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A large scale non-invasive genetic project: wolf population (*Canis lupus*) in Emilia Romagna region

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to my wife and to my sons

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ABBREVIATIONS

ADO	allelic dropout
BO	Bologna (Province)
bp	base pair
c.i.	confidence interval
DNA	desossiribonucleic acid
FA	false allele
GUS	guanidine thiocynate
H_E	expected heterozigosity
H_O	observed heterozigosity
HWE	Hardy-Weinberg equilibrium
LIFE	LIFE area ((Parco dei Cento Laghi (PR), Parco del Gigante (RE), Parco del Frignano (MO))
FC	Forlì-Cesena (Province)
LD	linkage disequilibrium
MO	Modena (Province)
mtDNA	mitochondrial DNA
N_A	mean number of alleles per locus
Ne	(genetic) effective poulation size
PCR	polymerase chain reaction
PCR+	positive amplifications
PR	Parma (Province)
PNFC	Parco Nazionale Foreste Casentinesi
RA	Ravenna (Province)
RE	Reggio Emilia (Province)
REM project	Emilia Romagna wolf project
RNSO	Riserva Naturale Statale dell'Orecchiella
scnDNA	single copy nuclear DNA
s.d.	standard deviation
SNP	single nucletoide polymorphism
STR	single tandem repeats (e.g. microsatellites)
STS	sequence tagged sites

LIST OF PAPERS

I. Santini A, Lucchini V, Fabbri E and Randi E (2007) Ageing and environmental factors affecting PCR success in wolf (*Canis lupus*) excremental DNA samples

II. Holm Andersen D, Fabbri E, Santini A, Paget S, Cadieu E, Galibert F, Andrè C and Randi E (2006) Characterization of 59 canine single nucleotide polymorhisms in the Italian wolf (*Canis lupus*) population

III. Fabbri E, Miquel C, Lucchini V, Santini A, Caniglia R, Duchamp C, Weber JM, Lequette B, Marucco F, Boitani L, Fumagalli L, Taberlet P and Randi E (2007) From the Apennines to the Alps: colonization genetics of the naturally expanding italian wolf (*Canis lupus*) population

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SUMMARY OF PAPERS

Paper I: Ageing and environmental factors affecting PCR success in wolf (*Canis lupus*) excremental DNA samples

Because DNA will degrade over time, the condition of the scat is important and samples should be collected as fresh as possible and preserved quickly (Foran *et al.* 1997)
Non-invasive sampling, combined with molecular genetic analysis, is one of the least intrusive of all sampling methods for data population, and allows access to species and populations which would otherwise be difficult to study (Taberlet *et al.* 1996 and 1997).
Most fresh faeces contain at least some sloughed rectal cells from the depositing organism. It's known (Kohn *et al.* 1995) as faeces contain substances that can interfere with PCR success. High bacterial content of scats, expecially if combined with an initially warm and darp environment, might cause rapid DNA degradation of any DNA which was present originally (Kohn *et al.* 1995). An effective sampling and conservation method is therefore of prime importance.
Using mainly faeces as source of DNA for population assessment implies that usually PCR success rate usually are lower, and genotyping errors higher than in standard population surveys (Pompanon *et al.* 2005), due to DNA degradation or contamination in aged field samples.

This study aimed to attempt how environmental factors (e.g. temperature, humidity, exposure to sun or rain; Nsubuga *et al.* 2004) and ageing affecting PCR succes rate and genotyping errors on wolf scat. Moreover, we tested the effects of four storage conditions on DNA stability over time.

7 fresh scats were collected in an enclosure at the Pistoia Zoological Garden (north Italy) where lived a pack of 11 wolves, regularly fed with rabbit carcasses. The night before sampling, the ground of the enclosure was cleaned from old scats to ensure that scats were no more than 12-18h old. As positive controls we also collected 7 blood samples from 7 different wolves (out of 11 present in the enclosure).
Each of seven wolf scats numbered from 1 to 7 were divided into eight aliquots (i=1-7) of about 2 g, which were used in 8 different experiments; four experiments (A-D) aimed to assess the effects of different environmental conditions on DNA integrity over time. The other four experiments (E-H) were performed to assess of four different storage conditions (Table 1).

Samples	Experiments	Aliquots	DNA extracts
scats 1-7	(A) indoor	A1-A7	49
	(B) outdoor on stones	B1-B7	49
	(C) outdoor on grass uncovered	C1-C7	49
	(D) outdoor on grass covered	D1-D7	49
	(E) stored in EtOH at -20°C	E1-E7	63
	(F) stored in EtOH at room temperature	F1-F7	63
	(G) stored dry at -20°C	G1-G7	63
	(H) stored in GUS at room temperature	H1-H7	63

Table 1 - experimental design for seven scat samples (1-7) subdivided

Each of first DNA extraction was performed within 6h from collection using a guanidine thiocynate protocol (Gerloff *et al.* 1995). DNA was purified from each aliquot at 3th, 7th, 10th, 14th, 21th, 28th, 35th, 90th and 180th day from collection. Singleplex PCRs were performed at 6 STR loci (three dinucleotides and three tetranucleotides of different length) for blood and scats and individuals genotypes obtained.

The consensus genotype obtained from scats amplifications exactly matched five distinct reference genotypes obtained from blood samples (see also Fernando *et al.* 2003, showing that the seven scats belong to five distinct wolves. In this and other studies the consensus genotypes obtained from excremental DNAs exactly matched distinct reference genotypes obtained from blood samples. Samples that were not collected for non-invasive genetic studies (probably not as fresh as possible) failed (Creel *et al.* 2003).

Only ageing had a significant ($P < 0.0001$) effect on PCR+ as already showed by others authors (Deagle *et al.* 2006; Broquet *et al.* 2007); treatments ($P=0.46$) and time-treatments interaction ($P=0.26$) showed no significant effect on PCR+. Just after 3 days from deposition PCR+ significantly decreases; PCR+ was about 30% lower in DNA extracted from scats aged for two weeks than in in DNA extracted from fresh samples.

The most effective preservation methods were storage in 95% ethanol at -20 °C according to other authors (Wasser *et al.* 1997; Murphy *et al.* 2002; Fernando *et al.* 2003) and in GUS buffer ($P < 0.0001$); however, GUS buffers are toxic and should be handled with care.

The average ADO and FA rates were, respectively, 12% and 2%, in samples kept outdoor. Ageing progressively disrupted DNA integrity, simoultaneously increasing ADO and FA error rates (Taberlet *et al.* 1996). DNA in faeces is probably affected by hydrolytic and oxidative damage, and enzymatic degradation (Linn 1981). this study conferms that DNA degradation can be reduced collecting scat samples as fresh as possible, just after the animal leaves it behind; moreover, genotyping errors are constantly present and it’s

necessary repeating PCR ("multitube approach" Taberlet *et al.* 1996) and controlling accurately the laboratory results, even though that means much more laboratory work.

Some authors (Kohn *et al.* 1995; Wasser *et al.* 1997) noted that the reproducibility of DNA analysis appeared to be reduced by the uneven distribution of intestinal cells in faeces. Repeated genotyping of the same samples at the same deposition age didn't present that problem (data not shown), accordingly to Flagstad *et al.* (1999).

We also experienced as variance in DNA quality among samples was significantly high, independent of experimental and storage conditions (ANOVA; $P < 0.0001$). DNA yeld is also largely unpredictable based solely on sample morphology or age, according to Morin (Morin *et al.* 2007).

Paper II: Characterization of 59 canine single nucleotide polymorhisms in the Italian wolf (*Canis lupus*) population

DNA purified from non-invasive samples is tipically at low concentration and fragmented. Many studies reported as DNA quantity and quality of boh mtDNA and nuclear DNA affect PCR success and genotyping errors, such as ADO and FA (Frantzen *et al.* 1998; Kohn *et al.* 1995). ADO and FA, if not considered, can have dramatic effect on population assessments (Creel *et al.* 2003; Paetkau 2003).
These negative factors can be overcome just by replicating independently PCR ("*multiple tube approach*" Taberlet *et al.* 1996; Morin *et al.* 2001) many times, consequently increasing times and costs.

Significant advances in non-invasive genotyping are expected from the amplification of DNA fragments as short as possible (Frantzen *et al.* 1998). In this sense SNP genotyping, which require the amplification of very short DNA fragment and the extension of single nucleotides , might overcome technical limitations embedded in microsatellites (Morin *et al.* 2004; Wayne & Morin 2004*;* Seddon *et al.* 2005).

DNA was extracted from 14 Italian wolf tissue-samples collected in north and central Italy. These samples were tested for SNPs for 76 primer pairs that are known to contain SNPs in domestic dogs (Guyon *et al.* 2003). All PCR fragments were sequenced in boh directions. We developed also new primer sets and analysed 15 SNPs by Pyrosequencing technology (Ronaghi *et al.* 1998)

Out of 76 STS tested, 49 reliably amplified. Twenty-one out of 49 STS contained from one to seven SNPs, with a total of 59 wolf SNPs, which were either polymorphic in the italian wolves (42 SNPs) or between woves and dogs (17 SNPs).
Significant deviation for HWE was found in one case for SNP189H18(247), while highly significant LD was found between SNP310M20(332) and SNP310M20(341)
For PID (the probability of identitiy) calculations were performed using only one SNP at each STS (the one with the highest frequency of the rare allele), with a total of 18 polymorphic markers. PID was found to be low enough to discriminate between individuals; PIDs (corrected for siblings; Waits *et al.* 2001) was 9,96x10E-4 and PIDu (corrected for small sample size; Paetkau *et al.* 1998) was 3,21x10E-7.

As expected, dog SNPs were usually found also in wolves. SNPs discovered unique in dogs or wolves are important for detecting hybrids between dogs and wolves.

The principle drawback of SNPs as markers compare to microsatellites is that a microsatellite tipically contains much more “genetic information” than a SNP; therefore, usually many SNPs should be used for common population assessments. In this study 18 not linked SNPs, selected from the most variable founded, have the same discrimination power of 10 microsatellite loci (Lucchini *et al.* 2002). A real benefit of SNPs using samples containing poor and degraded DNA include ease and efficency of genotyping (Seddon *et al.* 2005); hence, these characterized SNPs can be successfully be used in non-invasive genetic studies of wild canids. Here we provided an example developing a new primer sets for genotyping SNPs by Pyrosequencing.

Paper III: From the Apennines to the Alps: colonization genetics of the naturally expanding italian wolf (*Canis lupus*) population

Evene though wolf disappearing from the Alps in the 1920s, some non-invasive investigations reported as Alps are being recolonized by naturally expanding Apennine wolves (Lucchini et al. 2002; Valière *et al.* . 2003). We used a population genetic approach to elucidate some aspects of the recolonization pattern.
Dataset with all the unique genotypes obtained at 12 microsatellite loci aiming to assess:

- the strength of the bottleneck and founder effects during the onset of colonization;
- the rates of gene flow between source and colony;
- the minimum number of colonizers that are needed to explain the genetic variability observed in the colony.

In this study, we used. DNA extracted from 3068 tissue and scat samples collected in the Apennines and in the Alps between 1982 and 2005. DNA was purified according to Gerloff *et al.* (1995) or with the QIAGEN Stool kit (QIAGEN), Each unique genotype founded was charaterized either at 12 autosomal microsatellites and sexed: six dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12; Fredholm & Wintero 1995; C09.250; Ostrander *et al.* 1993), and six tetranucleotides (FH2004, FH2079, FH2088, FH2096, FH2132 and FH2137; Francisco *et al.* 1996) were used.

Unique wolf genotypes were splitted into four groups: wolves collected in the northern, central or southern Apennines, and in the Alps.
Afterwards, genetic variability analysis were performed to test population estimates (He, Ho, NA, HWE) the difference between groups (Fst, Fis, Fist, AMOVA). Moreover, we tried to detect the most probable population of origin, to infer cryptic population structure and simultaneously assign individuals to populations. A multivariate spatial autocorrelation was also used to detect spatial structuring through correlations between pairwise geographical and genetic distance matrix. Finally, we tested for bottleneck our dataset, aiming to estimate how many colonizers are needed to explain the genetic variability observed in the Alpine wolf population (see Paper III for detailed methods)

We identified a total of 435 distinct wolf genotypes, which showed that wolf population in the Alps:

- has significantly lower genetic diversity (heterozygosity, allelic richness, number of private alleles) than wolves in the Apennines;
- is genetically distinct using pairwise Fst values, population assignment test and Bayesian clustering;
- is not in genetic equilibrium (significant bottleneck test). Spatial autocorrelations are significant among samples separated up to 230 km, roughly correspondent to the apparent gap in permanent wolf presence between the Alps and north Apennines.

- the estimated number of first-generation migrants indicates that migration has been unidirectional and male-biased, from the Apennines to the Alps, and that wolves in southern Italy did not contribute to the Alpine population.

These results suggest that:

- the Alps were colonized by a few long-range migrating wolves originating in the north Apennine subpopulation;
- during the colonization process there has been a moderate bottleneck;
- gene flow between sources and colonies was moderate (corresponding to 1.25–2.50 wolves per generation), despite high potential for dispersal.
- bottleneck simulations showed that a total of c . 8–16 effective founders are needed to explain the genetic diversity observed in the Alps.

GOALS OF THE THESIS

The present PhD work was conducted at the Conservation Genetic Laboratory of the Italian Wildlife Institute (I.N.F.S), and is part of a long-term project on population and conservation genetics of wolves in Italy. The general aims of this project was to contribute for better understanding wolf biology in Italy.
The project was supported by Italian Institute of Wildlife Biology (I.N.F.S) and Emilia Romagna Region. All provinces where wolf is present (Forlì, Ravenna, Bologna, Modena, Reggio Emilia, Parma, Piacenza) and Regional Parks (Parco dei Cento Laghi (PR), Parco del Gigante (RE), Parco del Frignano (MO), Parco Nazionale delle Foreste Casentinesi (FC-AR), Riserva Naturale Statale dell'Orecchiella (LU)) have been involved into this project.
In addition, the regional project ("Monitoraggio della presenza del lupo in Emilia Romagna mediante indagini genetiche" march 2002-april 2005) took strong advantage by integration with a LIFE project ("Azioni di conservazione del lupo (*Canis lupus*) in 10 siti SIC di tre parchi della Regione Emilia Romagna LIFE00NAT/IT/7214 march 2001-may2004).

Briefly, we experienced a large scale non-invasive wolf genetic monitoring along the whole Emilia Romagna Apennines ridge (about 11500 hectares).

Objectives:

- developing reliable genetic tools for genetic analysis of degraded sources of DNA, such as field-collected faeces;
- population size, genetic variability, dispersion, sex-ratio, turn over and relatedness;
- carrying out, where possible, some hypoteses on pack dynamics;
- pointing out the entity of wolf-dog hybridization.

INTRODUCTION

WHY WOLF?

Nowadays, numerous top mammalian consumers are prime target of conservation efforts. (Soulè & Terborg 1999). Top predators live usually at small population size; hence, they are generally more prone to become demographically endangered than more abundant species (Schaffer 1987). Moreover, many life history traits, such as complex social system, large home range and low reproductive rate, contribute to their vulnerability (Cardillo *et al.* 2005). Wolves usually live at low densities (1-3/100 km2), more rarely at higher densities, and this contributes to making them more vulnerable to ill-planned harvest schemes (Mech 1970 and 1973; Peterson & Page, 1988).
The wolf (*Canis lupus*) is the mammal with the largest natural distribution, being adapted to virtually all terrestrial habitat types (Mech 1970); hence, it's not surprising that it does exist a large scientific literature that describe many aspects of its biology, most of its from American and Canadian investigations. Wolf adaptibility can substantially change its biology depending on different habitat conditions.
Wolf is also a perfect example of a so called “umbrella species”; that means that wolf conservation may require conservation of numerous other species in their environment (Simberloff 1998). Indeed, wolf possess large home range, thus its effective conservation encompasses extensive areas and habitats.
In addition, getting financial support for implementing large conservation studies is more feasible for wolf as a flagship species; a charismatic large vertebrate, capable of arising public interest and sympathy (Simberloff 1998), it's a symbol of wilderness.

ITALIAN WOLF POPULATION HISTORY AND LEGAL STATUS

The present European distribution of the species is greatly reduced if compared to the past one, in fact extermination efforts by man caused the species extinction in many countries through hunting, habitat destruction and the decrease of its natural prey (Delibes 1990). Now the largest European wolf populations live in Romania, Russia, Bulgaria, Poland, Balkan area. Three smaller sub-populations can be identified in the Iberian peninsula, in Scandinavia and in Italy/France: they appear to be relatively isolated from other wolf populations and are expected to remain distinct for long time (Boitani 1999; 2000; 2003).
Wolf is an historic component of Italian wildfauna and many investigations have been conducted so far for better understanding its biology in Italy (Zimen & Boitani 1975; Boitani, 1983; Corsi *et al.* 1999; Randi *et al.* 2000; Lucchini *et al.* 2002; Lucchini *et al.* 2004; Randi & Lucchini 2002; Verardi *et al.* 2006).
In the last two centuries, Italian wolf population suffered a severe decline and were confined south of the Alps since the turn of the last century. The first studies, conducted indirectly by interview (Cagnolaro *et al.* 1974; Zimen & Boitani, 1975), suggested that approximately 100 individuals surviving in at least two fragmented subpopulations in the central-southern Apennines; surviving of a small population into Foreste Casentinesi National Park it's still under debate (Cagnolaro *et al.* 1974).

Wolf disappered from the Alps in the 1920s, in Sicily before 1950, and the peninsular population, as in many other countries in Europe, was law-protected since early 1970s (Boitani & Fabbri 1983a).
A significant recovery of he population was registered already in the 1990s by Boitani (1992), when the wolf recolonized parts of its historical range alone the Apenine ridge. The factors affecting the wolf recolonization are to be find in the more effective legal protection and substantial changes in the ecology of mountain areas (e.g. decrease of human density and increase of wild ungulates).
The recolonization of the Southwestern-Alps was registered since 1992, in France since 1992 and in Switzerland since 1996 (Lucchini *et al.* 2002; Fumagalli *et al.* 2006; Valiere *et al.* 2003). Recent estimating (Boitani 2003) reported a number of more than 600 wolves inhabiting the Italian peninsula, but nowadays the population can count probably more than 800 individuals (Randi pers. comm.).
Nowadays, wolf is a strictly protected species in Italy, with law implementation fully delegated from the Ministry of Environment and the Ministry of Agriculture to the Regional Authorities; also responsible for compensation of damage caused by wolf on livestock, thus procedures and amount of compensation varies across regions.
Legal protection of the Italian wolf population was accorded first in 1971 and was completed in 1976 when the species was given fully protected status, a process stimulated by WWF International. The EC Habitats Directive (92/43 of 21.5.1992) lists the Italian wolf in Appendix II (needs habitat conservation) and D.P.R. 357 of 8.11.1997 of Habitats Directive in Appendix IV (fully protected) (Boitani 2000 and 2003; Genovesi 2002).

TAXONOMY AND LIFE HISTORY TRAITS

The grey wolf (*Canis lupus*) belongs to the order Carnivores and the family Canidae.
The wolf (*Canis lupus*) is the mammal with the largest natural distribution and its phenotype variation, in terms of size, color and weight, is remarkably high (Mech 1970; Boitani 1995 and 2000).
In 1921 Altobello first suggested for italian wolf the subspecies status “italicus”, based just on few phenotypic characteristics. Recent genetic and morphometric investigations (Randi *et al.* 2000; Randi & Lucchini 2002; Nowak & Federoff, 2002) seem confirm the status of subspecies.
The morphological and genetic peculiarity of Italian wolf population are the result of a long-term isolation, due to natural and anthropic barriers that limited or interrupted the genetic flow among IItaliantalian and other wolf populations. It’s today well-known as Italian, Balcanic and Iberic peninsulas were refugia-areas during Pleistocene ice age (Hewitt 1996; 2000).
Moreover, also big river can contributed consistently to population differentiation (Carmichael *et al.* 2001); Po river can limited the wolf during the *intraglacial age.* Probably antropic factors contributed to Italian wolf population isolation just for the last two centuries (Lucchini *et al.* 2004).
The Alps wolf recolonization has to be considered a great success in terms of conservation effort, because from Alps Italian wolves can be in contact with other european wolf populations.

Wolf is a typical social species, organized into packs, with one reproducing pair (the alfa male and the alfa female). The pack cooperate in hunting, reproducing and defending their territories (Mech 1970). A pack is fundamentally a family unit that originates when a pair establishes a territory and reproduces It is generally made up by a mated pair, its yearling

pups and by some other adults which are generally the offspring of the previous years remaining with the pack for a year or more, when new pups are born (Mech 1970; Rothman & Mech 1979). When a mated member lacks, it can be substituted by another wolf of the same pack or by a wolf coming from other packs or from other territories (Meier *et al.* 1995). The literature reports pack with 2-36 members (Mech 1970) but probably in Italy a tipical family group consist of 3-7 members (Boitani 1992; Lucchini *et al.* 2002; Apollonio *et al.* 2004). Pack-territory was related to prey density (Walters *et al.* 1981), on landscape, geographical and morphology features (Peterson, Woolington & Bailey, 1984; Peterson & Page 1988), and on human disturbance. Pack territory ranges from 80 to 2.500 km2 in North America and from 100 to 500 km² in Europe.

Factors triggering dispersal are scarcity of food and/or sexual competition. Some individuals can also disperse when they lose their status and are rejected by a pack (Mech 1995c and d). Wolves disperse from their pack as young as 5 months of age (Fuller 1989b), whereas others may remain with the pack for up to 3 years (Gese & Mech 1991) or occasionally longer (Ballard *et al.* 1987). A wolf is sexually active when it is two years old. The distances wolf disperse reflect the type of dispersal: some authors reported that younger the disperser is, the farther it disperses (Wydeven *et al.* 1995) and that the record dispersal lengths of males and females tent to be about the same (Ballard *et al.* 1983; Peterson, Woolington & Bailey 1984). A dispersal wolf can move from an adjacent territory to substantial dispersal distances; in fact dispersal distances of several hundred kilometres are common, and movements over 1000 km have been documented (Fritts & Mech 1981; Ballard *et al.* 1983; Fritts 1983; Mech *et al.* 1995; Wabakken *et al.* 2001).

It’s now well-known as dog represent the wolf’s domestic corrispective (Vilà *et al.* 1997; Savolainen *et al.* 2002); wild and free-ranging wolf and domestic or feral dogs can hybridize and introgression can be a real threat for wolf populations. Concern has been expressed that European population of gray wolves have extensively hybridized with domestic dog (Vilà & Wayne 1999). Nevertheless, significant introgression of dog genes into wild wolf populations has not yet documented (Vilà & Wayne 1998). Wolf-dog hybrid have been recorded in Italy (Boitani, 1983; Randi & Lucchini 2002; Verardi *et al.* 2006). The genetic data showed as large scale introgression between the two *taxa* has not yet occured in Italy (Verardi *et al.* 2006; Randi 2007).

NON-INVASIVE GENETIC MONITORING

It’s essential for conservation and management of wildlife populations collect information on parameters such as population size, demography, relatedness, gene flow, and population structure, but these parameters are difficult to obtain for species that are rare or elusive such as carnivores (Taberlet *et al.* 1996; Kohn *et al.* 1999; Creel *et al.* 2003). In addition, endangered populations are tipically at low densities.
About fifteen years ago are the first non-invasive genetic investigations (Taberlet & Bouvet, 1991; Taberlet & Bouvet, 1992; Morin *et al.* 1993; Taberlet *et al.* 1996 e 1997; Gagneux *et al.* 1997).
Non-invasive genetic techniques are methods that allow to collect biology informations from the population investigated using traces that the animal leave behind like scats, hairs, urine. All these non-invasive traces can be used as DNA source and properly analysed. Laboratory procedure for this kind of samples are not the same that for invasive samples: in fact, NGS (Non-invasive Genetic Monitoring) for giving reliable biology informations (Taberlet *et al.* 1996; Kohn *et al.* 1999; Morin *et al.* 2001; Miller *et al.* 2002), have to

overcome many problems related to low-quantity and -quality of DNA extracts (low PCRs success rate, genotyping errors, contamination). Laboratory techniques are therefore almost the same used in forensic genetics (Kohn & Wayne 1997; Piggott and Taylor 2003), requiring usually many times and costs to be implemented.

MATERIAL AND METHODS

LABORATORY PROCEDURES

Samples and DNA extraction

Samples were extensively collected in all over the study area. The area included most of the Emilia-Romagna Apennine Ridge and involved all Regional Provinces where wolf lives. In addition, two National and four Regional Parks (Fig. .1) partecipated to this investigation. This project was planned to rely on the use of DNA mainly from wolf faeces. 1665 presumed wolf scat samples were collected in the northern Apennines from March 2001 to April 2005 (Fig. 1).

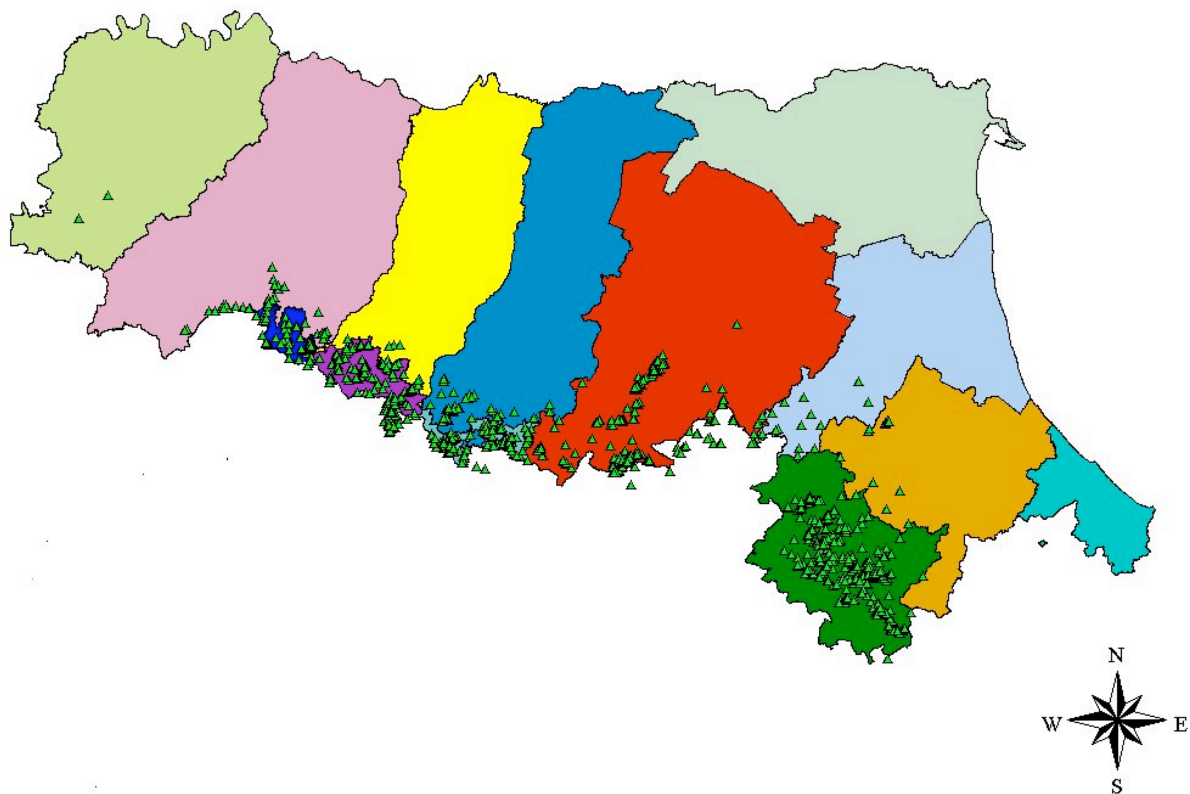

Fig. 1- study area: adminstrative boundaries and sampling (green dots) are indicated.

In addition, 8 invasive samples were also collected either from carcasses (accidentally died or illegally killed) or from animals captured as part of ongoing radio-telemetry projects. Scats were usually collected during transects, opportunistically chosen to maximise the penetrability of the areas. Many samples were collected in winter during snow-tracking sessions (references).

A preliminary study was conducted (see Paper I) to optimize the filed-samples collection and tested the best storage conditions on DNA stability over time.

Hence, a stricly sample-collection protocol were distributed to all field-researchers, with racccomandations to collect samples a fresh as possible. Samples were stored in ethanol 95% (one part of sample and three parts of alcohol), keep frozen at -20 °C until shipped to the laboratory.
Each scat was finally provided by field informations, such as date, track name, and scat apperance. For each faeces a Global Positioning System (GPS) reading was taken and transferred into a Geographic Information System (GIS) to establish their map locations.

In this study we followed all lab raccomandations for ancient or forensic samples (Taberlet *et al.* 1996; Budowle *et al.* 2005). To obtain reliable results, as accepted practice in treatment of such low/degraded DNA samples, we performed separately pre-PCR and post-PCR operations. Containment hoods, reagents, and pipettes were be dedicated to the pre-PCR or post_PCR area (Taberlet *et al.* 1996; Budowle *et al.* 2005). Negative and positive controls were always added, both in purification steps and in PCR.

Before any further manipulation, scats were deep-frozen at -80 °C for at least 2 days to avoid any risk of contamination by Echinococcus spp. (Guberti pers. comm.)
DNA of both scats and tissue samples were purified according to Gerloff *et al.* (1995) using a guanidinium thiocyanate and diatomaceus earth (guanidinium-silica) protocol (see Paper I).

Microsatellite genotyping, sex determination and DNA sequencing

Each unique genotype founded was charaterized either at 12 autosomal microsatellites and sexed (Table 1). Patterns of genetic variability were assessed also in paternal lines employing three Y-linked markers (Table 1).Microsatellites loci were selected among 18 canine microsatellites previously used in a study about Italian wolves (Randi & Lucchini 2002). For FH loci, new shorter primer pairs were designed.
For charaterizing all non-invasive samples collected six out of twelve STR loci has been selected: CPH2, CPH8, FH2004, FH2088, FH2096, FH2137. Infact, assessing population parameters from a non-invasive sampling such as genetic censuses should use the minimum number of loci required to attain a low probability of identity (PID; see Paetkau & Strobeck 1994) among samples from different individuals (Kohn *et al.* 1999; Waits *et al.* 2001; Flagstad *et al.* 2004). Table 2 shows the probability of identity (PID), computed for each locus estimated in a set of 100 Italian wolves using GIMLET v. 1.3.2. (Valière 2002).

MARKER	TYPE	RANGE (bp)	REFERENCE	REPEAT
CPH2	AUTOSOMAL	92-104	Fredholm & Wintero 1995	di
CPH4	AUTOSOMAL	139-147	Fredholm & Wintero 1995	di
CPH5	AUTOSOMAL	108-122	Fredholm & Wintero 1995	di
CPH8	AUTOSOMAL	195-209	Fredholm & Wintero 1995	di
CPH12	AUTOSOMAL	190-202	Fredholm & Wintero 1995	di
U250	AUTOSOMAL	127-141	Ostrander *et al.* 1993	di
FH2004	AUTOSOMAL	232-302	Francisco *et al.* 1996	tetra
FH2079	AUTOSOMAL	260-276	Francisco *et al.* 1996	tetra
FH2088	AUTOSOMAL	93-133	Francisco *et al.* 1996	tetra
FH2096	AUTOSOMAL	92-100	Francisco *et al.* 1996	tetra
FH2132	AUTOSOMAL	265-323	Francisco *et al.* 1996	tetra
FH2137	AUTOSOMAL	152-184	Francisco *et al.* 1996	tetra
MSY34A	Y-LINKED	170-174	Sundqvist *et al.* 2001	di
MSY34B	Y-LINKED	173-175	Sundqvist *et al.* 2001	di
MSY41B	Y-LINKED	114-126	Sundqvist *et al.* 2001	di
mtDNA CR	mtDNA		Randi *et al.* 2000	
ZFXY	XY		Lucchini *et al.* 2002	

Table 1 - markers employed.

MARKER	PID	PIDcorr	PIDsibs
CPH2	2.83x10-1	2.88x10-1	5.57x10-1
CPH4	5.12x10-1	5.17x10-1	7.23x10-1
CPH5	2.23x10-1	2.27x10-1	5.09x10-1
CPH8	2.34x10-1	2.39x10-1	5.18x10-1
CPH12	3.73x10-1	3.76x10-1	6.06x10-1
U250	1.83x10-1	1.88x10-1	4.82x10-1
FH2004	1.77x10-1	1.82x10-1	4.78x10-1
FH2079	3.69x10-1	3.71x10-1	5.93x10-1
FH2088	1.94x10-1	1.97x10-1	4.74x10-1
FH2096	3.25x10-1	3.28x10-1	5.72x10-1
FH2132	3.32x10-1	3.35x10-1	5.81x10-1
FH2137	6.75x10-2	7.1x10-2	3.7x10-1
TOTAL	3.45x10-11	4.78x10-11	2.43x10-5

Table 2 - probability of Identities (PID, PIDcorr, PIDsibs) for each locus used in this study estimated in a set of 100 Italian wolves using GIMLET v. 1.3.2. (Valière 2002).

PID is the probability of identity for individuals randomly chosen within the same population, PIDcorr is the probability of identity corrected for small population size, PIDsibs is the probability of identity corrected for siblings. PIDsibs < PiIDcorr < PID. Each total probability was computed by multiplying single locus probabilities, assuming that loci are independent, as suggested by the microsatellite linkage map of the domestic dog (Neff *et al.* 1999). The overall PIDsibs is the upper limit of the possible ranges of PID in a population and thus provides the most conservative number of loci required to resolve all individuals, including relatives.
An empirical evaluation about the wolf population-size inhabiting the study area has been conducted prior the present investigation. Based on field observations, an estimating of about 100 wolves present in the whole study area (Randi & Lucchini 2002; Lucchini *et al.* 2002) were conducted. Moreover, as wolves in a pack are known to be partially related, sharing alleles which are identical by descent (Wayne *et al.* 1995) it was necessary to achieve PIDsibs values < 0,01 . For the set of six microsatellites chosen in this study the PID sibs was $1.38x10^{-2}$< 0.01; hence, samples with the same multilocus genotype across all six loci were interpreted as representing the same individual. If in a population PID is not zero, some individuals cannot be detected (shadow effect) leading to a population size underestimation (Mills *et al.* 2000).

To increase resolution power for subsequent data analysis (levels of genetic variability, assessment of immigration, and relationship analysis) we genotyped each of the identified individuals across six additional autosomal microsatellite loci (Table 1).

Only one sample per individual was used for genotyping of these additional loci. Before accepting a consensus genotype we applied the same stricly criteria as described.
Each unique multilocus genotype (e.g. each individual) was also sexed using ZFXY locus followed by digestion of PCR product with TAQ I restriction enzyme.

103 faeces extracts were also sequenced at a 350 bp mtDNA CR fragment (Randi *et al.* 2000). This kind of analysis was performed at early stages of the project mainly for checking the quality of field sampling. The mtDNA contol region is a straightforward marker for Italian wolf population (Randi *et al.* 2000); it does exist only one aplotype (W14) that is unique among European wolf population and it doesn't exist in dog. Hence, mtDNA CR can be used both as indicator of appartenence of an individual to Italian population or

MARKER	METHOD
CPH2	(94°C 2m) [(94°C 30 s - TD 63°-55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
CPH4	(94°C 2m) [(94°C 30 s - TD 63°-55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
CPH5	(94°C 2m) [(94°C 30 s - TD 63°-55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
CPH8	(94°C 2m) [(94°C 30 s - TD 63°-55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
CPH12	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
U250	(94°C 2 m) [(94°C 30 s - 56°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
FH2004	(94°C 2m) [(94°C 30 s - TD 63°-55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
FH2079	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
FH2088	(94°C 2m) [(94°C 30 s - TD 63°-55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
FH2096	(94°C 2m) [(94°C 30 s - TD 63°-55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
FH2132	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
FH2137	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
MSY34A	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
MSY34B	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
MSY41B	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
mtDNA	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)
ZFXY	(94°C 2 m) [(94°C 30 s - 55°C 30 s - 72°C 30 s) x 40] (72°C 5 m)

Table 3 - PCR protocol for each marker used

Microsatellites were PCR-amplified (Randi & Lucchini, 2002; Lucchini *et al.* 2002) separately in 10 µl of volume, using 4 µl of DNA solution, 1 µl of PCR Buffer 10X (1,5 mM of MgCl2), 2 µg of BSA (Bovine Serum Albumin), 0,4 µl of dideossinucleotides (dATP, dCTP, dTTP, dGTP) 2,5mM, 0,15 µl of each primer 10 µM, 0,25 units of Taq. Sequences were PCR-amplified accordingly to Lucchini *et al.* (2002).
Table 3 summarized all PCR-protocols. All PCR products were run on an ABI 3100 instrument (Applied Byosistems, Forster City, California) and fragments were resolved with GENESCAN and GENOTYPER (Applied Byosistems). Sequences were analyzed with SEQSCAPE (Applied Byosistems).

To obtain reliable results and detect genotyping errors, a multiple-tube approach was taken into account (Taberlet *et al.* 1996) for multilocus genotyping. The number of replicates to obtain a reliable multilocus genotype was computed using the software RELIOTYPE (Miller, Joyce & Waits, 2002). All samples that could be not reliably typed at all loci after 8 amplifications were discarded.

After scat DNA purification, the total amount of DNA available for genetic typing can be very low, and is often in the picogram range (Taberlet *et al.* 1999) or can contain ihnibitors of PCR (Monteiro *et al.* 1997). If the project is large, many authors (Kohn *et al.* 1999; Morin *et al.* 2001; Creel *et al.* 2003; Flagstad *et al.* 2004; Lucchini *et al.* 2002) suggested that every non-invasive genetic protocol takes strong advantage by screening the samples for DNA quality. This procedure has been implemented by mtDNA amplification (Lucchini *et al.* 2002), by real time PCR (Morin *et al.* 2001) or by STR amplification (Flagstad *et al.* 2004). In the present investigation the screening consisted in amplifying each sample four times at 2 microsatellite loci (FH2096 and FH2137) chosen, for their high PCR success and their low dropout and false allele rates, among the first 6 microsatellite used for the individual identification (Lucchini *et al.* 2002; Paper III). Only those samples that give positive PCRs major than 50 % pass the screening.

DATA ANALYSIS

To avoid typing errors, consensus genotypes were performed with GIMLET v. 1.3.2 (Genetic Identification with MultiLocus Tags) (Valière 2002). The program was also be used to compare all the consensus genotypes. This software gives as output both 100% matches all over loci considered (on our case six) and the pairs of genotypes where only one allele or two alleles are different between the genotypes. In this way it is possible to re-check these genotypes by re-looking at the electropherograms and obtaining some indication for eventually repeating PCRs at some loci.

Wolf faeces can be quite easily confused with those from dogs and red foxes. Therefore, we verified putative wolf faeces in two ways: sequencing mtDNA CR (Randi *et al.* 2000) and/or performing Bayesian clustering analysis implemented in STRUCTURE v. 2.1 (Pritchard *et al.* 2000; Falush *et al.* 2003). STRUCTURE allows to assign to an individual a posterior probability *q-value* that its multilocus genotype originates from either of the population under study; hence, it can also be used efficay in hybrids detection (Pritchard *et al.* 2000; Vilà *et al.* 2003; Randi 2007) revealing cryptic population structure or in detecting immigrants (see also Paper III; Flagstad *et al.* 2004). A genotype sampled in one population but with a higher posterior probability of originating from another may be considered a migrant. We run STRUCTURE with a burn-in period of 30000 Markov Chain

Monte Carlo (MCMC) cycles to reach the stationary phase and 1000000 additional cycles from which the results were extracted.

Genotyping errors such as allelic dropout (ADO) and false alleles /FA) were assessed from a subsample of 100 scats using GIMLET v. 1.3.2 (Genetic Identification with MultiLocus Tags) (Valière 2002)

Levels of genetic variability were described across all 12 loci by the number of alleles, and heterozigosity (Nei 1987). Population estimation was measured in terms of Fst (Weir & Cockerham 1984). All population parameters (allele frequencies, H_E and H_O, F estimators) were calculated with GENEPOP (reference) FSTAT (reference) and GENETIX v. 4.02 (Belkhir *et al.* 2001). All individuals were also analysed with GENETIX v. 4.02 (Belkhir *et al.* 2001), that performs a factorial corrispondence analysis.

Once obtained the allele frequencies in the study area, simulations were performed with the software CERVUS (Marshall *et al.* 1998) to compare the power of six loci (the six chosen for individual discrimination) and twelve loci. CERVUS allows to estimate the relationship power of loci used, based on their allele frequencies of population, percent of individuals sampled and number fo individuals to be tested. Our first assumption was that we sampled 100% of individuals live in the study area during the investigation period.
We obtained the r (relationship index) distributions from KINSHIP (Goodnight % Queller 1999) for parent-offspring, and full-sibs and not related. All simulations were performed with 10000 simulated pairs. To determine the probability that a dyad of one type of relationship would be missclassified as belonging to another type of relationship, we used the midpoint between (Blouin *et al.* 1996) between the means of the two distributions as the cut-off value for classification.
Pairs parents-offsprings were identificated with PARENTE (Cercueil *et al.* 2002). No field informations exept GPS readings and date of sampling were used for relationship analysis.

In this investigation we treated a successfully analyzed faeces sample as one trapping event and simply recorded how many individuals were trapped once, twice, three times, and so on. Population size estimation can be based on mark-resight methods to genetic data (Taberlet *et al.* 1997: Creel *et al.* 2003) if individuals are sampled sufficiently often to estimate resighting probabilities (Otis *et al.* 1978; Seber 1982). Recently, the use of capture-recapture studies based on non-invasive genetic sampling has rapidly increased (Lukacs, 2005; Lukacs & Burnham, 2005).
In the present study heterogenous capture probabilities were expected; this is suggested by the fact that sampling effort varied greatly among localities and periods, and because it's easier to have been collected scats leaved behind by the dominant individuals (the alfa male and alfa female) of the pack. Most directly, the number of distinct genotypes is an estimate of the minimum population size, which can be identified by the asymptote of a curve relating the number of distinct genotypes to the number of samples (Kohn *et al.* 1999).

SNPs DETECTION

An non-invasive genotyping investigation is usually time- and costs-consuming.
Most of problems related to non-invasive genotyping, such as low PCR success rate, repeating PCR , ADO and FA, could be at least partly overcome by substituting STR markers with markers as short as possible, like SNPs (Frantzen *et al.* 1998; Morin *et al.* 2004; Wayne & Morin 2004; Seddon *et al.* 2005).

Starting from previously identified SNPs on dog genome (Guyon *et al.* 2003), we resequenced in both directions 76 fragments for 14 Italian wolf tissue-samples (for details, see Paper II). Sequences are resolved with SEQSCAPE (Applied Byosistems, Forster City, California). Here we also provided an example developing a new primer sets for genotyping SNPs by Pyrosequencing.

RESULTS

A total set of 1665 scat samples of presumed wolf were collected in this study (Table 1). In addition, during the study period 7 invasive samples (blood/tissue) were also collected.

	BO	FC	MO	LIFE	RNSO	PR	PNFC	RA	RE	TOT
before march 2002	65	0	105	22	0	1	0	0	0	193
april 2002-march 2003	76	13	9	283	25	12	86	4	17	525
april 2003-march 2004	88	11	16	197	20	2	197	4	19	554
april 2004-march 2005	136	6	28	5	0	0	174	23	21	393
TOTAL	365	30	158	507	45	15	457	31	57	1665

Table 1 - samples collected and analyzed during the whole study period in the Emilia-Romagna Apennine Ridge. See Abbreviations for acronyms.

Performance and reliability of the applied methodology

1116 samples (67%) out of 1665 samples were positively screened and for 663 (40%) we obtained a reliable indivdual genotype.

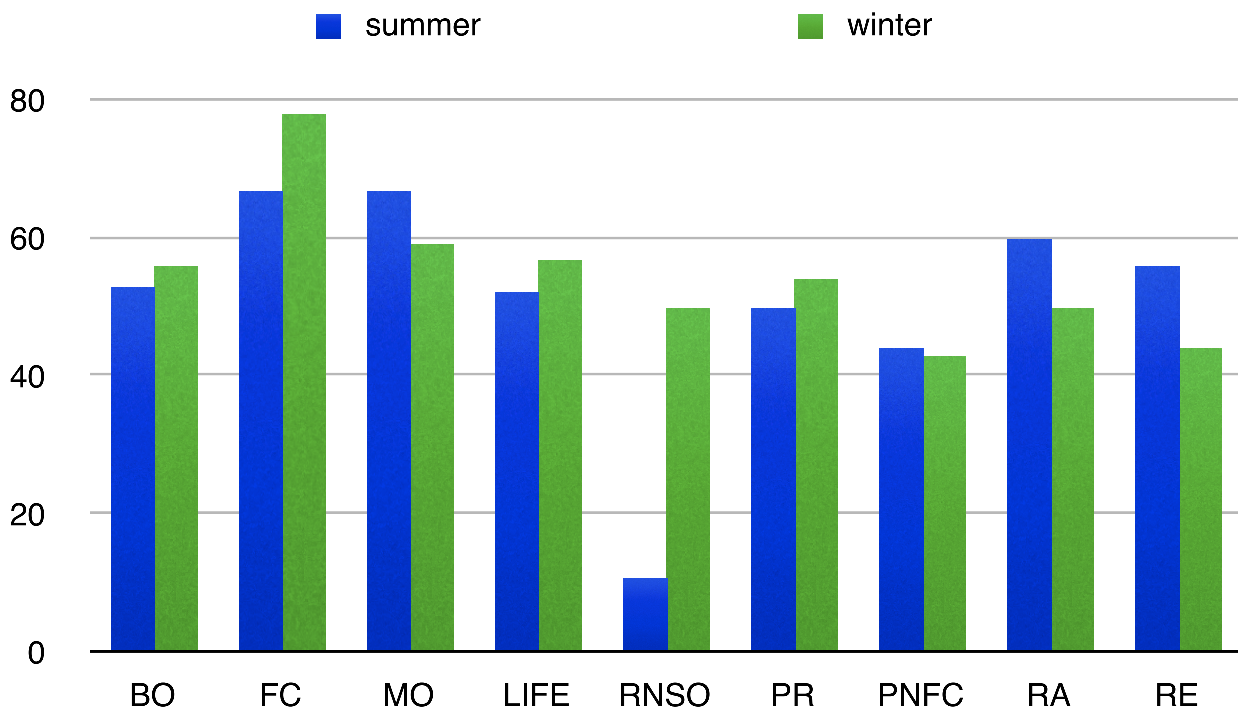

Fig. 1 - winter and summer genotyping success obtained for the whole project period for every study area.

Species identification was possible for 753 samples (45%). No significant divergence was found (P = 0.7734) between genotyping success in every study area of samples collected during winter (november-april) and summer (may-october) periods, even though summer sampling was tipically low (Fig. 1).
7 blood/tissue samples were also analysed; in some cases, the 12 loci genotype matched a gentoype precedently and non-invasively recorded.

Genotyping errors

After screening all the 6 loci used for individual identification showed high rates of positive PCRs, ranging from 66 % (CPH8) to 98 % (FH2096), ADO per locus varied from 10,1 % (CPH2) to 35,2 % (CPH8) and false allele rates per locus varied from 0 % (CPH2) to 8,1 % (FH2137) (Table 2).

	2004	**2088**	**2096**	**2137**	**CPH2**	**CPH8**	**MEAN**
allelic range	106-176	93-133	92-100	154-182	92-104	195-211	
PCR success (%)	84	94	98	86	92	66	87
ADO (%)	24,8	15,8	13,9	26,7	10,1	35,2	21,1
FA (%)	4,4	2,9	3	8,1	0	2,1	3,4

Table 2- PCR success, ADO and FA observed in the 6 loci for individual/species identification.

ADO (r = 0,98) and FA (r = 0,39) correlates positively with allelic range of the locus.

Species identification

Using Bayesian clustering analyses and/or mtDNA CR sequencing following by BLAST, species identification was possibile for 753 scat samples: 52 were of domestic dog, 4 of fox, 3 wolf-dog hybrids (Table 3) and 694 of wolf. Resampling rate for dogs resulted just in three cases.

individual ID	**Sex**	***q-value***	**mtDNA CR**	**resampling**	**Area**
WR48	M	0,789	N.A.	NO	Parma Province
WPG108	M	0,847	W14	NO	Gigante Park
WFC259	F	0,753	N.A.	YES	PNFC

Table 3 - wolf-dog hybrid sampled

Six loci performed well in species determination with STRUCTURE (Pritchard *et al.* 2000) in all but 10 samples species assignment with *q-value* > 0,9 were possible.
A posterior test with 12 microsatellite loci was conducted for each individual to check the correct attribution of six loci assignment; all the attributions previously obtained using 6 loci were confirmed through a better assignment probability, 7 out of 10 uncertain six-loci assignments resulted to belong to the Italian wolf population while the other 3 uncertain individuals, that presented an admixture clustering resulted to be real hybrids, probably of second generation (Table 3).

Individuals, sex ratio and sampling rate

193 different italian wolf genotypes, corresponding presumably to 193 wolves were totally found regrouping the 663 reliable genotyped obtained (mean one new individual every 3,44 scats analysed). Samples analyzed allowed to detect 106 males and 83 females with a sex ratio among detected individuals greater than one (1,28M vs 1,00F). Considering individuals sampled for more than a year, we found 24 males and 24 femals, suggesting as the sex ratio between stantial individual was 1:1.
Sampling rate varied greatly among individual. Just 95 out of 193 individuals (49%) were sampled more than once (Fig. 2) and resampling rate was higher for females (55%) than for males (43%). Moreover, if we considered the time interval during which genotypes were sampled, only 53 individuals (27%) were observed for a period of at least one year. This could indicate that turn-over is particurarly high.

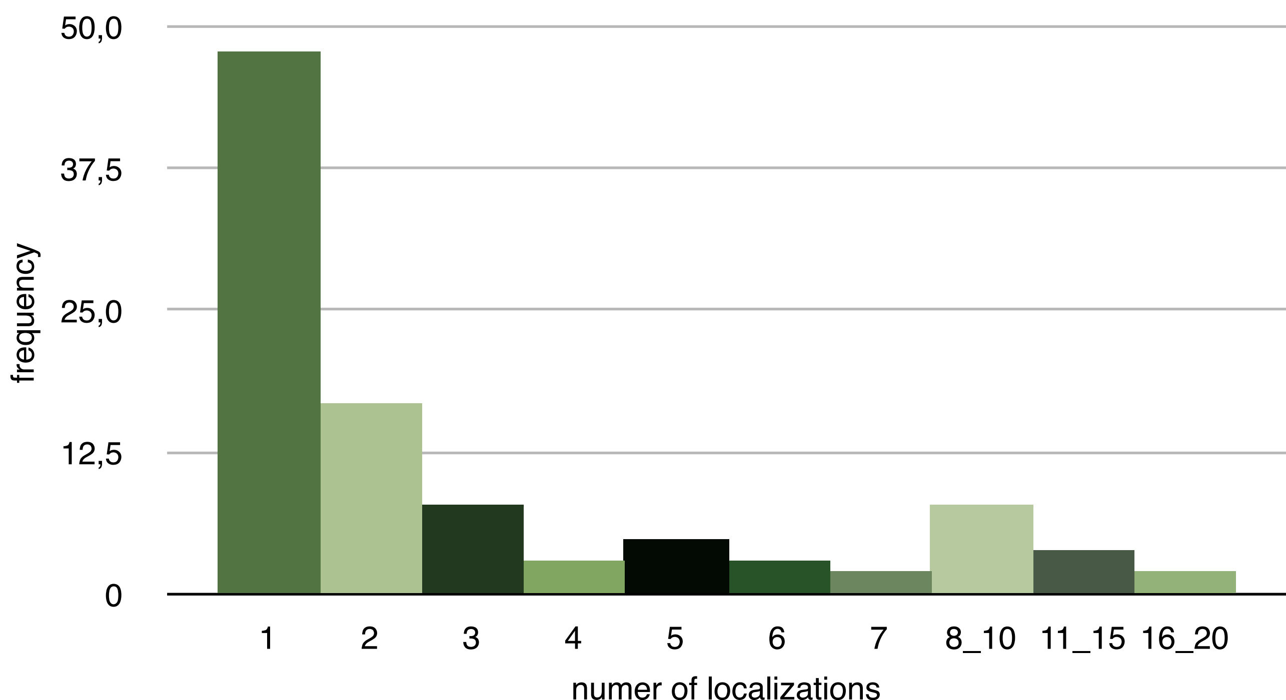

Fig. 2 - proportion of individuals sampled from once to 20 times during the study period.

Population genetic data

In the Emilia-Romagna wolf population PIDsibs over six loci used for individual identification was acceptable (6,691e-03; $P < 0,01$) for the present investigation.
All 12 loci were polymorphic, showing high values of heterozygosity ($H_{O\ mean} = 0,61$; $N_{A\ mean} = 5,83$; Table 4) and 7 private alleles (N_P) were found (see also Paper III), even thoug at low frequency (< 0,02). No significant deviation from HWE was found (Fis 0,019; $P > 0,05$). Moreover, a highly significant multilocus Fst= 0,015 ($P < 0,01$) was found between Emilia Romagna and Italian wolf population.

Differentiation between Emilia-Romagna wolf population and the other Italian wolf populations (Alp, Central and Northern Apennine wolf populations) was assessed also by Analysis of MOlecular VAriance (AMOVA), as indicated in Paper III. A significant average multilocus Fst = 0.09 ($P = 0,01$; computed from AMOVA) indicated that genetic diversity was significantly partitioned among the four wolf groups.

LOCUS STR	N_A	H_O	H_E
2004	6	0,61	0,61
2079	4	0,57	0,64
2088	5	0,63	0,65
2096	3	0,63	0,65
2132	11	0,65	0,7
2137	12	0,75	0,79
CPH2	6	0,6	0,56
CPH4	4	0,39	0,39
CPH5	4	0,62	0,6
CPH8	6	0,75	0,7
CPH12	3	0,45	0,44
U250	6	0,69	0,66

Table 4 - genetic diversity in Emilia-Romagna wolves genotyped at the 12 unlinked STR loci. H_O = observed heterozygosity, H_E = expected heterozygosity, N_A = number of alleles.

Mapping pack localizations

All single and temporal localizations were plotted on a regional map using GIS (Geographic Information System). Starting from individuals most resampled, it was possible to detect the territory where each individual was considerably stable in the time. Overlapping these individual areas and comparing the sampling periods of the individuals observed in them, it was possible to perform preliminary hypotheses of packs living in the study area. Integrating field observations (snow-tracking, wolf- howling) we identified 22-25 different possible wolf packs in all over the Apennine ridge sampled. (Fig. 3).

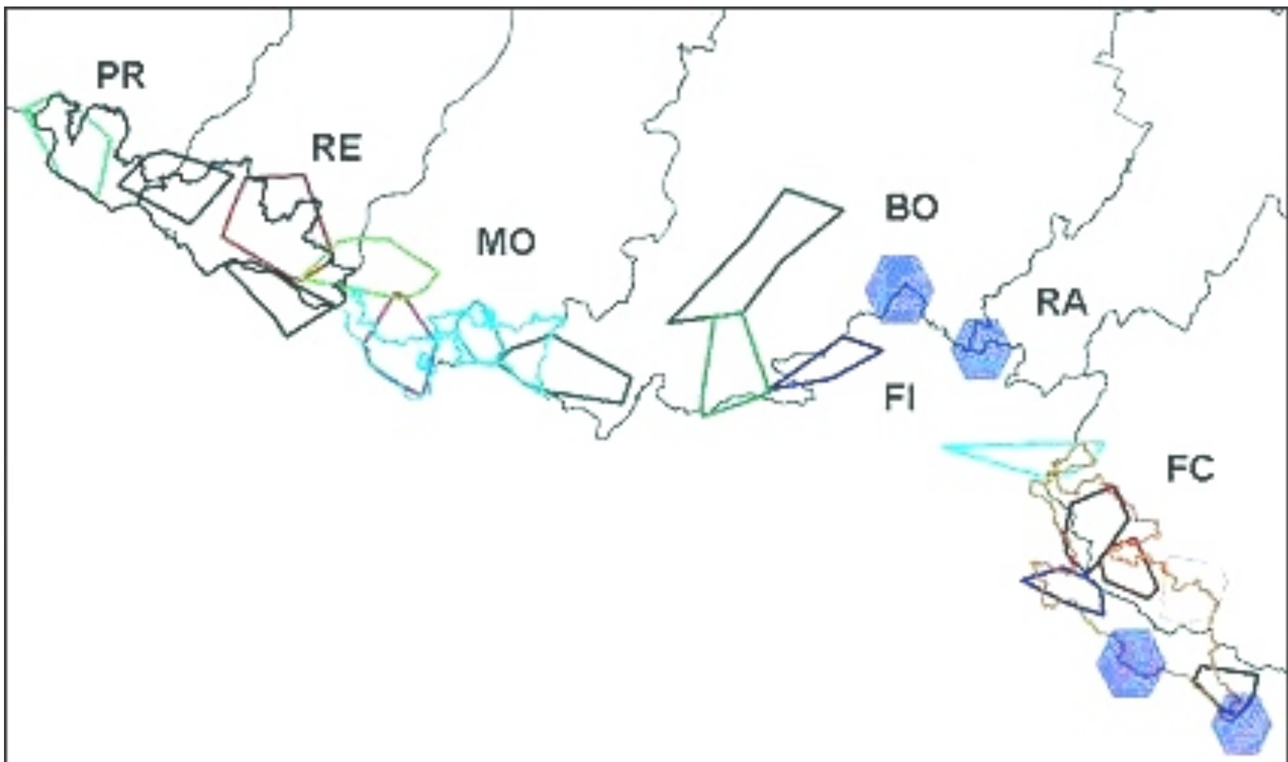

Fig. 3 - a possible scenario for wolf pack inhabiting the study area, obtained from genetic and field observations

Dispersion

We detected 7 cases of presumed dispersal events (Table 5). We recorded successive localizations on linear distances of more than 25 kilometres (corresponding to longer real distances on the territory). All of them are males.
For two individuals (WBO16M, WFO47M), it was possible recording other localizations in the presumed new territory areas. In addition, the male WPR3M was captured as part of ongoing radio-telemetry project, and its successive localizations registered: it migrated from Parma Province to France and back to Italy, where died for unknown causes.
Mean of linear dispersion distances was 72 kilometers and 5 out of 7 events showed a east-ovest pattern. Fig. shows two examples, with the two longest distances monitored.

ID GENOTYPE	SEX	SOURCE	DATE	DESTINATION	DATE	DISTANCE (km)
WBO10M	M	BO	23/feb/01	PR	02/feb/03	114
WBO16M	M	BO	06/giu/02	Gigante Park	18/mag/03	66
WFO15M	M	PNFC	11/mar/02	Cento Laghi Park	22/gen/04	142
WPR3M	M	Frignano Park	06/dic/03	Cento Laghi Park	15/mar/04	53
WRE4M	M	Gigante Park	30/dic/02	Frignano Park	01/gen/03	27
WFO47M	M	PNFC	17/feb/04	BO	31/gen/05	25
WRE6M	M	Gigante Park	17/gen/02	BO	23/feb/03	76

Table 5 - successive localizations on linear distances of more than 25 kilometers.

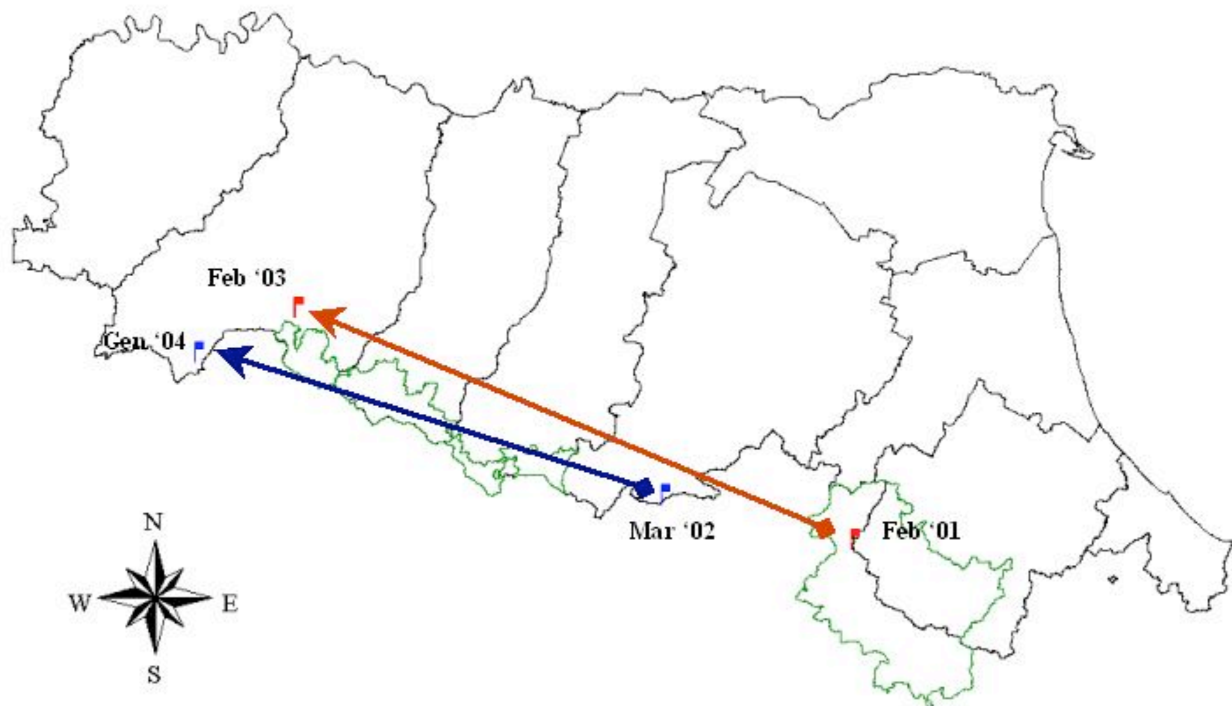

Fig. 4 - dispersal event of individual WBO10M and WFO15M.

Relationship analysis

Simulations were performed with the software CERVUS (Marshall *et al.* 1998) to compare the power of six loci (for individual discrimination) and twelve loci, in relationship analyses (Fig. 5). Relationship success depends also on sampling rate of candidate parents. We calculated with CERVUS the success rate in relation with 10, 20 and 30 candidate parents (Fig. 5).
We obtained the r (relationship index) distributions from KINSHIP (Goodnight % Queller 1999) for parent-offspring, and full-sibs and not related (Fig. 6). All simulations were performed with 10000 simulated pairs. To determine the probability that a dyad of one type of relationship would be missclassified as belonging to another type of relationship, we used the midpoint (r = 0,248) between the means of the two distributions as the cut-off value for classification.

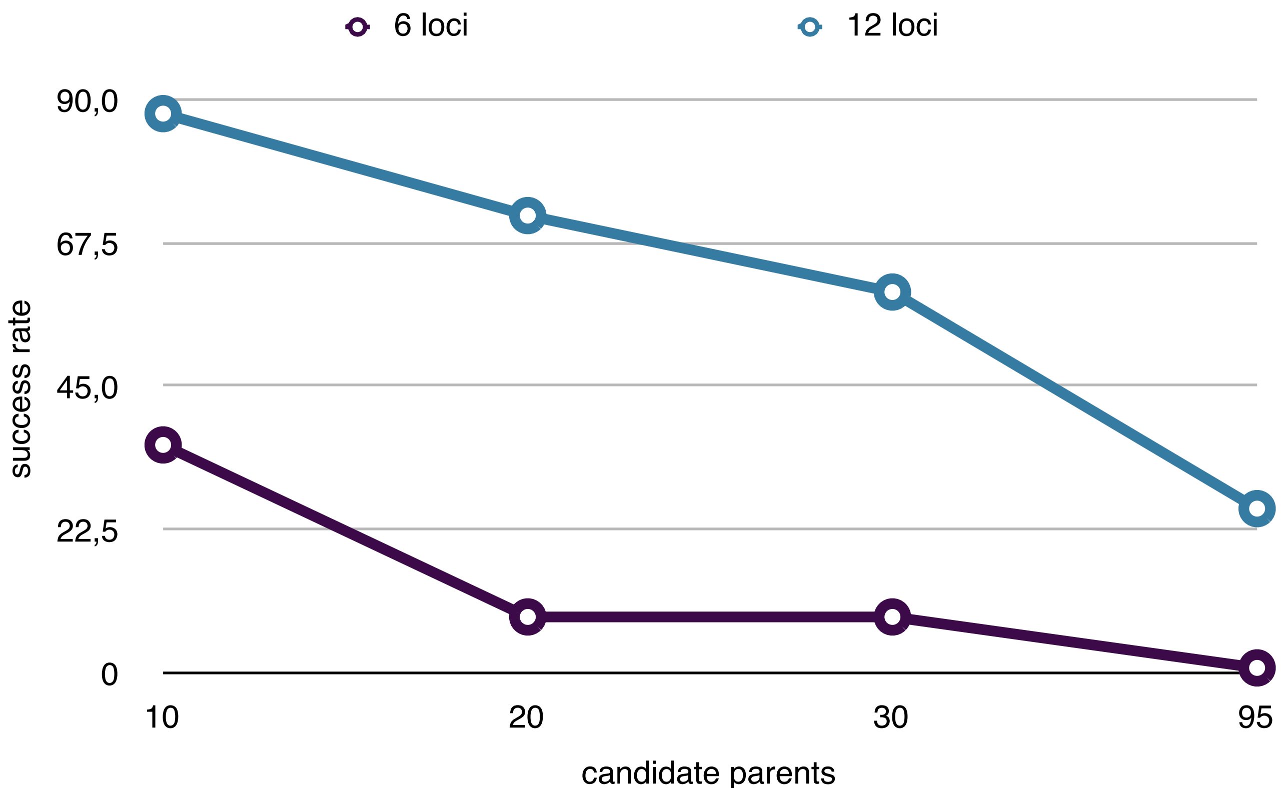

Fig. 5 - plotting of relationship success rate for 10000 simulated pairs, assuming 100% of candidate parents sampled

We didn't know which proportion of related parents where sampled for every presumed wolf pack taken into account: moreover, sampling effort varied greatly among localities (Fig. 7). These facts suggested a caution approach to evaluate every pair parents-offsprings. In addition, age of analyzed individual was not known and it's difficult therefore if not impossible to distinguish between parent-offspring relationships and those between full siblings, according to Flagstad *et al.* (2004). We accepted parent-offspring relationships only when both putative parents were sampled (Fig. 6).

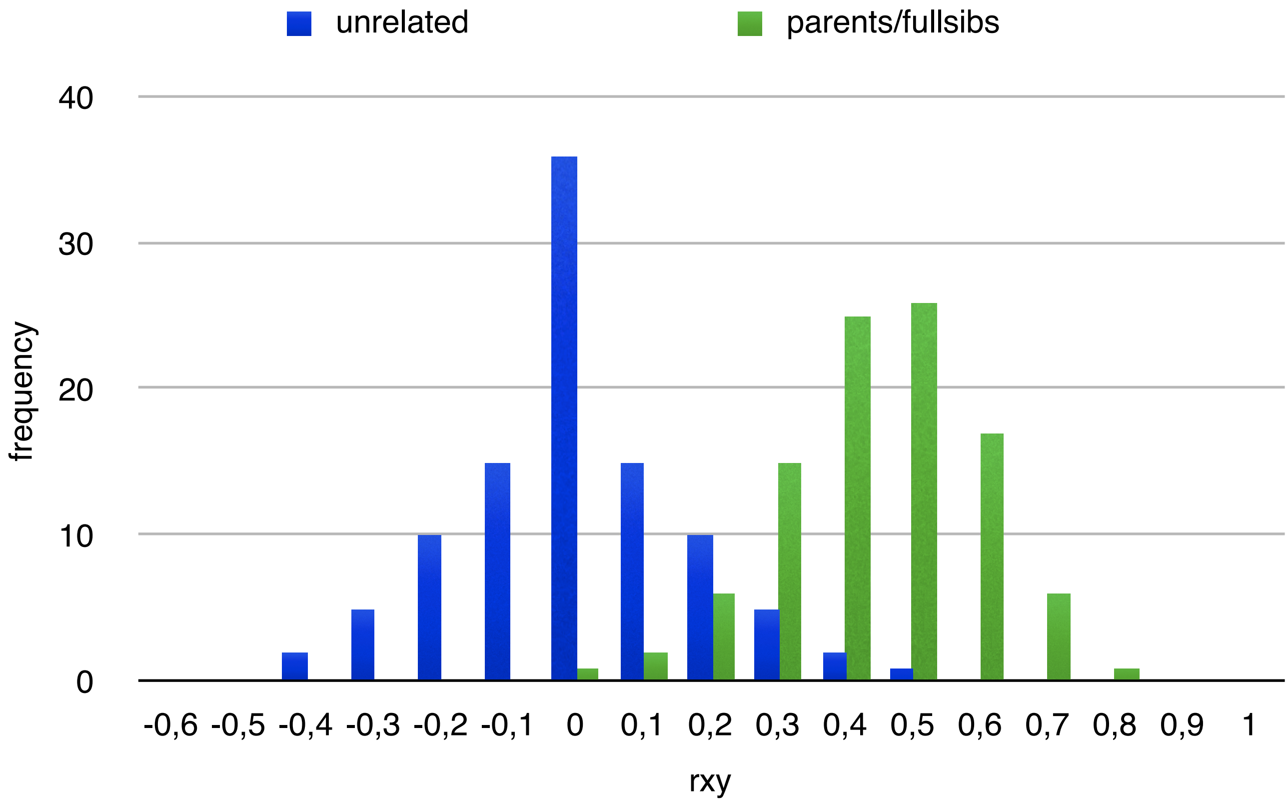

Fig. 6 - distribution of “r” (relationship index), after 10000 simulated pairs

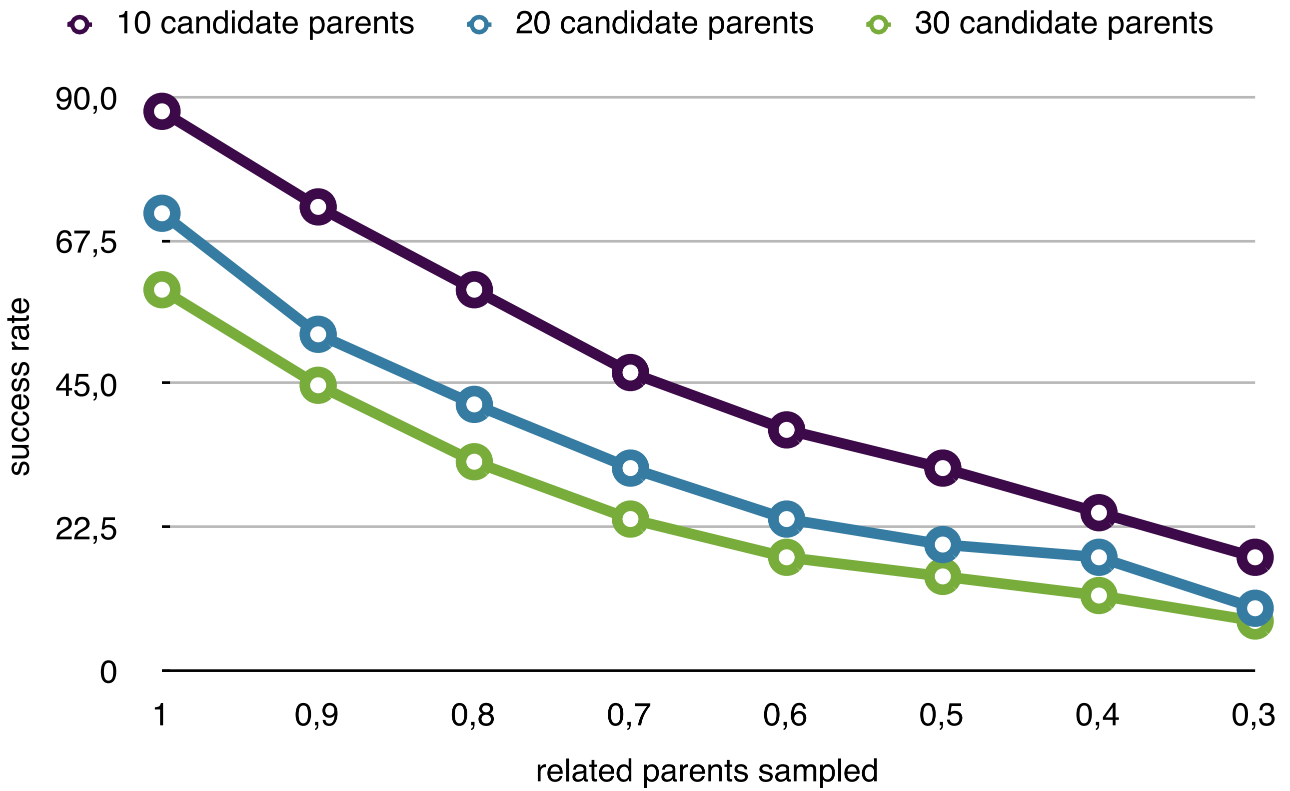

Fig. 7 - relationship success rate vs percent of related parents sampled

Population size estimation

Multilocus genotypes, obtained analysing non-invasively collected samples, can be used to estimate population size in several ways (Sloane *et al.* 2000). In this study we treated a successfully analyzed scat sample as one trapping event (Flagstad *et al.* 2004).
Given that several groups of field collectors were involved in sampling and that sampling effort varied among localities, heterogeneus capture probabilities were expected.
Resampling rate (Fig. 2) showed a pattern which just 51% of individuals was trapped just once.
Table 6 shows the number of samples analyzed and unique genotypes detected during the different years of the study period: it seems correct refer to those data as minimum population data estimations (Taberlet *et al.* 1996; Kohn *et al.* 1999; Flagstad *et al.* 2004;).

	faeces analysed	individuals identified
before march 2002	193	39
april 2002-march 2003	525	88
april 2003-march 2004	554	79
april 2004-march 2005	393	64
TOTAL	1665	193

Table 6 - number of samples analyzed and genotypes detected during the different years of the study period

Mark-resight methods of estimating population size can be applied to genetic data to estimate resighting probabilities if the individuals are sufficiently sampled (Otis *et al.* 1978; Seber 1982). An mark-recapture method for open populations was implemented during this study (Caniglia, PhD Thesis), with a total mean value of 123,95 individuals (95% CI 80,83-174,01)

DISCUSSION

The non-invasive methods, already tested on wolf in Northern Italy in quite small areas (Lucchini *et al.* 2002), has substantially confirmed its efficacy in the present large-scale investigation for approaching elusive animals such as wolf. Despite the vastity of areas sampled and the great logistic effort to standardize the sampling procedure (see also Paper I), the “field-laboratory” cooperation was successful. This work showed the presence of wolf along the whole Apennines ridge, also with packs adapted to live, when human disturbance is low and preys abundant, quite close to cities. In most cases, supposed adiacent packs showed to have not-overlapping territories. 22-25 different probable wolf packs were genetically identified and a substantial according to field informations was found.
Sampling effort resulted very different among the areas, even though all administrations (provinces/parks) actively cooperated: this could probably explained as recapture rates were so variable among the different detected individuals. On the other hand, wolf marking behaviour affects the patterns of defecation on trails (Peters & Mech, 1975; Vilà *et al.* 1994; Kohn *et al.* 1999), were likely samples from dominant individuals that frequently mark the territory. Genotypes sampled only once or twice might be juveniles or individual that disperses to look for new territories where they can found new packs of their own. Just 24% of individuals were sampled for more than one year, suggesting that just a small part of population can be considered stable and presumably contribute to the effective population size; therefore, turn over seemed particularly high. Among stantial individuals sex ratio was 1:1, according to other studies (Mech 1970 and 1975; Lucchini *et al.* 2002).

A large scale genetic census in a population of known size, using microsatellite genotypes obtained from faeces (Creel *et al.* 2003: Kohn *et al.* 1999) was not feasibly; small size census such as a pilot study at the Pistoia Zoological Garden (see Paper I) and a pack of known size (Monte Sole; data not shown) allowed to validate the standard genetic protocol used.

The STR set of loci selected for individual/species discrimination performed well, even in some cases we had to genotype more loci to solve some uncertain assignments. Increasing statsitical power genotyping all unique genotyped at 12 STR loci was useful both for population and relationship analysis.

Three wolf-dog hybrids of presumed second generation were found; they represented only 1,5% of wolf identified in all over the whole study period, indicating clearly as wolf-dog hybridization can occasionally occur, but that it doesn’t represent a real risk of introgression for wolf population, according to Randi *et al.* (2000) and Randi & Lucchini (2002) and Verardi *et al.* (2006). This evidence is also confirmed by the fact that wolf in Italy is still largely outnumbered by feral or free-ranging dogs (Genovesi & Duprè 2000). Scat samples can be confused with those from dogs and foxes; dog genotypes weren’t sampled more than twice, indicating that no ranging dogs were constantly present in the study area.

A total of 7 male-based dispersal events were monitored. For three individuals new territory colonizing were confirmed by successive field/lab evidences. 5 out of 7 events showed a east-ovest migration. All these results confirm patterns of colonization observed in Paper III, that wolf migration were unidirectional from the Apennines to the Alps and were male-biased. In fact, Emilia Romagna region acts as a natural narrow ecological

corridor along the ridge of the north-western Apennines linking the central-northern Apennine Mountains with the western Alps.

A review of molecular scatology studies shows that on average 31% of faecal samples did not yield scnDNA or mtDNA, even after repeaed extractions (Frantzen *et al.* 1998); 33% of samples were discarded after screening.
Total genotyping success was 40%, significantly lower than 84% in Lucchini *et al.* (2002), obtained previously in the same laboratory: similar estimates were from studies of Tabelet *et al.* (1996). Allelic dropout (ADO) was found at all loci of all independent replicates of inferred heterozygotes; mean was 21,1%, higher than values reported in previous studies: 5% (Kohn *et al.* 1999), 11,1% (Morin *et al.* 2001), 9,8% (Flagstad *et al.* 2004), but substantially comparable with 18% in Lucchini *et al.* (2002). Misprinting rate (FA rates) was 3,4%, comparably to 5,6% (Morin *et al.* 2001), but higher than <0,5% calculated by Flagstad *et al.* (1999). According to other authors (Frantzen *et al.* 1998; Taberlet *et al.* 1999; Morin *et al.* 2001) PCR success and ADO were significantly related with allele size.

Emilia Romagna wolf population showed high level of polymorphism at loci investigated (H_O = 0,61; 3 < N_A < 12) and resulted to be HWE TableTable A total of 7 alleles never found in the rest of the Italian wolf population (private alleles); this is probably due to different sampling.

Non-invasive genetics approach is known to be cost- and time-consuming. As reported by Taberlet *et al.* (1996), the total cost of the laboratory work can be five to ten times higher than when using blood or tissue samples. Therefore, planning a broad non-invasive genetic project with many people involved in collecting samples needs to be standardized as much as possible, aiming to implement a reliable laboratory procedures.
Nowadays, prescreening of extracts for DNA quantity is recommended for sorting of samples for likely success and reliability (Taberlet *et al.* 1996: Morin *et al.* 2001) and certanly reduced logistic effort and genotyping errors (Creel *et al.* 2003; Morin *et al.* 2001). Repeating PCR seems the unique way to take into account genotyping errors and hence avoiding errors on estimates of population parameters.
A possible improving for non-invasive investigation could be planned preliminary study aiming to assess the relationship between DNA quantity of the species under study by real-time PCR and genotyping errors (see Morin *et al.* 2001 and 2007); repetition of results could be remain extensive for analysis from low starting amounts of DNA, but is reduced for those with higher DNA content (Morin *et al.* 2001).
On the other hand, markers such as SNPs , even though they are only biallelici markers, can be quite easily characterized amplifying short sequences and extending single nucleotides (Morin *et al.* 2001; Abecasis *et al.* 2002). SNPs genotyping should increase PCR success and reduce the allelic dropout and false allele rates. These advantages 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 usually allows to develop several of them in a single locus of a few hundred base pairs, so that SNPs could represent a more reliable and faster genotyping method. In our study (see Paper II) 18 not linked SNPs, selected from the most variable founded, have the same discrimination power of 10 microsatellite loci (Lucchini *et al.* 2002).

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