Perkin Elmer), is equipped with a Four Tip Arm (500 µl syringes), Versa Tips for pipetting, a Vacuum Manifold and a gripper. To obtain a uniform DNA concentration we applied the DNeasy® 96 Blood & Tissue KIT (QUIAGEN) according to the extraction protocol (www.quiagen.com).

The first step of the procedure consists of cutting a small amount of tissue (50 mg) or scat material (80 mg) into minute pieces. Scalpels and pincers are sterilized by flame with repeated dipping in ethanol. The cut amount is placed in a labelled 2.0 ml safe lock tube. After adding 20 µl of proteinase K and 180 µl of ATL Lysis Buffer (warmed up at 56 °C), the solution is mixed by vortexing and incubated in rotation at 56 °C until completely lysed. Usually the digestion took 30 min for scats and tissues samples and for hairs overnight. Vortex by centrifuge at room temperature for 10 minutes than the supernatant transferred in a new 1.5 ml collection tube for scats samples otherwise directly in a sterilised U-bottom QUIAGEN tube. The automated phase setted previously programming the software and preparing the bench with all the needed support and solutions, so transfer, aspiration, dispense and vacuum phases were activated and inactivated directly and consequently, samples can be ready in just one hour after lysis. The DNA solutions from the QUIAGEN tubes set on the robot's platform is mixed by pipetting and moved into the Binding Plate were the DNA is selectively bound to the DNeasy membrane as contaminants pass through. Than 410 µl of AL/E Lysis Buffer (warmed up at 57 °C) were added by the pump to each tube containing digested sample solutions to isolate the DNA; the remaining contaminants and enzyme inhibitors are removed in two efficient wash steps: in the first add 500 µl of AW1 Washing Solution and perform 10 minutes of vacuum; and than addition of 500 µl of AW2 Washing Solution followed by 10 minutes of aspiration. At the end 300 µl of AE, elution solution, warmed at 70°C, were added to each sample re-suspending the DNA linked to the silica-based membrane of the binding plate. The DNA solution transferred in a new tube and stored in freezer at -20 °C is ready to use.

3.3 DNA amplification

A single copy DNA sequences made up of a few dozen or thousands nucleotides can be amplified effectively up to 10 million times in a few hours thanks to Polymerase chain reaction (Mullis *et al.* 1986). PCR occurs by reconstructing the chemical conditions necessary to obtain DNA synthesis in vitro. First, it is necessary to identify the target gene or DNA sequence to amplify. The sequence to be amplified is flanked on both side by sequences that must be at least partially known, in fact to start off PCR it is necessary to chemical synthesize a pair of oligonucleotides (20-30 bp) "primers" that are complementary to the flanking sequences and can bind to flanking regions starting the duplication process of the target sequence. In PCR we utilize single stranded DNA as a template and, by the action of DNA polymerase enzyme, a complementary strand is synthesized over and over again, until extensive quantities are produced. Every PCR consists of a cycle, repeated many times, made up of the following steps: **denaturation** of the DNA sample at temperatures up to 90-95°C; **annealing** of the primers to the flanking sequences: it occurs at temperatures which vary from 40°C and 55°C, depending on the length of the primers and their base sequence; **extension** of the primers through the enzymatic action of a thermoresitant DNA polymerase (Taq Polymerase) which catalyses the extension of the primers: it occurs at 72°C end ends in the complete replication of both strands of the target sequence.

3.3.1 Amplification and sequencing of Mc1r and Agouti genes

As a first step towards a more complete understanding of the black coated colour determination, for the mutation of the **Mc1R gene** partially related to black in Doberman we tested a subset of ten individuals representative for the five categories, to evaluate the genetic relatedness of genotypes belonging to same or different categories.

We utilized as forward primer: 5'-GGTCATTGCTGAGCTGACAC-3' and as reverse (primer G): 5'-GAGATGCTGTCCAGTAGTCCC-3' as described in Newton *et al*. 2000.

The PCR was of 40 cycles at the conditions of 94°C/30 s, 60°C/60 s, 72°C/120 s.

To investigate linage of ***Agouti*** to black coat colour as described in Kerns et al 2004 we searched for mutations by PCR amplifying and sequencing fragments that contained each of the four exons of the **Agouti gene.**. We examined a sub set of 30 samples containing dog and wolf both black and non black. We PCR-amplified and sequenced fragments that contained all the four amplicons of AGOUTI as follows: exon 1, a 261 bp, forward primer 5'-CACCCAACACACTTCTGCG-3' and reverse primer 5'-TACCATACCAAAACATCTGC-3'; exon 2, a 265 bp, forward primer 5'-AGCCTGACTGCTTCTCTGTTCC-3'and reverse 5'-TGTTTGACATCCTGTAGCCCTT-3'; exon 3, 176 bp, forward primer 5'-AGCTCGTGGCCTCCAAAAACA-3' and reverse 5'-GTTCCTAGCTAGAATTCCT-3'; exon 4, 620 bp, as forward and reverse primer, respectively: 5'- GATGTCTGGTCTGGAGCCTC-3' and 5'-CCTTCTCAAAGTCCCATCTC-3'.

The cycling protocol conditions for each marker was 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and finished with a 4 min extension at 72°C on a thermocycler.

We focused mostly on identifying the missense alteration in exon 4, C427T that predicted to cause an Arg to Cys substitution at codon 96 (R96C). This single nucleotide variant in German Shepherd Dogs is likely to account for recessive inheritance of a uniform black coat.

3.3.2 Analysis of the K locus

In 2007 Candille *et al*. of the Stanford University reported that melanism in dogs, is caused by a different component of the melanocortin pathway, the K locus (for blacK), in which a beta-defensin protein encoded by CBD103 acts as an alternative ligand for the MC1R.

After that we collaborate with Stanford University and the presence of the KB mutation was still investigated at the same time on other individual especially wolves (Anderson et al. 2009). The only location in which we are aware of melanistic wolves' presence outside North America and southern Canada is Italy. The wolf and dog CBD103 (acronym of Canids Beta Defensin) mature coding

regions (the location of the KB ΔG23 mutation) were analyzed in a single amplicon by the sequencing primers *S54*, forward: 5'-TGTCTTCATCCCTGTGAGGT-3' and reverse: 5'-CCAGGAGGCATTTTCACACT-3', 396 bp, (Kerns et al 2007). A touchdown PCR protocol was used with the following conditions: (1) 94°/90 sec; (2) 94°/30 sec; 65°/30 sec (-0.5°/cycle); (4) 72°/60 sec; (5) repeat steps 2 – 4 x 20; (6) 94°/30 sec; (7) 55°/30 sec; (8) 72°/60 sec; (9) repeat steps 6 – 8 x 20; (10) 72°/5 min.

Direct sequencing of amplified product after primer hydrolysis with ExoSap-IT™ (GE healthcare) was carried out with standard fluorescent dye terminator technology on a ABI3130 Genetic Analyzer (Applied Byosistems) sequencer.

Candille *et al*. 2007 identified a three-nucleotide deletion in Betadefinsine gene on chromosome 16 related to black phenotype in dogs. We drawn with PRIMER 3 the couple of primers CBD103ΔG23 amplifying a short region including the discovered mutation. To identify the deletion we utilized the principle of fragments analysis that allows the electrophoretic separation of the two segments differing in three bases (Fig. 5). We utilized the forward primers 5'-TCCGGCACGTTCTGTTTT-3', labeled with 6-FAM dye, and the reverse primers 5'-TTCGGCCAGTGGAAGAAC-3'. Amplification conditions were 5 minutes at 94°C followed by 30-40 cycles of 15 sec at 94°C, 15 sec at 55°C, and 30 sec at 72°C, then 10 min at 72°C, and 4° for 10 min.

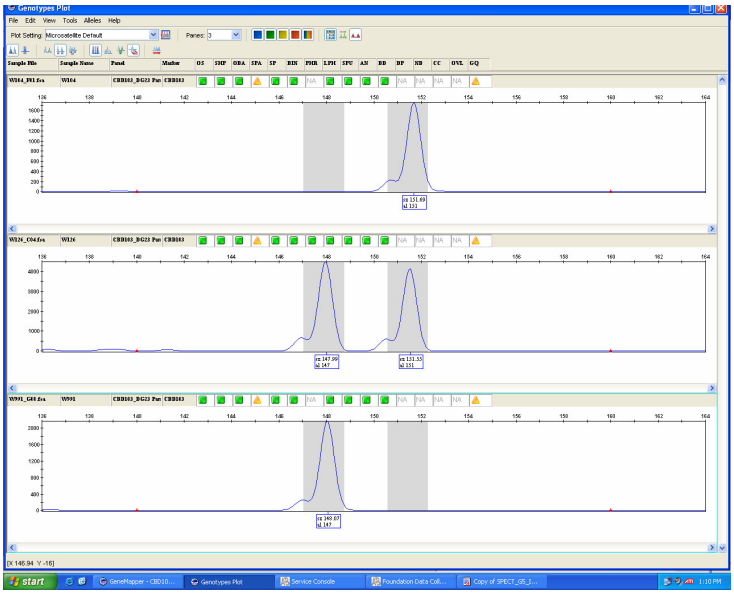


Figure 5 Example of K locus patterns, utilizing the primers *CBD103*ΔG23, outputs of three individuals showing respectively homozygosity (W991) for the 3 bases deletion, heterozygosity (W126) and homozygosity for the absence of the mutation (W104).

3.4 Microarray analysis

DNA microarray technology is a high throughput method for gaining information on gene function. Microarray technology is based on the availability of gene sequences arrayed on a solid surface (i.e. nylon filters, glass slides), and it allows parallel expression analysis of thousands of genes. Microarray can be a valuable tool to define transcriptional signatures bound to a pathological condition to rule out molecular mechanisms tightly bound to transcription. Since our actual knowledge on genes function in high eukaryotes is quite limited. Microarray analysis frequently does not imply a final answer to a biological problem but allows the discovery of new research paths which let to explore it by a different perspective. The DNA microchip technology consists of synthesized oligonucleotides containing all the genetic variation (SNP) set on hard supports and used to analyse a sample's genome (Fig. 6).

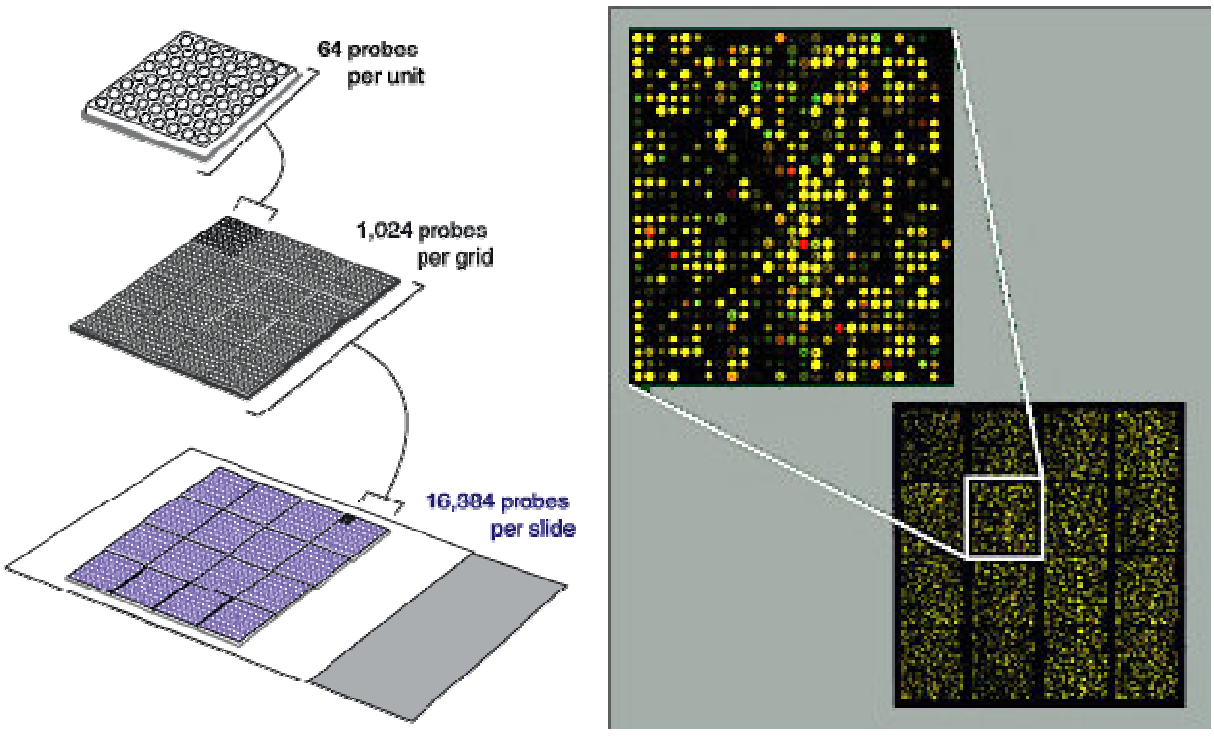


Figure 6 An example of high density of thousands of probes set on the slide surface (left). Visualisation of luminescent high density microarray slide (right). Both images are provided with enlarged inserts to show details.

It allows to identify mutations in the genome by hybridisation with DNA sequences. Through a robotic and automated method, single-stranded DNA drops are set on an optical microscopy slide in order to obtain a grid containing a different genic fragment in each cell. As many oligonucleotides as the investigated mutations are placed on the slide, each one having a specific sequence. Then the sample's DNA is made fluorescent through the chemical link with a molecule capable to glow when excited by apposite light source. Afterwards, the genic material needs to incubate on the slide to allow the mutations to link to the correspondent nucleotide. A washing is then performed in order to remove all the material that has not linked. The slide is analysed by a computer that is able to perform a scanning of each cell, sending the luminous information received to a computer (Fig. 6).

For microarray analysis the starting DNA samples should be diluted to 50 ng/ul with TE (at least 20ul) furthermore DNA must be double stranded, free of PCR inhibitors, not contaminated with other genomic DNA and not highly degraded.

The Broad Institute (http://www.broad.mit.edu/) of MIT and Harvard (Cambridge, MA, USA) has developed a custom canine SNP array in collaboration with Affymetrix (Santa Clara, CA, USA). The goal was a SNP array useful in many breeds to perform genome-wide association mapping using at least 15,000 SNPs. Affymetrix GeneChip® microarrays enable large scale, genome wide linkage analysis and association studies using high-density arrays (Fig. 7).

Utilizing photolithographic technology adapted from the semiconductor industry, arrays are produced with hundreds of thousands of unique probes packed at an extremely high density. Researchers are able to obtain high quality, genome-wide data using minimal amounts of starting DNA template. Affymetrix GeneChip DNA analysis software allows for rapid and accurate genotype calling with minimal user manipulation. The Broad Institute has implemented the use of the BRLMM genotype calling algorithm for the Gene Chips. The initial assembly, CanFam1.0, released July 2004, has ~27,000 high quality SNPs is a robust and easy to use array for gene mapping. Version 2 has a larger number of high-quality SNPs (~50,000).

The version 1 array is a 5um, 100-format, PM/MM probe strategy, Whole Genome Sampling Assay (WGSA) design which can detect a total of 66K canine SNPs. These SNPs were chosen from the 2.5 million SNP map generated as part of the dog genome project. A "v1 platinum" set of 26,578 SNPs was selected to include accurate and robust SNPs, using a panel of more than ten diverse breeds.

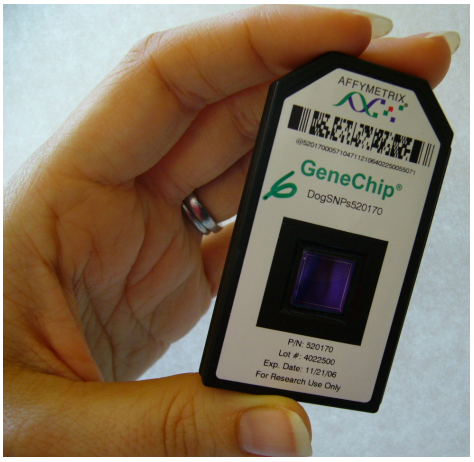


Figure 7 In 1 cm^2 we have thousands of probes.

The library file (DogSNPs520170P) has masked out the SNPs that are not of high quality and thus only will show results for the "v1 platinum" SNPs.

Name	Chr	ChrPos	Genome Allele	Read Allele	Breeds
BICF2S2345117	1	3000644	T	G	English Shepherd
BICF2S2411814	1	3001108	C	G	India Grey Wolf
BICF2S2411815	1	3001150	T	A	India Grey Wolf
BICF2S2411816	1	3001354	A	T	India Grey Wolf
BICF2S2411817	1	3001576	T	A	India Grey Wolf
BICF2S2411818	1	3001583	T	C	India Grey Wolf
BICF2G630707752	1	3002119	T	G	Boxer Assembly
BICF2S23018596	1	3007636	C	G	Rottweiler
BICF2S23018597	1	3007638	G	C	Rottweiler
BICF2S23018598	1	3007681	C	A	Rottweiler
BICF2S23018599	1	3007689	T	A	Rottweiler
BICF2S23027238	1	3009085	G	A	Rottweiler
BICF2P1489644	1	3010274	T	C	Standard Poodle
BICF2P1489645	1	3010310	G	A	Standard Poodle

Table 3 Example list of the first 14 SNPs for Dog Chromosome1, online available.

In this study we utilized the Current Assembly CanFam2.0, released May 2005, is a 5 um, 100-format, PM probe only WGSA design, which can detect a total of 127K SNPs. These SNPs were chosen the dog genome project and include the majority of the v1 platinum set SNPs. A "v2 platinum" set of 49,663 SNPs was selected to include accurate and robust SNPs. The library file has masked out the SNPs that are not of high quality and thus only will show results for the "v2 platinum" SNPs. Also available is the library file for all SNPs (Tab. 3).

The SNPs on the X chromosome are not treated differently than SNPs on the autosomes. In other words, SNPs on the X chromosome will be called in diploid mode. Because of this, the genotyping software will not assign a gender.

The DNA microchip technology consists of synthesized oligonucleotides containing all the genetic variation (SNP) set on hard supports and used to analyse a sample's genome.

It allows to identify mutations in the genome by hybridisation with DNA sequences.

Through a robotic and automated method, single-stranded DNA drops are set on a optical microscopy slide in order to obtain a grid containing a different genic fragment in each cell.

As many oligonucleotides as the investigated mutations are placed on the slide, each one having a specific sequence.

Then the sample's DNA is made fluorescent through the chemical link with a molecule capable to glow when excited by apposite light source (Fig. 8).

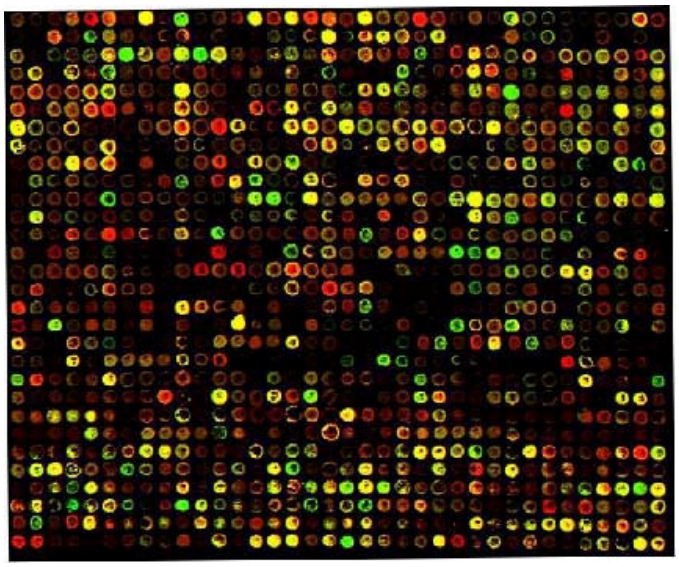


Figure 8 Fluorescence of a hybridised DNA microarray.

Afterwards, the genic material needs to incubate on the slide to allow the mutations to link to the correspondent nucleotide. A washing is then performed in order to remove all the material that has not linked. The slide is analysed by a computer that is able to perform a scanning of each cell, sending the luminous information received to a PC (Fig. 9).

Only the arrays with a call rate of 75% or higher were utilised, usually the call rate is between 70 and 85%, in human genetics only a value over 95% is take into consideration. Each call has p value indicating call reliability. Lowering the p value cut-off improves the call quality but decreases the call rate. We generally use $p<0.01$.

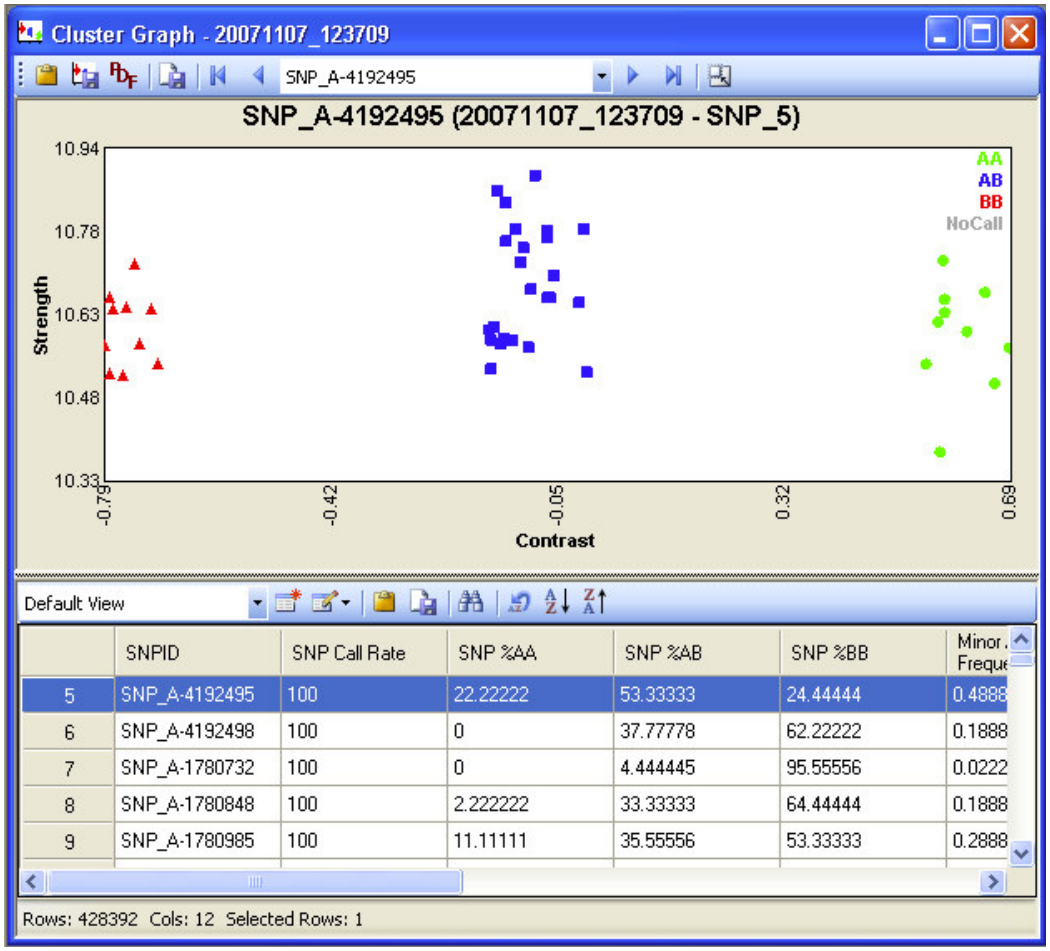


Figure 9 An example of SNP call rate output.

A long process take place in three different laboratories, step 1 to 3 pre PCR room than 4 to 9 post PCR room. The final steps 10 to 12 were carried on in the core laboratory of human genetics department in the school of medicine at UCLA (Fig. 10, 11).

All the process details are explained in Affymetrix GeneChip System Protocol, and the principal steps (www.affymetrix.com) consist of:

Stage 1: DNA Preparation and quantification

Stage 2: STY1 Restriction enzyme digestion (2.5 h)

Stage 3: Ligation of adaptors (3.5 h)

Stage 4: PCR (2.5 h)

Stage 5: PCR product purification and elution (3 h)

Stage 6: Quantitation and Normalization

Stage 7: Fragmentation (0.75 h)

Stage 8: Labelling (4.5 h)

Stage 9: Quality Control

Stage 10: Target hybridization (overnight up to16 h)

Stage 11: Washing and staining (2 h)

Stage 12: Scanning

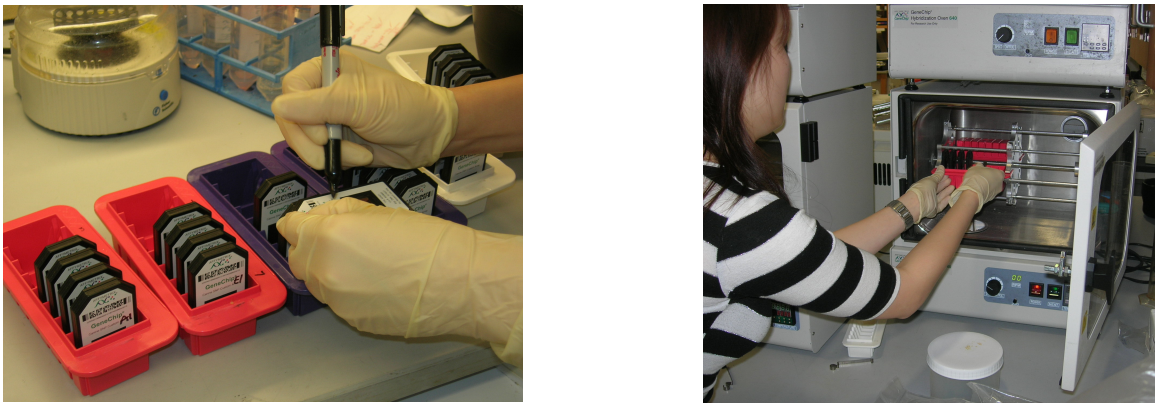


Figure 10 The final step performed at the core lab: Marking of the Chip (left) before the hybridisation stage 10 (right).

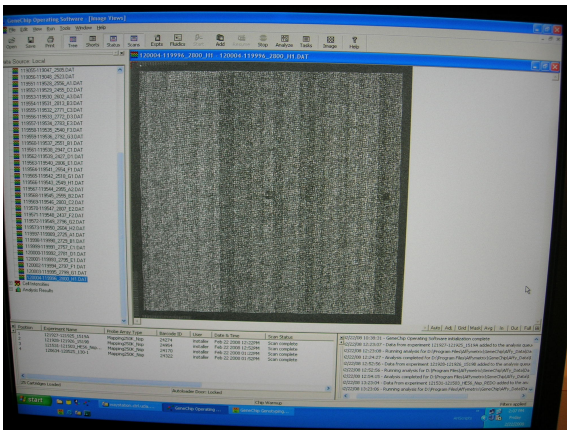


Figure 11 Bubble removal (left) after the washing phase, stage 11 and before scanning, stage 12 (right), charging of the chip in the scanner (above right) and row data scanned (below right).

3.5 *Genetic variability analysis*

The aim of population genetics is to describe the genetic composition of population and to understand the causes that determinate evolutionary changes. The five principal evolutionary forces that can cause change are: natural selection, genetic drift, non-random mating, migration and mutation.

Genetic variability in population is described through allele frequencies. Allele frequencies at each locus can vary in the course of generations under the pressure of evolutionary forces.

The different combinations of alleles present at each locus determine individual genotypes, whose frequency in populations can be calculated.

In an ideal population, in which population forces are not active, genotype frequencies remain constant from one generation, to the next. An important value to observe is heterozygosity, proportion of loci heterozygous in an individual or in a population.

Another important measure of genetic structure is F-statistic (F_{ST}) developed in 1969 – 1978 by Sewall Wright. This is related to statistical analysis of variance (ANOVA). The F_{ST} is the proportion of the total genetic variance contained in a sub population relative to the total genetic variance. The values can vary from 0, low differentiation among population to 1, considerable degree of differentiation.

In this study individual 13 loci microsatellite genotypes were recorded in Excel and the software **GENALEX** V. 6.0 (Peakall & Smouse 2006) a package for population genetic analyses that runs within Microsoft Excel was used in order to perform genetic analysis variability.

We carried out admixture analyses with **STRUCTURE** 2.1 (Pritchard *et al.* 2000) a multi-locus genotype data to investigate population structure in command line option in order to run SNP data.

The software **DISTRUCT** (Rosenberg 2004) used to graphically display results produced by the genetic clustering program STRUCTURE.

We performed additional analysis with **PLINK** (Purcell *et al*. 2007) a useful whole genome association analysis toolset able to describe variability, heterozygosis rate and clustering of the data.

The R packages **MCLUST** a model-based clustering and normal mixture modelling including Bayesian regularization (Fraley & Raftery 2006).

The software **SIGMA PLOT**® 8.0 a graphic tools was utilized to represent distance data.

4 – RESULTS

4.1 The melanism in Italian wolves

4.1.1 Agouti and Melanocortin receptor 1

We first verified whether variation in *Agouti* or *Mc1r* was associated with different pigmentary pathways. None variant of the detected polymorphisms appear to be related with coat colour. We utilised a subset of 27 individuals representing all the phenotypic categories: wild type wolf (WT), black wolf (BW), hybrid or anomalous (H), dog (D), and black dog (BD).

At first we tried to find a mutation in the exon four of AGOUTI that Kenrs *et al.* (2004), identified in German shepherd dog. In that work a missense alteration in exon 4, C427T (R96C) appears to be correlated to black colour. One of the AGEX4 alleles (R96C) in domestic dogs causes a solid black coat colour in homozygotes. Although some wolves are melanistic, this phenotype does not appear to be caused by this same mutation (Schmutz *et al.* 2007).

This was absent in Italian wolves and dogs but sequencing the four exons we found a remarkable mutation in the same exon: SNP A450G (Table 4). This mutation in Italian wolf appeared not to be related with coat colour but seems informative for wolf-dog hybridization.

Group	n	AA	R	GG	TOT
WT	9	6	2	0	8
WB / H	9	3	2	2	7
BD	4	0	0	3	3
D	5	0	0	5	5

Table 4 Results for the SNP A450G.

The second gene tested was the MC1R, Newton et al. in 2000 compared colour patterns in 167 domestic dogs, and found a mutation C306T related to MC1R loss-of-function, and related to melanic hair colour individual. We did not found the same mutation in Italian samples but we found other 4 SNPs: G304A, C467A, A781R, C907T all unrelated with colour but fixed in wolves and very variable in dogs, as shown in Table 5.

Group	n	G304A	C467A	A781R	C907T
WT	6	6 GG	6 CC	6AA	6 CC
WB	7	7 GG	7 CC	7 AA	7 CC
BD	10	6 GG 2 R 2 AA	6 CC 4 AA	3 AA 5 R 1 G	7 CC 1 Y 1 T
D	4	4 GG	3 CC 1 AA	2 AA 2 R	2 CC 1 Y 1 T

Table 5 Results for the four SNPs identified for the gene MC1R.

4.1.2 The K locus mutation

The melanistic *K* locus mutation in the Italian in the North American in the Canadian Arctic and in the Yellowstone National Park wolves probably derived from domestic dogs (Fig. 12), but has returned to the wolf population and risen to high frequency under positive selection. Moreover the same mutation also causes melanism in coyotes (*Canis latrans*), and Italian wolves.

The evolutionary relationships and history of the *K* locus in canids is presented in Figure 12. It suggests the timeline scenario for *K* locus evolution in dogs and wolves, in which ancestral *ky* chromosomes are indicated in orange, derivative K^B chromosomes in gray and recombinant chromosomes as an orange-gray checkered pattern. The *ky* to K^B mutation may have overlapped or even predated domestication, but introgression of K^B into North American gray wolves is more recent.

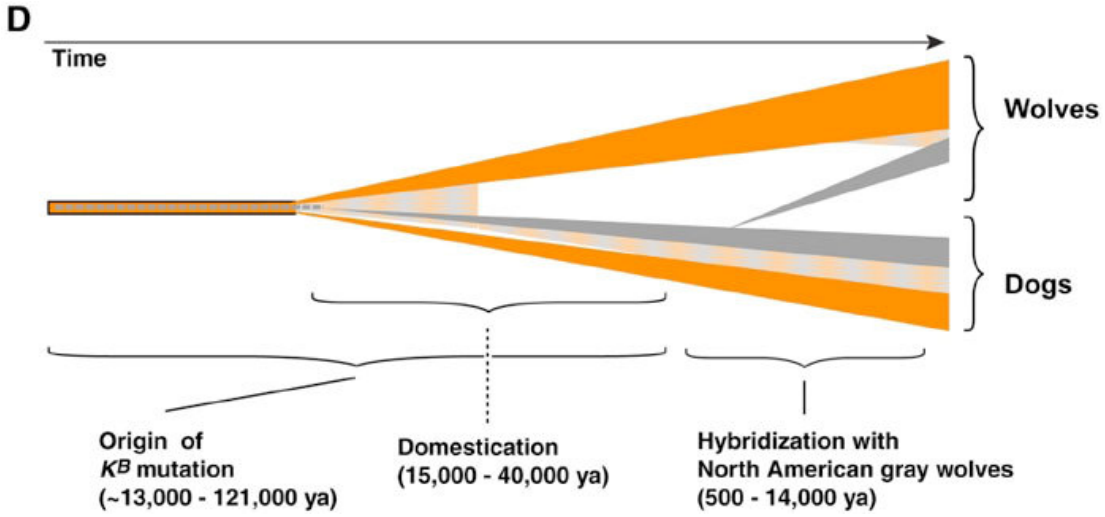


Figure 12 Time scale of the K^B mutation in Canids (Anderson *et al.* 2009).

The K_B mutation was estimated to be at least 46,886 years old (95% confidence limit: 12,779 - 121,182), therefore it is not possible to distinguish whether K_B arose before or after domestication. However, if K_B arose in Old World wolves prior to domestication, the data collected from the Stanford University indicate that it must have been lost from the gene pool and subsequently reacquired in North America.

On the other hand, according to the molecular clock based on the difference of mutations around the K locus in dogs and wolves the hypothetical period of introgression of the K_B mutation from dog into North American wolf's gene pool was attested around 14,000 years ago.

The 160 kb area surrounding the K locus was analysed. In Figure 13 are shown the haplotypes surrounding *CBD103* in five Italian black wolves, each row represents a K_B- or k_y-bearing chromosome, blue and yellow squares represent the major and minor alleles, respectively, grey squares represent missing data. Wolf haplotype structure was inferred on the basis of 36 SNPs. Much greater diversity is apparent among k_y than K_B haplotypes.

The Italian haplotype looks pretty different from the American one so we suspected that the dog introgression follows a different history. Probably the Italian melanism is due to a more recent hybridisation event (Fig. 13).

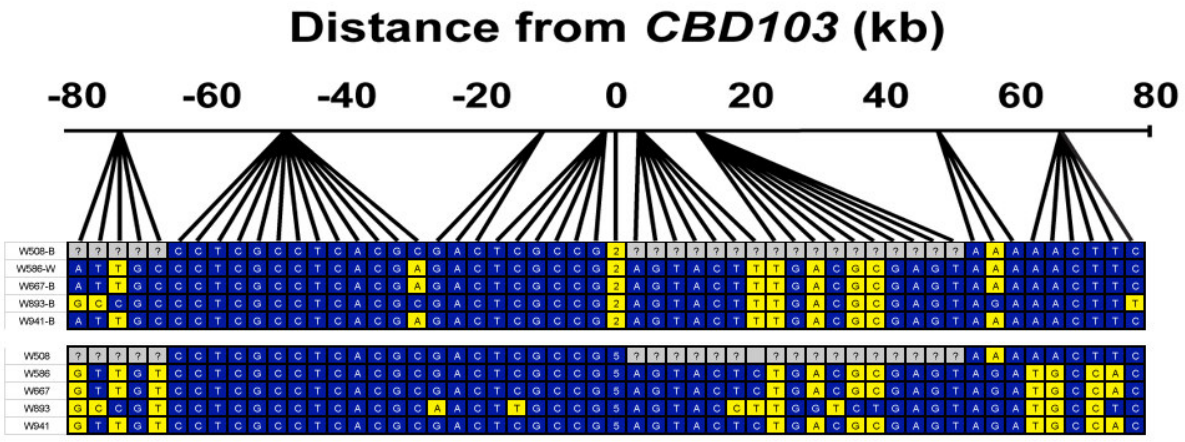


Figure 13 Haplotypes surrounding *CBD103* in five Italian black wolves. The first five rows represented the K_B bearing chromosome and below the *ky* chromosomes. In blue the more common alleles in yellow the rare one and in grey missing value.

We sequenced and amplified a region of 396 bp with primer *S54*, for invasive good quality samples we were able to obtain the sequences but for the noninvasive samples the results was not enough clever. We verified the presence of the 3bp deletion in Italian black wolves' tissue samples (Table 6, Figure 14). We analyse additionally wild type wolf and black and non-black dogs and in this case we found a completely concordance between phenotype and K locus results.

ID	Region	Date	seqS54	CBD103	mtDnaCR	Aplo.
W334M	Central Italy (Abruzzo)	1997	ky/ky	ky/ky	W14	I
W356F	Central Italy (Tuscany)	?/11/1994	ky/*KB*	ky/*KB*		
W357F	Central Italy (Tuscany)	?/11/1994	?	?		
W508F	Central Italy (Tuscany)	04/12/1998	ky/*KB*	ky/*KB*	W14	
W586F	Central Italy (Tuscany)	01/12/2000	ky/*KB*	ky/*KB*	W14	
W667M	North Italy (Emilia-Romagna)	20/10/2002	ky/*KB*	ky/*KB*	W14	U
W893F	North Italy (Emilia-Romagna)	28/10/2005	ky/*KB*	ky/*KB*		
W929F	North Italy (Emilia-Romagna)	02/02/2007	?	ky/*KB*		
W941M	Central Italy (Tuscany)	03/04/2007	ky/*KB*	ky/*KB*		U

Table 6 Hairs and tissue samples of Black wolves Italian carcasses, all phenotypes were described as black except for W667M, black with white breast and W941M, dark with white nails.

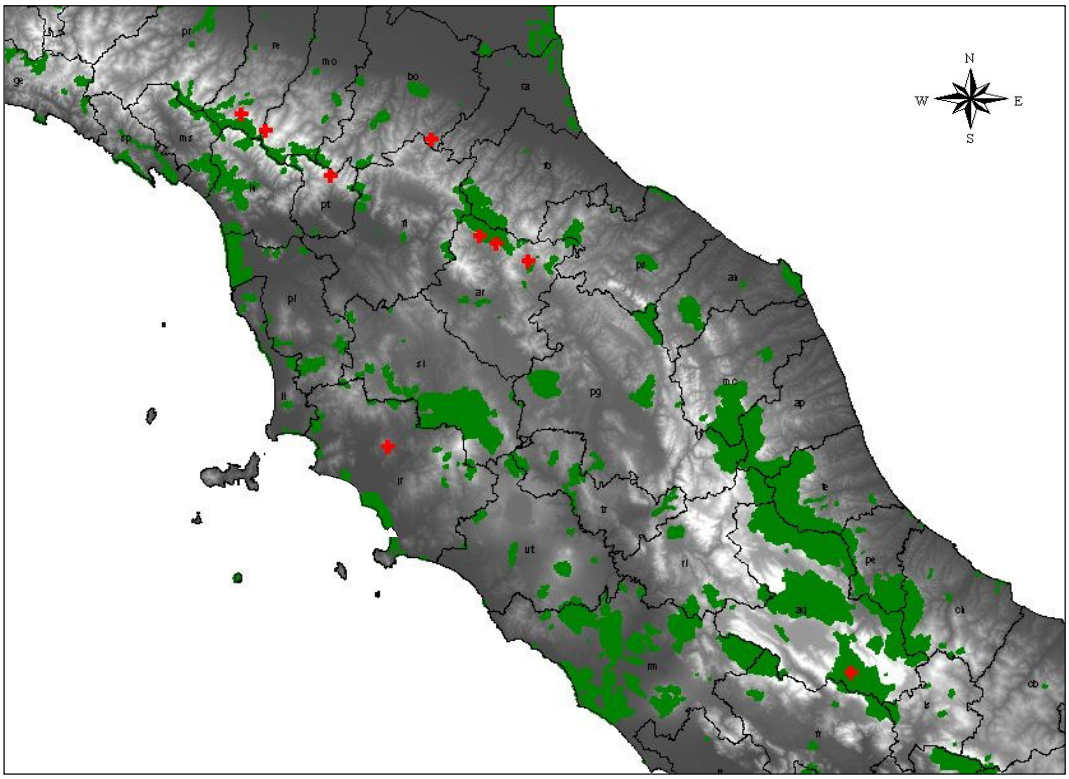


Figure 14 Areas of the Italian Apennines were the nine black carcasses (red cross) were collected. In green the protected areas.

In 2008 we designed a specific oligonucleotide primers pair with PRIMER 3, as described in chapter 4, (CBD103ΔG23) and we analysed the Italian samples with particular attention to the noninvasive sampling. We tested the noninvasive genotypes starting from the areas were evidence of presence of black individuals were collected. So we started from the province of Bologna, Forlì, Arezzo, and Florence in the northern Apennines between Emilia-Romagna and Tuscany and also in the Maremma National Park in the province of Grosseto in Tuscany. Thanks to personal communications, sighting and photos we identified that areas as the most involved from the melanistic phenomenon.

We analysed a larger number dataset, 175 tissue samples referring to 149 different individuals, 89 dogs, 57 wolf (48 grey and 9 black) and 3 hybrid, as reported in Table 7.

Type	n	*ky*	ky/*K^B*	*K^B*	Not assigned
WT	48	48	0	0	0
BW	9	1	7	0	1
D	89	51	28	8	2
H	3	3	0	0	0

Table 7 Italian individuals analyzed for the presence of the K mutation.

In 46 wolves the 3 bp deletion was absent. Except for 2 the 9 carcasses described as black or dark were heterozygous for the mutation. One of these two samples was W357F unreliable, probably the tissue collected in 1994 was too much degradated. One individual W334M, the unique black wolf collected from Abruzzo in Central Italy, despite the black phenotype was *ky/ky* maybe that the individual described as black from by the collector was not a completely black individual but only a dark one. The samples W535 was not only dark but presented the spurs on the posteriors legs. Two pictures of black individual carcasses (Fig. 15) founded respectively in Modena in 2005 W893F and in Reggio Emilia in 2002 W667M, these individual presented a visibly large white spot on the breast.

Figure 15 Carcass W667M (left), Duccio Berzi and individual W893F, Fontana Riccardo (right).

We also analyzed a large set of feral or free range dogs from Emilia Romagna, Abruzzo and Calabria but many of them were not provided of phenotypic description. We analysed some pure breed black individuals one Toy Poodle, two Labrador Retriever and one Bavarian Shepherd, they resulted all homozygous for the deletion K^B / K^B except for the shepherd dog that was heterozygous. Additionally we examined one black and than one completely black and one wild type dog from Emilia Romagna and one wild type dog from Marche. As shown in Table 8 and Figure 16 from the random sampling of feral dogs we found that in 27 (33%) individuals was presented the heterozygous mutation *ky*/KB and in 5 (6%) cases the mutation was homozygous.

	N	***ky/ky***	***ky/ K^B***	***K^B / K^B***
Tot	81	49	27	5
Abruzzo	26	19	6	1
Calabria	31	17	11	3
Toscana	24	13	10	1

Table 8 Italian feral or free range dog analyzed for the black mutation.

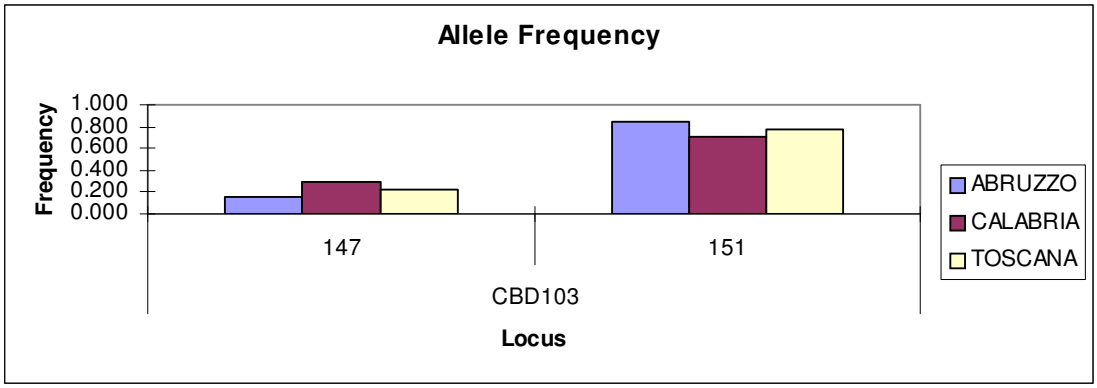


Figure 16 The K locus frequency in dogs Italian samples 147 (for K^B) and 151 (for *ky*).

Then we proceed analysing the noninvasive wolf samples. We analysed 234 different samples corresponding to 197 wolf genotypes, in 147 individuals, representing the 75% of the total, the mutation was absent but 21 (11%) individuals were heterozygous for the deletion. The rest of the samples (29) were unreliable. The scats collected from the presumably black individuals were collected in the areas showed in the map (Fig. 17).

We performed STRUCTURE analysis to verify if the previous detected wolf-dog hybrid were better defined adding the k locus information.

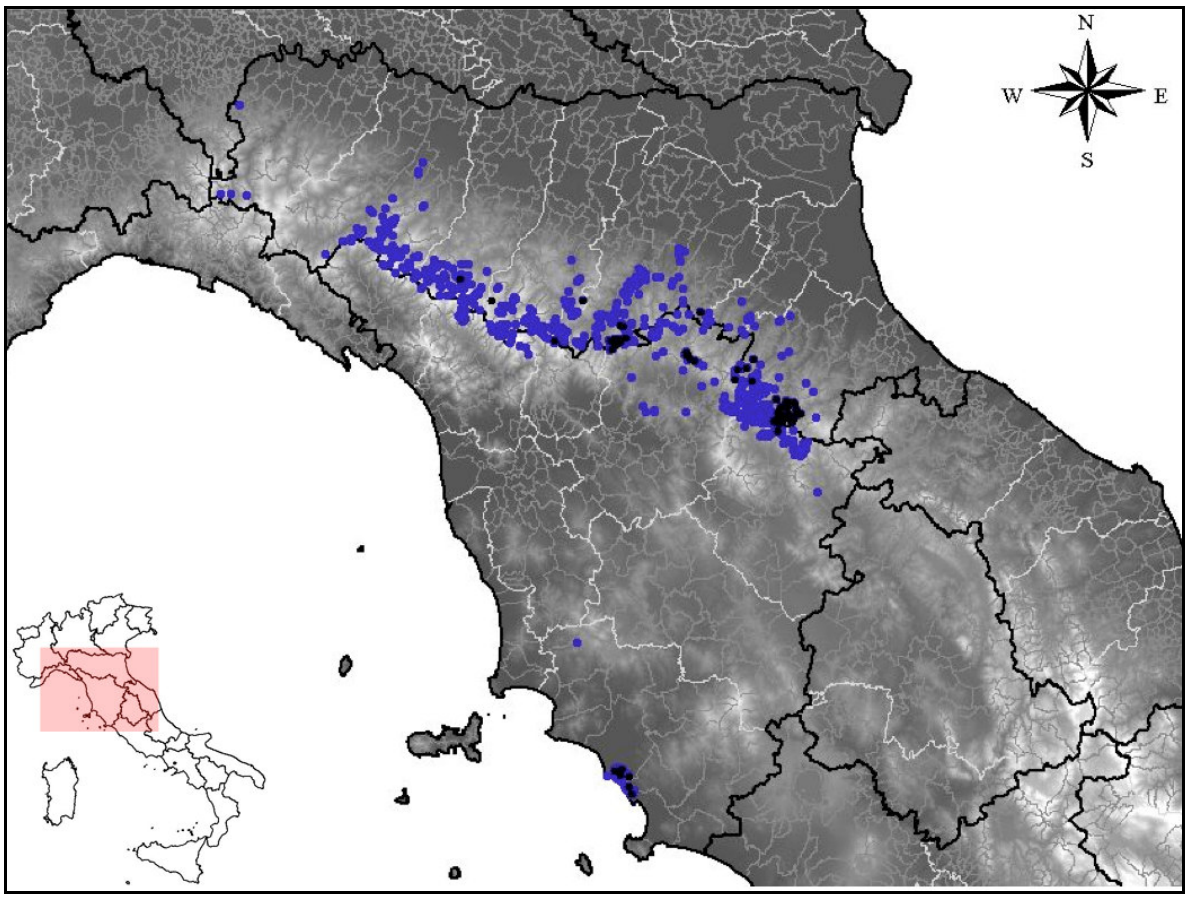


Figure 17 Italian distribution of the noninvasive wolf sampling (in blue dot) and of all the detected genotypes presenting the back mutation.

As these are noninvasive samples it's almost impossible to attribute the correct phenotypic information to the related genotypes. Despite that in areas intensively monitored (Fig. 18) we may have additional information that support the genetic data as in some case of studies presented below.

In the Park of Suviana and Brasimone lakes (blue circle in Figure 18) the only black individual detected was the dominant alpha male. It was observed and photographed on several occasions and most likely corresponds to the genotype WBO41M that presented the K locus mutation (Fig. 19). There is a two minutes movie of that black individual with other two grey wolves a female and a juvenile (Renato Fabbri).

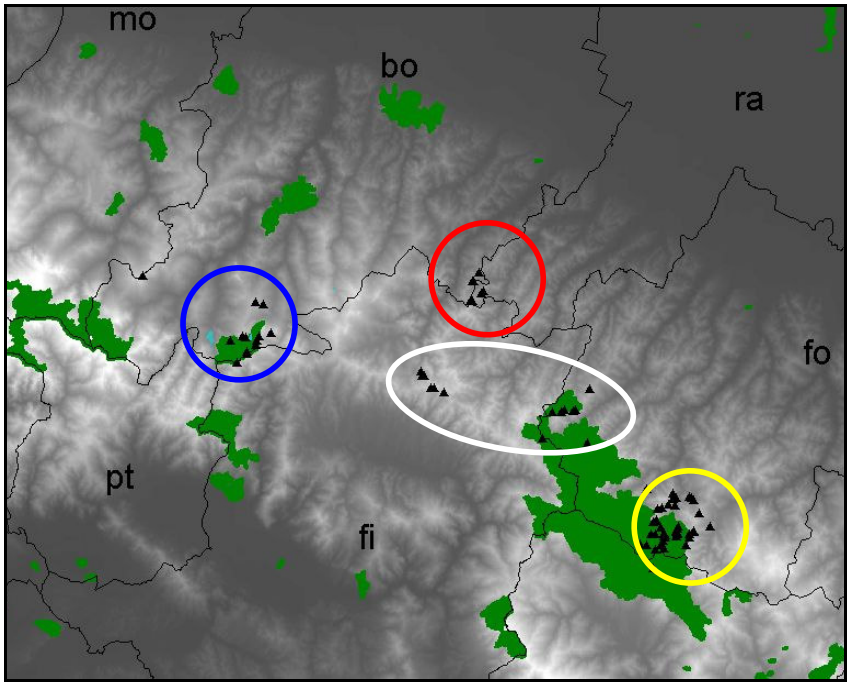


Figure18 Location of the detected black individuals (black triangles) in the four principal areas in Emilia Romagna. The presence of black mutation was identified starting from the NGS.

Figure 19 Picture of the completely black α male of Suviana and Brasimone WBO41M, dated 30.12.2005 (Fabbri Renato).

We had also additional information in other areas as Sasso Fratino (yellow circle in Figure 18) in the Casentinesi Forest National Park were we first identify a female bearing the heterozygous mutation WFO9F so we analyzed the related genotypes present in the area and we found the presence of the mutation also in WFO1F and in other five genotypes WFO59F, WFO60M, WFO62M, WFO63F, WFO80M. Then we performed a kinship analysis with PARENTE (Cercueil *et al.* 2002) and we found that the female WFO9F was probably the dominant female from 2002 to 2005, and some of her cubs results characterized from the presence of the same mutation, the α male WFO2M since 2002 to 2007 remain the same and did not present the black mutation.. From that area we had the sighting of at least tree black cubs in 2004 (P. Simoncini, pers. comm.). The female WFO9F resulted daughter of WFO1F (2002-2003), a female that was present in the area between 2000 and 2002.

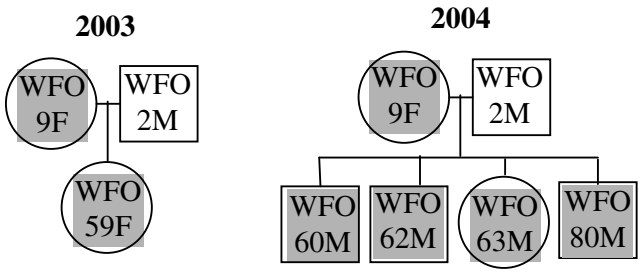


Figure 20 Pedigree of Sasso Fratino packs only for two years was possible to reconstruct the probably kinship of the individuals. In grey the black individuals.

Moreover, the carcass W929F of a juvenile black female was found in February 2007 in Castel del Rio (Imola Province). It was heterozygous for the K locus mutation and its genotype (13 loci microsatellites) resulted in kinship relation with two individuals, WRA2F and WRA6M, identified in the non-invasive study. The male WRA6M resulted heterozygous for the K mutation. The area occupied from these individuals is presented in red circle in Figure 18.

At the beginning of February 2009, a camera trap recorded, in a closer area on the boundary between Bologna and Ravenna, the presence of a black wolf and a grey one. Less than a month after, another record showed two black individuals at the same time and at the 18th of march four individuals two black and two wild type were recorded (D. Errani, pers. comm.).

An area with reported presence of black individuals was between Florence and Forlì (in white circle in Figure 18) in this area we identified WFI1F, WFI5M and WFI6M all presenting the heterozygous black mutation. In both provinces at the beginning of 2009 were recorded by camera trapping videos showing one black individuals (D. Berzi, E. Centofanti).

Another well studied area in Tuscany is the Maremma National Park (Tuscany) that area was occupied from a wolf pack in 2004; some of the individuals that in general presented anomalous phenotype were completely black. We found that the dominant male, WGR14M, did not present the mutation but the alpha female, WGR13F, had the heterozygous K locus mutations and 7 of 12 of the cubs of three reproductive season (Fig. 21) presented the same mutations, these data are completely concordant with the field work data collected.

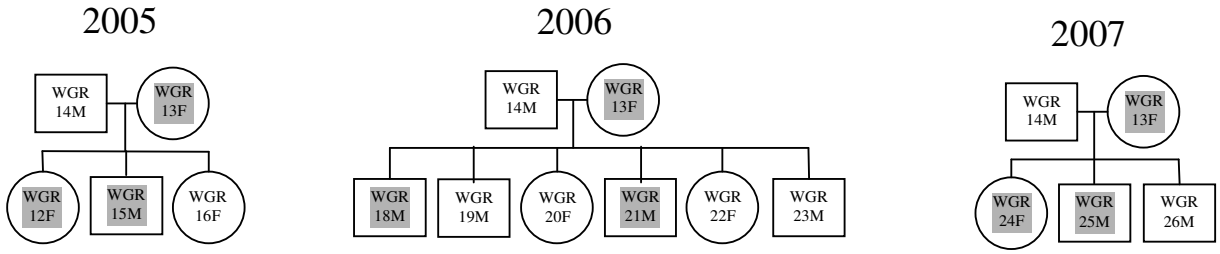


Figure 21 Hypothesis of pedigree of 3 different litters of the Maremma pack. In grey the black individuals.

4.2 Characteristics of the black phenotype

To better understand the main areas interested from the phenomenon we collected information from rangers, biologists and photographer. And we obtained information about sighting and in some cases pictures. The oldest sighting we were able to collect was of one black isolated individual following a Roe Deer (*Capreolus capreolus*) in Romagna near Lama Forrest, locality Forconali, Fonte Murata (Forlì province) in the Central Apennines, the period was 1982-1983 (G. Crudele, pers. comm.). Additionally we collected reports on the presence of black individuals in 1950 in the Alps (Pyrenees). On the northern boundaries we found in Switzerland the only reference on the

presence of black wolves comes form Tschudi F. (1959) how cited Gessner (XVI): “it was said that in the time of Gessner, that black wolves were existing in the Rhine valley and in the Grisons”. Tschudi mentions also the presence of black wolves in the Pyrenees, but without giving his source (Tschudi 1858).

The older images collected was from Graziano Tortelli. The picture dated 1.01.1998 portrayed a black individual, at 8.00 am, in locality Poggio Segaticcio, Capanna Maremmana close to Camaldoli (AR) in the Casentinesi Forrest National Park (Fig. 22).

Figure 22 Picture of a black wolf in the forested area of Tuscany, Capanna Maremmana 1.01.1998 (Graziano Tortelli).

This black individual presented one white spot on the breast. The photographer noticed the individual following two Red Deer (*Cervus elaphus*), and reported that in the areas lived at least other two grey wolves (G. Tortelli, pers. comm.).

Besides, we learned that two completely black individuals had been sighted in several occasions between 1997 and 2000 in Reggio Emilia, Park of Gigante (A. Meriggi, pers. comm.).

We had additional information on other possible sighting but if the description was uncertain we decided not t consider the data. For example, Corradino Guacci; a historian gathered information about past killings. He collected around 47 pictures of wolves captured and killed between 1921 and1970, none of these individuals were black. He also found the documentation regarding the killing of an individual shot in Sannicardo Garganico the 24 September 1904 with the black coat. But the age of this animal was estimated around 8 months and if it is correct this wolf born period is not concordant with the Italian wolf's born in May or June.

In general the black individuals identified in this study despite the solid black appearance uniformly dark are characterized from long black coloured guard hair but we verified that undercoat tend to be light, in some case we observed white spot on the breast and on the muzzle.

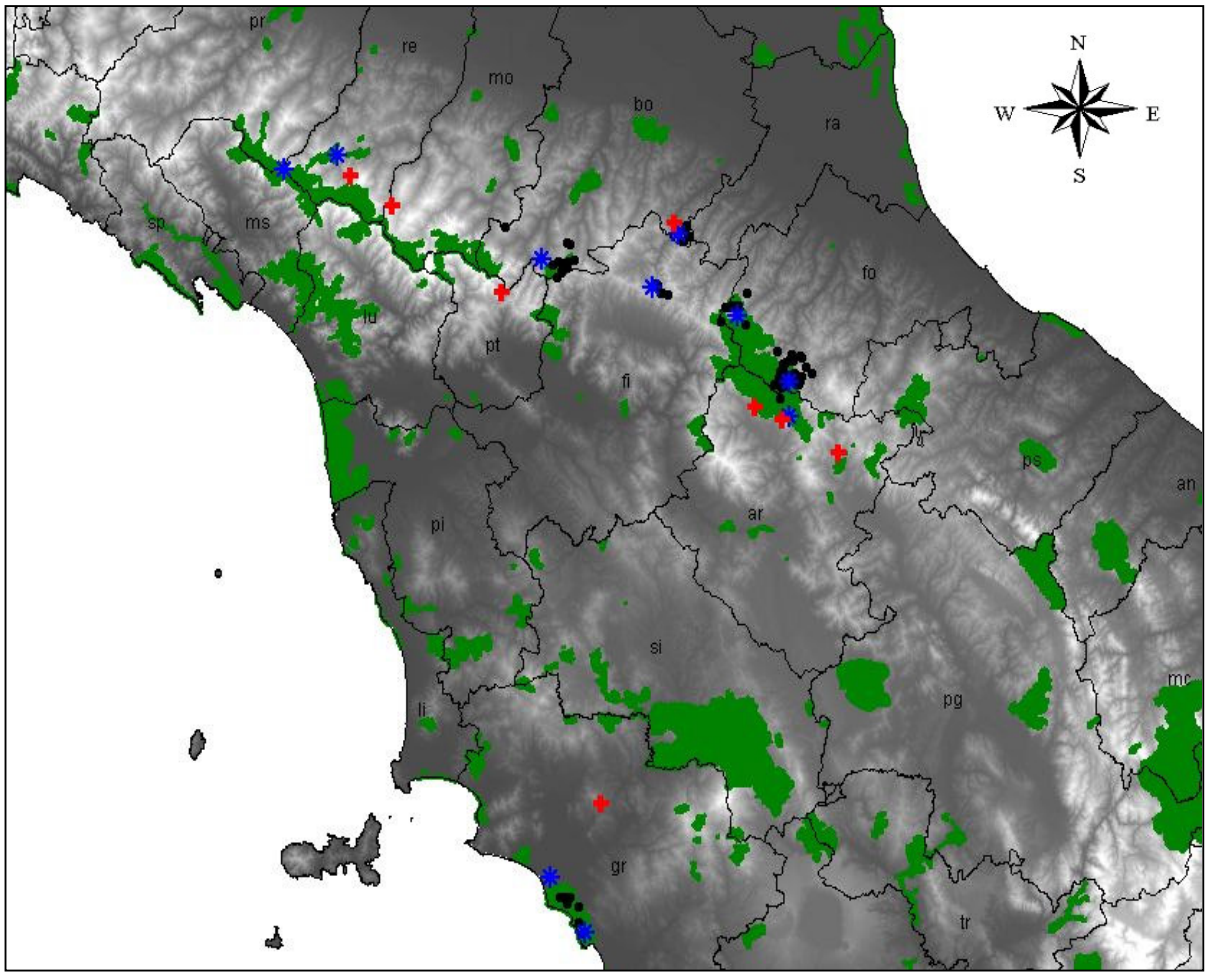
The Italian melanistic wolves are characterized by a uniform dark brown colour shading to black but not completely black. They present shade and in some case small white spot with a high individual variability, a pattern of intermingled white hairs on some part of the body. In particular white spot were observed on the frontal legs, on the breast or on the muzzle. The undercoat, down hairs, and the basis of hairs are pale creamy white despite the top coat, awn hairs and guard hairs, are deep black, most of the visible coat is made of this kind of hair and conferred to those individuals a black appearance. The characteristic mask on the muzzle is absent or extremely reduced. Moreover the black coat turn in grey or it dilute with age.

We collected the hairs of the black carcass W929F and we examined the colour and the shape of hairs collected from different part of the body: muzzle, neck, breast, leg and tail.

Finally the overall distribution of Italian melanism is shown in Figure 23 and derived from the overlapping of all the available data: carcasses founding (red cross), sighting, video and camera trapping (blue star) and noninvasive genotypes (black dot) characterized by the presence of the K locus mutation.

In the majority of the areas turned into light (Fig. 23) we had confirm of the effective presence of black individuals thanks to occasional observation and to comparative study of both genetic and field data.

Figure 23 Map of the Italian wolf distribution (blue, right), the area interested from the melanism highlighted in a red square (enlarged above).
The above map showed the distribution of black invasive (red cross) and noninvasive (black dot) samples compared with the localizations of sighting, video and camera trapping (blue star) collected in the last ten years.



4.3 Canine DNA microchip results

Around 107 European wolves, from 15 different countries were analyzed.

The Italian wolves were 28 included 8 individuals presenting trace of hybridization with dog, this were representative for all the Italian peninsula as showed in Table 9 and were compared to the previous chip data from American and European wolf and suspected hybrids and tent of different dog breeds such as Boxer, Poodle, Mastiff, Akita, Basenji, Labrador, German Shepherd, Basset, Collie, Whippet and many others (approximately 85).

UCLA ID	INFS ID	SP	COUNTRY	N S C	PROV
2756	W087	CLU	ITALY	NORTH	SP
2781	W509	CLU	ITALY	NORTH	GE
2800	W764	CLU	ITALY	NORTH	CN
11354	*794	CLU?	ITALY	NORTH	TO
11355	*801	CLU?	ITALY	NORTH	TO
2725	W004	CLU	ITALY	CENTRAL	FC
2730	W028	CLU	ITALY	CENTRAL	CH
2742	W055	CLU	ITALY	CENTRAL	AQ
2748	W067	CLU	ITALY	CENTRAL	RI
2751	W070	CLU	ITALY	CENTRAL	PG
2755	W085	CLU	ITALY	CENTRAL	FI
2757	W131	HYBRID	ITALY	CENTRAL	AQ
2770	W387	HYBRID	ITALY	CENTRAL	AR
2771	W391	HYBRID	ITALY	CENTRAL	SI
2772	W392	HYBRID	ITALY	CENTRAL	SI
2795	W586	CLU	ITALY	CENTRAL	PT
2796	W587	HYBRID	ITALY	CENTRAL	GR
2797	W667	CLU	ITALY	CENTRAL	RE
2803	W936	HYBRID	ITALY	CENTRAL	FI
11353	*792	CLU?	ITALY	CENTRAL	PI
2729	W027	CLU	ITALY	SOUTH	CS
2747	W066	CLU	ITALY	SOUTH	RC
2753	W074	CLU	ITALY	SOUTH	MC
2767	W377	HYBRID	ITALY	SOUTH	(BA)
2785	W525	CLU	ITALY	SOUTH	PZ
2792	W538	HYBRID	ITALY	SOUTH	FG
2794	W572	CLU	ITALY	SOUTH	BA
2799	W755	CLU	ITALY	SOUTH	BA

Table 9 Complete list of the 28 individuals collected from Italy and analyzed by the DNA microchip.

Initially we performed preliminary analysis on the starting raw data, just to verify the power of the resolution of statistical analysis and the possibility to detect species origin and hybridization.

We performed multiple STRUCTURE analysis and utilizing K, number of population up to 42.

The analysis performed with K equal to 42 based on 49,000 SNPs was conduced on 146 dogs 2 up to 4 representing 43 different breeds, 70 Eurasian wolves, 13 Italian wolves and 10 hybrids.

The Eurasian samples were collected from Belarus, Croatia, Lithuania, Poland, Russia, Slovakia, Spain, Turkey and Ukraine.

The Italian cluster (22) resulted the better identified with the higher value of mean Fst equal to 0.6311 despite only 0.3959 for the Eurasian wolves that appeared all included in the same cluster (27). The Fixation Index (Fst) is a measure of population differentiation based on genetic polymorphism data. Fst values can range from 0 to 1 and high Fst implies a considerable degree of differentiation among populations. Than we performed Structure analysis assuming K equal 20, with 56,500 loci for 145 dogs, 95 Eurasian wolves adding Bulgarian, Chinese, Indian, Israeli, 13 Italian wolves and 10 hybrids. But the results showed a unique cluster for all the Eurasian wolves included the Italian one. We observed that the best resolution was K=16 as showed in Figure 24.

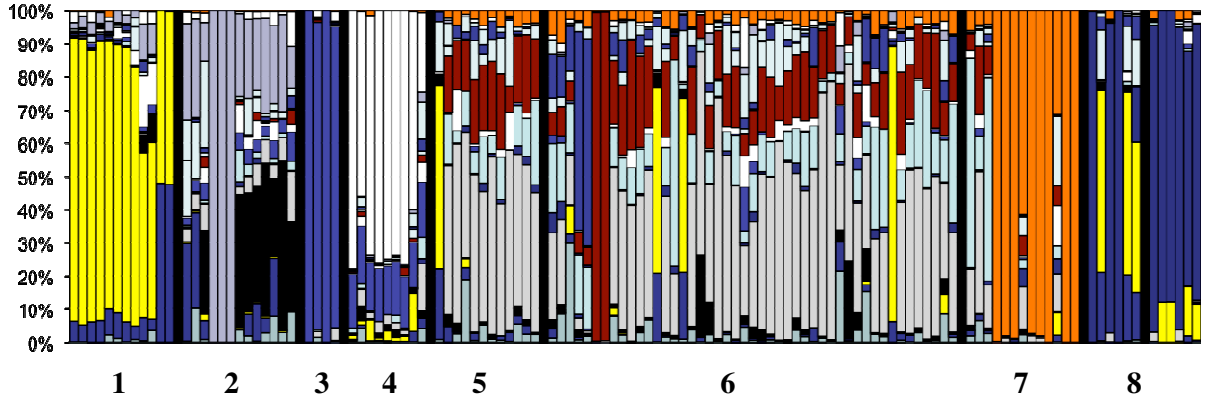


Figure 24 The STRUCTURE results, bayesian clustering assuming K equal 16.

Utilizing twelve ancestral dog breeds (1) as represented in Figure 24 and all the Eurasian wolf samples, we obtained the correct identification of the Italian wolf population (8) and also of the Chinese (2), Indian (3), Israeli (4), Belarus (5), and Spanish (7) ones. The rest of the eastern European populations (6) were not correctly identified. Furthermore, all individuals suspected to be hybrids - thanks to microsatellites analysis - were confirmed. The yellow portions in Figure 24 represent the dog cluster.

We utilized the software EIGHENSTRAT for the detection of population stratification using principal components analysis (PCA) and, as shown in Figure 25 the first two principal components divided in three separated cluster: in blue the dog cluster containing two dogs as case control and also some European wolf-dog hybrids, in green the European wolves and in red the Italian population completely separated (Fig. 25).

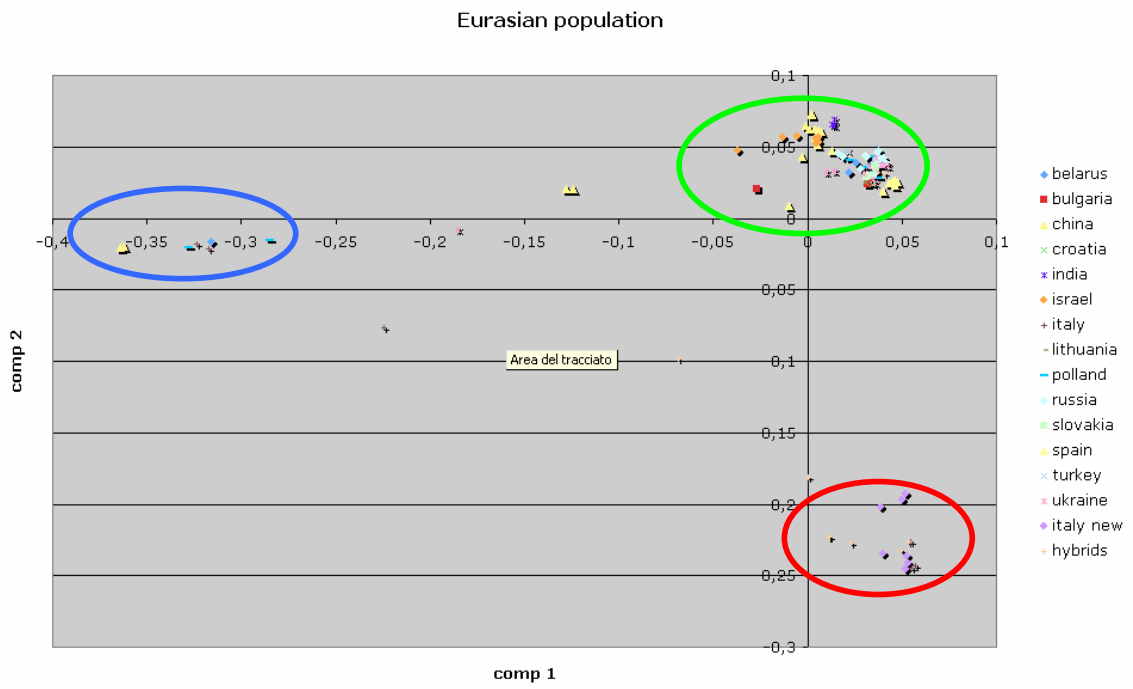


Figure 25 Graph of Principal component analysis results obtained by EIGHENSTRAT.

After these preliminary results, we decided to focus on the Italian wolf population by selecting a subset of individuals and by reducing the number of SNP markers.

Quality filtering was performed to ensure that only high quality data were utilized. Consequently a subset of 46,036 high quality SNPs instead of 127,000 was checked by the software MAGIC V 2 of Cornell University, and used for the analysis. This software was realized for a better resolution avoiding errors, performing a quality control filtering the analysed SNPs and obtaining a smaller number of reliable SNPs.

We selected a sub set of 152 samples: 85 dogs representing each breed already processed on the microchip, 30 European wolves, 21 Italian wolves and 17 European suspected hybrids.

We used PLINK software to determine population stratification, to cluster the SNP data and to obtain a matrix of genome-wide pairwise identity-by-state (IBS) distances. We performed multidimensional scaling analysis on the matrix of IBS distance. Plotting the first component value against the second component, in a bi-dimensional representation we obtained a scattered plot in which each point represent one individual and we were able to identify the clusters. We generated a visualization of the substructure in the sample by using a statistical package MCLUMP to obtain the bi-dimensional scaling plot of the fist two genetic distance value C1 and C2 (Fig. 26).

As shown in Figure 26 we identified three different clusters: in blue (1) represented the dog breeds, in pink (2) the European wolves and in yellow (3) the Italian wolves. Except for the German Shepherd dog that clustered with European wolves and for 7 European wolves 3 from Spain, 2 from Croatia and 2 from Ukraine that seems very close related to dog's cluster we had a clever distinction for the three clades.

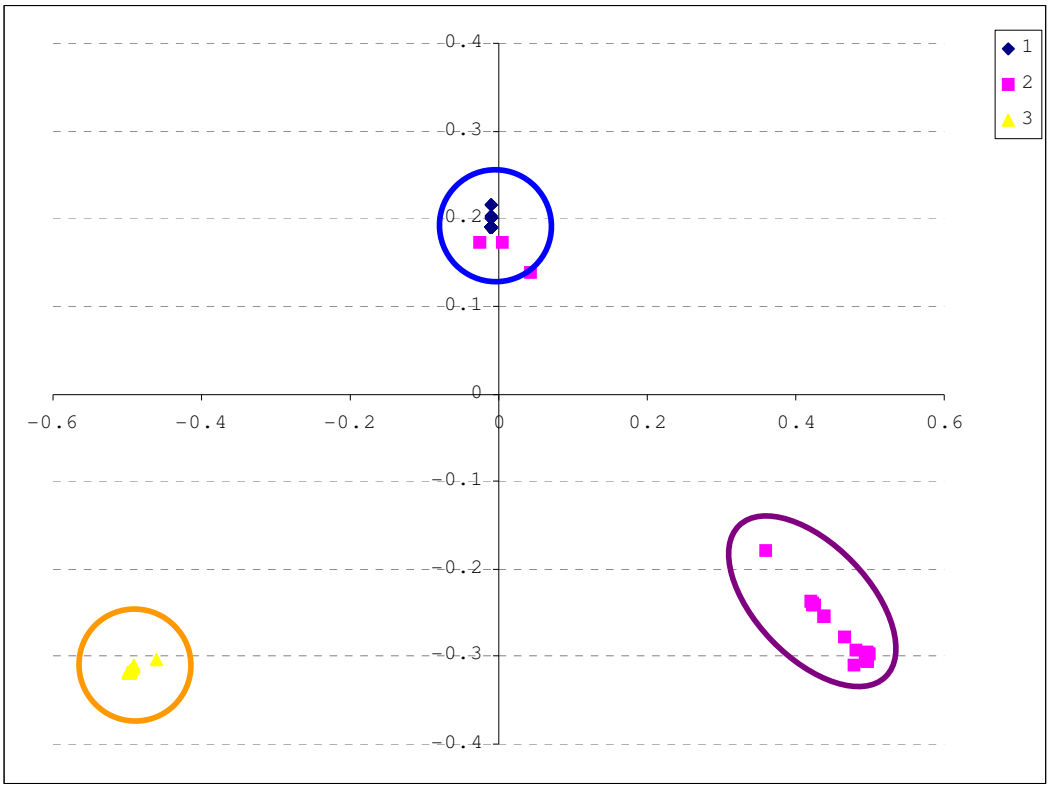


Figure 26 The plot of PLINK clustering data performed with MCLUST.

Afterwards, instead of using each individual as the unit of analysis we utilized each point as cluster and we calculated with Plink cluster option the distance between known clusters and the average distances of all individuals in those clusters. Than utilizing the three genetic distance value C1 C2 and C3 of PLINK IBS distance we draw in SIGMAPLOT a three-dimensional graph as illustrated in Figure 27. As in the bi-dimensional representation the Italian wolves cluster is well defined and separated from the other two clusters.

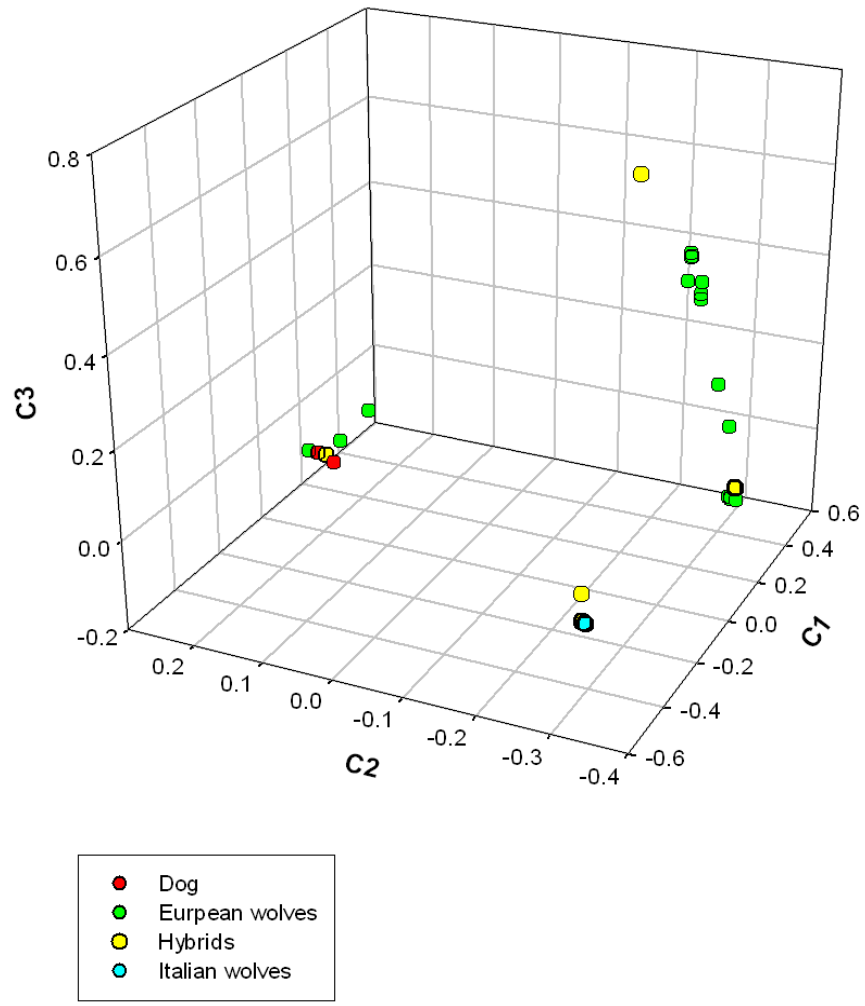


Figure 27 Plot of PLINK clustering data performed with SIGMAPLOT.

We performed multiple sessions of STRUCTURE analysis to identify the number of genetically distinct clusters that maximize the likelihood of the data, and to assign the individuals to the clusters, using only genetic information. Each session was performed considering a different number of populations (K), from 2 up to 6. Drawing the likelihood curve, we observed that the most representative K value was 3. We presented in Figure 28 the results plot of K=3. We performed four repetitions of 10^4 iterations following a burn-in period of 30,000 iterations. Each individual is represented as a vertical line partitioned into three coloured segments, whose length is proportional to the individual coefficients of membership in the three clusters.

In the Figure 28 on the left side there are all the 85 dogs separated by the black line from the European wolves. On the right side all the European wolf population in alphabetical order from left to right. We set the Italian samples at the right end of the graph (in orange) the last six individuals were the Italian hybrids. The Italian wolf and hybrids appeared separated from all the other European wolf populations.

The European wolves that presented a yellow component resulted hybrids. Seventeen hybrids were previously detected by microsatellites analysis but we found three additional individuals presenting dog introgression two were Italian samples previously analyzed from UCLA laboratory and one was Croatian. This sample due to the very high level of yellow component appeared more likely a dog than a hybrid.

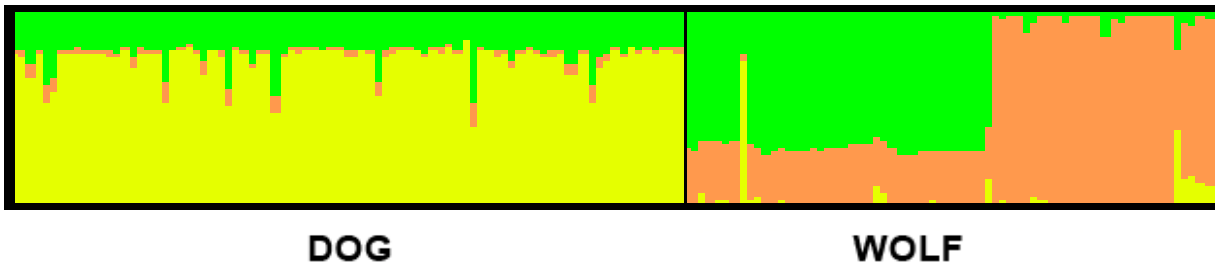


Figure 28 STRUCTURE results for K equal 3.
In yellow the Dog, in green the European wolves and in orange the Italian wolf.

We verified, according to previous studies, that the Italian population is separated not only from dog and North American wolves but also from the rest of the European wolf populations and that the suspected hybrids were set in an intermediate position between wolf and dog clade.

To conclude all the results obtained can be applied to several different genetic studies.

Checking the Italian samples results, 28 individuals of the Italian wolf's population, we were able to identify a panel of more than 70 highly polymorphic SNPs chosen from the 127.000. These SNPs could be utilized in individual identification.

5 - DISCUSSION

The historical process leading to the decline of the wolf in Italy after World War II became far more critical with an uncontrolled increase of free-ranging and feral dogs. As a consequence, the population decreased to less than one hundred individuals in the 1970s. Such a severe decline urged a consistent and accurate monitoring of the population. In fact, introgressive hybridization may have compromised wolf's gene pool.

Since 2000 the Italian Wildlife Institute has been coordinating large scale wolf monitoring projects in order to ascertain the loss of genetic diversity, the introgression and to monitor any genetic or behavioural changes.

The overall project aimed to identify genetically pure or hybrid populations in order to verify and possibly to preserve the genetic integrity of natural populations of carnivores. These entailed noninvasive genetic analysis based on microsatellites, mtDNA and SNPs markers.

In the framework of such study, during my three-year PhD research I developed innovative methods for the study of the wolf population investigating both functional genes and SNPs performing microarray analyses. The extremely interesting results achieved are described as follow.

5.1 The K locus allows to identify black wolves across the Italian peninsula

We focused our research on the debated question of the origin of melanism in the Italian wolf. As a first step, we searched for differences in genes involved in colour determination in birds, mice, cattle, and dogs. The agouti gene regulates the amount of red and yellow pigments in skin, feather and hairs. Melanocortin 1 receptor (Mc1r) is involved in black and brown colour determination.

Analyzing wolf and dog samples including phenotypic description, we ascertained that there were no correlation between black phenotype and genetic mutations in agouti and Mc1R. It ensues that Agouti and Melanocortin genes are the main actors involved in the genetic determination of pigmentation for many dog breeds and other species, but the wolf.

Since 2003 focusing on disease and pigmentation, Prof. Gregory Barsh research group published interesting papers on relationship between particular mutations that appeared to be strictly related to black coat colour in several dogs (Kerns *et al*. 2003, 2004, 2007).

Kerns et al. (2003, 2005) found evidences, through DNA studies, that the "dominant black" in wolves is not caused by an agouti allele. Finally, in 2007, Barsh and his research group determined that the loss of three bases of the genetic code in another gene, called K locus, determined the black coat in at least fifty breeds such as Labrador Retriever, Great Dane and German Shepherd dogs. Afterward, Barsh's team started to investigate the presence of the mutation in wild canids, particularly wolves and coyotes (Candille *et al*. 2007).

The recently discovered mutation CBD103ΔG23 belongs to Beta Defensin genes and allows identifying also black wolves. This family of genes was already known to play an important role in immunity in humans, mice and other animals, but this is the first time that a defensin gene has been shown to affect pigmentation pathway. A genetic study turned into light the gene variant that determines the black coat.

We analysed samples of wolves, feral dogs and suspected hybrids from the entire Italian peninsula. The relevant rate of occurrence of the black mutation in feral and free range dogs gave a hint of the possibility of introgression of such mutation into the Italian wolf population in case of inbreeding.

We verified that sightings and carcasses founded proved the increasing trend of the phenomenon in different areas in the northern Apennines between Tuscany and Emilia Romagna as well as in Maremma National Park. In Maremma, for example, eight on fourteen individuals were black. Nevertheless, this pack, settled in the area from 2004, is suspected to be hybrid. This is not surprising as Maremma is a new area of colonization for the species never described in the past ten years.

As Apollonio *et al.* reported in 2004, there were no reliable sightings before 1982 from the Central Apennines (G. Crudele, pers. comm.). The first Italian carcass of a black wolf was found in

1994 in the Province of Arezzo (Tuscany) and, until today, only eight more black carcasses were found. In Italy, direct evidence of interbreeding with dog has been obtained at least once, when a radio-tracked wolf female bred with a feral dog and gave birth to six cubs. Four of them were black with spotted frontal legs and two were phenotypically wolves like (Boitani 1992).

We collected additional historical information of the presence of melanistic wolves. All the collected carcasses, samples from museums, and sighting reported, were only of grey individuals presenting the classical or light dark phenotype. Only in the Pyrenees we discovered evidence of the presence of black individuals in the middle of the last century (Tschudi 1858).

Our genetic results highlighted that not only black coated dogs and wolves share the same gene variant, but also that the DNA surrounding the K locus was quite similar in all black individuals. On the contrary, it was quite different from grey wolves' haplotype. The variability observed in a 160,000 bp region surrounding the KB mutation showed that it is a highly conservative area (with only 52 SNPs detected). This region in black wolves and coyotes is less variable than in black dogs.

These results suggested that the mutation was introduced into wolves by interbreeding events with domestic dogs. The truncated K locus also in black coyotes was assumably inheredited from dogs.

For the first time we found evidence that a gene mutation originated in a domesticated species in all probability was transferred to and become very common in a closely related wild species.

As reported in chapter four, the black coat colour mutation came into worldwide wolf's population likely by interbreeding with black dogs thousands of years ago. In North American wolves the mutation occurred around 14,000 years ago. The Italian black wolf's haplotype surrounding the K locus mutation is more variable than the North American one. Consequently, the interbreeding episode might be more recent.

Contrary to the North American study, in Italy we had no suspect that the black colour could be advantageous for specific habitat selection because black individuals are reported from dry areas near sea level to mountain forests.

Drawing specific primers for the short area surrounding the three bases deletion, we succeeded in analysing non-invasive samples. We detected 19 additional black individuals (heterozygous) and we determined a larger area of presence were the melanism occurred.

We ascertained the heterozygous mutation in Italian wolves only in samples pertaining to individuals described as phenotypically black or collected in areas were black sightings were reported. The mutation was not observed in phenotypically wild type wolves. We found that in dogs the heterozygous mutation was very common and rarely homozygous.

We obtained additional information on genotypes and we performed parentage analysis. The presence or absence of the deletion was a useful support to the correct genotyping of some individuals and the identification of packs. We were thus able to describe the black phenotype, evidencing the major morphological traits and hairs structure.

Up today, this PhD research may well be considered the more relevant study on the Italian melanism phenomenon. Previous studies (Apollonio *et al.* 2004, Randi & Lucchini 2004) turned into light the presence of several black individuals. Both articles evidenced the difficulties to determine the dog gene introgression into black wolves using microsatellite analysis and only one individual was identified as a possible hybrid.

5.2 The microchip analysis: genetic diversity in the Italian population

Thanks to a customized Canine array, we were able to analyse a consistent number of SNPs. This allowed comparing the Italian wolf samples with dogs of thousands of different breeds and with wild Canids processed by Prof. Wayne's research group. We analysed over 100,000 canine genes in 21 Italian wolf and 8 suspected hybrid samples.

We ascertained that the Italian wolf population is enough differentiated to guarantee a correct identification utilizing clusters and principal component analysis. According to previous studies (Randi *et al.* 2000, Randi and Lucchini 2002, Verardi *et al.* 2006), the Italian population resulted separated not only from the dog and north American wolves but also from the rest of the European

wolf populations. Moreover, the suspected hybrids were correctly identified and resulted in an intermediate position between wolf and dog clade.

The microchip analysis is only applicable to invasive good quality and high concentrated samples. Moreover, it is expensive and requires a long procedure for preparing each sample. It follows that also several good quality samples failed. Despite the high cost of the analysis, we acknowledged the high power of resolution of such method. Compared with previous works on standard markers like microsatellites and mtDNA and also on SNPs, we found a higher number of reliable data (Holm Andersen *et al*. 2006).

The study opens the path to numerous future research lines: among these, in my opinion, the most interesting ones focus on knowledge on the hybridisation between dog and wolf and explore the genetic diversity among the European populations.

At first, we analysed the whole dataset with hundreds of individuals and all the 125 K markers. The difficulties encountered to manage such a huge dataset suggested performing the analysis on a reduced one. This circumscribed our research to the Italian samples, a small number of European wolves and 85 dog breeds. We selected and analyzed only the most reliable SNPs. Consequently, we focused only on the Italian wolf's genetic differentiation. In some of the analysis the results were affected by the low number of individuals not representative for the other European population.

Our data proved the reliability of the microarray analysis method. Both dogs and European wolves were correctly identified. Furthermore, hybrids were detected and three wolves previously identified as pure wolf with microsatellites loci resulted hybrids. This highlighted the better power of resolution of such technique.

Compared to previous studies we detected a higher power of resolution of mixed ancestry. For this reason, we are currently working on the identification of past generation backcrosses. Such study is complicated since first generation hybrids are extremely rare and usually second or third generation hybrids are detected.

In the past studies, genetic analyses showed that the Italian wolf population has distinctive genetic traits. A unique mtDNA CR haplotype (named W14 by Randi *et al*. 2000) and microsatellite allele frequencies are sharply different from any other wolf population and dog breed genotyped so far (Randi & Lucchini 2002). The factorial correspondence analysis (FCA) and structure clustering of microsatellite genotypes indicate sharp distinctions between wolves and dogs (Randi 2007). All wolves genotyped in Italy, including the admixed individuals, presented the Italian wolf mtDNA CR haplotype. Furthermore, all the admixed wolves identified so far also showed wolf Y haplotypes (E. Fabbri & E. Randi, unpublished). This indicates that they were not first-generation hybrids, but probably backcrosses. No hybrid wolves were found among 130 distinct wolf genotypes noninvasively identified in the Alps (Fabbri *et al*. 2007).

Moreover, due to the founder effect, the loss of genetic diversity is higher in the Alpine population descending from the Italian one.

Previous population genetic studies did not reveal large-scale introgression of dog genes in European wolf populations, hybrids have been detected in several countries in Europe (Vilà & Wayne 1999, Randi *et al*. 2000; Andersone *et al*. 2002; Randi & Lucchini 2002; Vilà *et al*. 2003).

Research studies indicate that introgressive hybridization can be locally pervasive in populations of carnivores, and that conservation plans should be enforced to preserve the integrity of their gene pools. The small number of genetic markers used in earlier research projects showed a limited power of hybrid detection after the first generation of backcrossing.

The presence of anomalous morphological characters such as black coat colour, dewclaws or white nails has been observed in several wolves in Italy. These characteristics are common in some dog breeds but not in wolves. As Randi reported in 2007 morphological and molecular traits should be used together to identify admixed individuals in order to perform more detailed analysis.

6 - CONCLUSIONS AND PERSPECTIVES

Despite the decline of the Italian wolf in the past, nowadays the population is growing rapidly across the whole peninsula. Nevertheless, the species is still threatened by genetic pollution, loss of genetic variability and low connectivity with other European populations.

By means of genetic analysis methods, we gathered data on the threatened Italian wolf population. Such information constitutes a solid basis to support the ongoing conservation and management projects at both local and national level.

This PhD project focused, on one hand, on the debated Italian wolf melanism phenomenon. The research dealt with a considerable amount of heterogeneous data which outlined a detailed framework of information proving the relatively high occurrence of black individuals.

We detected the genetic mutation responsible for the black *pelage*, K locus, in a consistent number of dogs and in several wolves.

Our future purpose is to investigate also the other European populations searching for the melanistic mutation. We also intend to identify which dog breeds contribute mainly to the introgression to the black wolf phenotype in Italy.

On the other hand, this study proved that microarray technique is a powerful genetic tool for future functional genomic studies. Following and based on this comprehensive study we intend to perform other genomic investigations focusing on olfactory and further functional genes. This represents the preliminary study to determine the efficiencies and reliability of the chip SNP data. Moreover SNPs detected for the Italian population proved to be informative genetic markers. These will likely substitute microsatellites. We have already identified a panel of highly polymorphic SNPs useful to perform individual identification.

Noninvasive DNA analyses are frequently prone to genotyping errors due to DNA fragmentation and degradation. The use of single nucleotide polymorphisms (SNPs), which requires

amplification of short DNA sequences, may allow more efficient genotyping of noninvasive samples (Seddon *et al*. 2005, Holm Andersen *et al.* 2006).

To conclude, our next goal will be to investigate in depth the genetic differentiation among the European wolf populations.

The possibility of extensive hybridization between declining wolf populations and widespread free-ranging domestic dogs in Europe has been a main concern for conservation biologists over the past 30 years (Boitani 1984, 2003, Randi & Lucchini 2002). In fact, wolves and domestic dogs are isokaryotypic, fully interfertile, and they could mate successfully in captivity and in the wild when they co-occur as described from Wayne *et al*. (1995), Vilà & Wayne (1999).

This research study is of utmost importance for the future conservation of both the Italian and European populations to verify and constantly monitor the signals of hybridization and introgression with free-ranging domestic and the integrity of the wolf gene pool, as well as to detect the presence and distribution of hybrids.

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REFERENCES

Abecasis GR, Cherny SS, Cookson WO, Cardon LR (2002) Merlin-rapid analysis of dense genetic maps using sparse gene flow trees, *Nat. Genet*. **30,** 97-101.

Altobello G (1921) Fauna d'Abruzzo e Molise. Ed. Colitti, Campobasso. Mammiferi. Carnivori, **IV**, 38-45.

Anderson TM, VonHoldt BM, Candille SI, Musiani M, Greco C *et al.* (2009) Molecular and evolutionary history of melanism in North American gray wolves. *Science*, **323**, 1249-1390.

Andersone Z, Lucchini V, Randi E, Ozolins J (2002) Hybridization between wolves and dogs in Latvia as documented using mitochondrial and microsatellite DNA markers. ***Mammalian Biology***,**67**,79-90.

Apollonio M, Mattioli L, Scandura M (2004) Occurrence of black wolves in the Northern Apennines, Italy. *Acta Theriol.* **49** (2), 281-285

Boitani L (1984) Genetic consideration on wolf conservation in Italy. *Boll. Zool*. **51**(3), 367-373.

Boitani L (1992) Wolf research and conservation in Italy. *Biological Conservation***, 61,** 125-132.

Boitani L (1995) Ecological and cultural diversities in the evolution of wolf-human relationship. In: *Ecology and Conservation of Wolves in a Changing World* (eds. Carbyn LN, Fritts SH, Seip DR), pp. 3–11. Canadian Circumpolar Institute, Occasional Publication n. **35**. Edmonton.

Boitani L (2000) Action Plan for the Conservation of Wolves in Europe (*Canis lupus*). *Nature and environment*, 113 pp.

Boitani L (2003) Wolf conservation and recovery. In: *Wolves. Behavior, Ecology, and Conservation* (eds Mech LD, Boitani L), pp. 317–340. University of Chicago Press, Chicago.

Breitenmoser U (1998) Large predators in the Alps: The fall and rise of man's competitors. *Biological Conservation*, **83,** 279-289.

Brunetti R (1984) Distribuzione storica del lupo in Piemonte, Valle d'Aosta e Canton Ticino Rivista Piemontese di Storia Naturale, **5**, 7-22

Brumfield RT, Beerli P, Nickerson DA, Edwards SV (2003) The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology and Evolution,* **18,** 249-256.

Cagnolaro L, Rosso D, Spagnesi M, Venturi B (1974) Inchiesta sulla distribuzione del lupo in Italia e nei Cantoni Grigioni e Ticino (CH) Ricerche di Biologia della Selvaggina, **59**, 1-75

Candille SI, Kaelin CB, Cattanach BM, Yu B, Thompson DA *et al*. (2007) A b-Defensin Mutation Causes Black Coat Color in Domestic Dogs. *Science*, **318**, 1418.

Cercueil A, Bellemain E, Manel S (2002) PARENTE: computer program for parentage analysis. *Journal of Heredity*, **93**, 458-459.

Chen X, Sullivan PF (2003) Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput. *Pharmacogenomics Journal,* **3,** 77-96.

Ciucci P, Boitani L (1998) Il lupo, elementi di biologia, gestione, ricerca. Istituto Nazionale per la Fauna Selvatica "Alessandro Ghigi", Documenti tecnici, **23,** 114 pp.

Collins FS, Guyer MS, Charkravarti A (1997) Variations on a theme: cataloguing human DNA sequence variation. *Science*, **278**, 1580-1581.

Cooper DN, Krawczak M (1989) The mutational spectrum of single base-pair substitutions causing human genetic disease: patterns and predictions. *Hum. Genet.* **85**, 55-74.

Coppinger R (1983) The domestication of evolution, Environmental Conservation.

Coppinger R and Coppinger L (2001) Dogs, a startling new understanding of canine origin, behaviour & evolution, Scribner.

Corsi F, Dupré E, Boitani L (1999) A large-scale model of Wolf distribution in Italy for conservation planning. *Conservation Biology*, **13,** 150-159.

ESRI, ArcView® GIS 3.2 a geographical informative systems produced by Environmental Systems Research Institute, Inc.

Fabbri E, Miquel C, Lucchini V, Santini A, Caniglia R *et al*. (2007) From the Apennines to the Alps: colonization genetics of the naturally expanding Italian wolf (*Canis lupus*) population. *Molecular Ecology***, 16,** 1661-1671.

Fraley C, Raftery AE (2006) MCLUST Version 3 for R: Normal Mixture Modeling and Model-based Clustering, Department of Statistics, University of Washington, Technical Report, 504.

Guberti V, Francisci F (1991) Cause di mortalità di 60 lupi raccolti in Italia dal 1984. In: Spegnesi, M. & Toso, S. (eds.), Atti del Secondo Convegno Nazionale dei Biologi della Selvaggina. *Suppl. Ric. Biol. Selvaggina,* **XIX**, 599-603.

Hewitt GM (1996) Some genetic consequences of ice ages and their role in divergence and speciation. Biological Journal of Linnean Society **58**, 247-276.

Hewitt GM (1999) Post-glacial re-colonisation of European biota. Biological Journal of the Linnean Society 68: 87-112.

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature **405**, 907–913.

Holm Andersen D, Fabbri E, Santini A, Paget S, Cadieu E, *et al.* (2006) Characterization of 59 canine single nucleotide polymorphisms in the Italian wolf (*Canis lupus*) population. *Molecular Ecology Notes*, **6,** 1184-1187.

IUCN (2000) The 2000 IUCN Red List of Threatened Species. Gland, Switzerland: IUCN World Conservation Union.

Kerns JA, Olivier M, Lust G, Barsh GS (2003) Exclusion of melanocortin-1 receptor (mc1r) and Agouti as candidates for dominant black in dogs. *J. Hered*. **94**, 75–79.

Kerns JA, Newton J, Berryere TG, Rubin EM, Cheng JF, Schmutz SM, Barsh GS (2004) Characterization of the dog Agouti gene and a nonagouti mutation in German Shepherd dogs. *Mamm Genome*,**15**, 798-808.

Kerns J, Cargill E, Clark L, Candille S, Berryere T, *et al.* (2007) Linkage and segregation analysis of black and brindle coat color in domestic dog. *Genetics***, 176,** 1679-1689.

Leonard JA, Wayne RK, Wheeler J, Valadez R, Guillén S, Vilà C (2002) Ancient DNA Evidence for Old World Origin of New World Dogs. Science, **298**, 1613.

Li WH, Ellsworth L, Krushkal J, Chang JH, Hewett-emett D (1996) Rates of nucleotide substitution in primates and rodents and the generation time hypothesis. *Mol. Phylogenet. Evol.* **5**, 182-187.

LCIE (2007) Guidelines for Population Level Management Plans for Large Carnivores (Final draft, April 2007). Istituto di Ecologia Applicata, Rome.

Lovari S, Sforzi A, Scala C, Fico R (2007) Mortality parameters of the wolf in Italy: does the wolf keep himself from the door?. *Journal of Zoology,* **272**, 117-124.

Lucchini V, Galov A, Randi E (2004) Evidence of genetic distinction and long-term population decline in wolves (*Canis lupus*) in the Italian Apennines. Molecular Ecology, **13,** 523-536.

Martinez-Arias R, Calafell F, Mateu E, Comas D, Andres A, Bertranpetit J (2001) Sequence variability of a human pseudogene. *Genome Res*. **11**: 1071-1085.

Marucco F, Avanzinelli E and Gazzola A (2005) Stato, distribuzione, e dieta del lupo in Regione Piemonte, in Report 2005 Progetto Lupo Piemonte.

Mech LD (1970) The wolf: the ecology and behaviour of an endangered species. New York, *Natural History Press*.

Miller R, Pui-Yan Kwok (2001) The birth and death of human single nucleotide polymorphisms: newest experimental evidence and implication for human history and medicine, *Human Molecular Genetics*, **10**: 2195-2198.

Newton J, Wilkie A, He L, Jordan S, Metallinos D, Holmes N, Jackson IJ, Barsh G (2000) Melanocortin 1 receptor variation in the domestic dog. *Mamm Genome*, **11**, 24-30.

Nowak RM, Federoff NE (2002) The systematic status of the Italian wolf. *Acta Theriol,* **47**, 333-338.

Olsen SJ and Olsen JW (1977) The Chinese wolf, ancestor of new world dog. Science, **197**, 533-535.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes***, 6**, 288-295.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics,**155**, 945-959.

Promberger C, Schröder W editors (1993) Wolves in Europe: Status and perspectives. Munich Wildlife Society, Ettal, Germany.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR *et al.* (2007) PLINK: toolset for whole-genome association and population analysis. *American Journal of Human Genetics*, **81**.

Randi E (2007) Detecting hybridization between wild species and their domesticated relatives. *Molecular Ecology,* **17**, 285-293.

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. *Conservation Genetics,* **3**, 31-45.

Randi E, Lucchini V, Christensen MF, Mucci N, Funk SM, Dolf G, Loeschcke F (2000) Mitochondrial DNA variability in Italian and east European wolf: detecting the consequence of small population size and hybridization. *Conservation Biology***, 14**, 464-473.

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes,* **4**, 137-138.

Savolainen P, Zhang Y, Luo J, Lundeberg J, Leitner T (2002) Genetic Evidence for an East Asian Origin of Domestic Dogs. *Science,* **298**, 1610-1613.

Schlötterer C (2004) The evolution of molecular markers just a matter of fashion? *Nat Rev Genet* **5**:63–69.

Schmutz SM, Berryere TG, Barta JL, Reddick KD, Schmutz JK (2007) Agouti Sequence Polymorphisms in Coyotes, Wolves and Dogs Suggest Hybridization. Journal of Heredity.

Seddon JM, Parker HG, Ostrander EA, Ellegren H (2005) SNPs in ecological and conservation studies: a test in the Scandinavian wolf population. *Molecular Ecology,* **14**, 503-511.

Sillero-Zubiri C, Hoffmann M, Macdonald DW (2004) Canids: Foxes, Wolves, Jackals, and Dogs. Status Survey and Conservation Action Plan. Gland, Switzerland: IUCN/SSC Canid Specialist Group. 430p.

Sokolov VE, Rossolino OL (1985) Taxonomy and variability. In: *The wolf: history, systematic, morphology, ecology*. (ed. Bibikov DI), pp: 21-50. USSR Acad. Sci. Nauka, Moscow.

Sulkava S and Pulliainen E (1999) Canis lupus In: Mitchell-Jones AJ *et al*. (eds), The Atlas of European Mammals. Academic Press, London.

Syvanen AC (2001) Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nature Genetics Reviews*, **2,** 930-942.

Tsuda K, Kikkawa Y, Yonekawa H, Tanae Y. (1997) Extensive interbreeding occurred among multiple matriarchal ancestors during the domestication of dogs: evidence from inter- and intraspecies polymorphisms in the D-loop region of mitochondrial DNA between dogs and wolves. *Genes genet Syst.* **72,** 229-238.

Tschudi F (1858) Le monde des Alpes ou description pittoresque des montagnes de la Suisse et particulierement des animaux qui les peuples Tome II, editè par IMP Jules - Gme Fick.

Verardi A, Lucchini V, Randi E (2006) Detecting introgressive hybridisation between free ranging domestic dogs and wild wolves (*Canis lupus*) by admixture linkage disequilibrium analysis. *Molecular Ecology*, **15,** 2845-2855.

Vignal A, Milan D, SanCristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. *Genet. Sel. Evol*, **34**, 275-305.

Vilà C, Savolainen P, Maldonado JE, Amorim IR, Rice JE, *et al.* (1997) Multiple and Ancient Origins of the Domestic Dog, Science, **276**, 1687 – 1689.

Vilà C, Wayne RK (1999) Hybridization between wolves and dogs. *Conserv. Biol.* **13**, 195-198.

Vilà C, Walker C, Sundqvist A-K *et al.* (2003) Combined use of maternal, paternal and bi-parental genetic markers for the identification of wolf-dog hybrids. *Heredity*, **90**, 17–24.

Wang DG, Fan JB, Siao CJ, Berno A, Young P, *et al.* (1998) Large-scale identification mapping and genotyping of single-nucleotide polymorphisms in the human genome. *Science***, 280**,1077-1082.

Wayne RK, Vilà C (2001) Phylogeny and origin of the Domestic Dog. In: *The genetics of the dog* (ed. Sampson J), pp. 1-13 CABI, Wallingford, UK.

Wayne RK, Lehman N, Fuller TK (1995) Conservation genetics of the gray wolf. In: *Ecology and Conservation of Wolves in a Changing World* (eds. Carbyn LN, Fritts SH, Seip DR), pp. 399-407. Canadian Circumpolar Institute, Occasional Publication no. **35**. Edmonton, Alberta.

Zimen E (1981) The wolf: a species in danger. Delacorte Press, New York.