The use of microsatellites in the study of social structure in large mammals: Italian wolf and fallow deer as case studies

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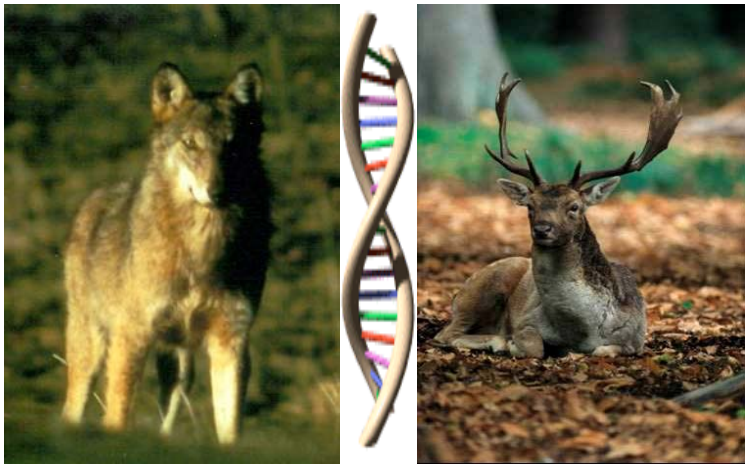

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- Summary -

The present thesis deals with the application of microsatellite analysis to the study of two species of large mammals, referring to some aspects of their social and mating systems. The Italian wolf (*Canis lupus*) and the fallow deer (*Dama dama*) were chosen as case studies, since genetic investigations on their populations result, for different reasons, problematic.

The wolf in Italy is a particularly protected species, recovering throughout the peninsula from the effects of a recent bottleneck. Sampling wolves may not rely on capturing or killing them, therefore an alternative, non-invasive, approach was adopted in my study. Scats, shed hairs and blood drops collected on the snow represented the main source of DNA for the analysis. Methodological cares were necessary to obtain reliable wolf genotypes. A set of ten canine microsatellites was employed to achieve unique multilocus genotypes in the population. Fifty two individuals were typed in the period 1998-2003. In some cases, pack composition was determined, confirming that familiar bonds are at the basis of wolf social units. An unpredictable high local differentiation was found among geographic areas. Early dispersal seem to be common in the population, but its effects on the gene flow are not relevant, at least at the study scale. I proposed that most of this dispersal may be unsuccessful or over long distances. The study population, indeed, seem to have reached a high level of local saturation, with clumped pack territories and high reproductive rates, and thus possibly represents a source, from which wolves disperse toward sink areas.

The second study involves an enclosed population of fallow deer. Lekking is seldom observed in mammals, and among them, it is more common in ungulate species. Fallow deer is one of the most studied lekking ungulate and the study population has been object of long-term researches on male mating strategies. Mature bucks in the population join into leks during the breeding season: a costly strategy, which apparently does not guarantee high direct benefits (fitness). In this study, for the first time, I used a molecular approach to verify the existence of a genetic basis to lek formation. The recourse to microsatellites enabled to overcome the strong limitation due to the extremely monomorphism of the species, documented in several studies. Though the low variability even at microsatellite loci, the hypotheses of kin selection (territorial bucks in a lek are relatives) and of heterozygosity advantage (territorial bucks in a lek have a high overall heterozygosity) were tested and resulted not supported by data. Hence, future studies should be addressed towards phenotypic variation and consider in detail physiological and ecological factors, in order to clarify the reasons why lekking takes place in fallow deer.

- Acknowledgements -

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In the fallow deer study, genetic data were compared to observational data collected at the two leks, during several breeding seasons. With regard to this effort, I express my profound gratitude to Simone Ciuti, Siriano Luccarini, Irene Di Vittorio, Sara Luchetti, Sara Davini, Giuseppe Caleo, Paolo Bongi and many students and volunteers contributing to observation sessions at leks. Concerning sample collection, to the mentioned people I should add my acknowledgements to the warders of the San Rossore estate for their contribution in catching and manipulating animals.

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- Thesis layout -

This thesis is aimed to verify the application of microsatellite analysis to the knowledge of social and mating systems. The manuscript consists of a first chapter describing the nature of microsatellites as molecular markers, their structure and evolution. Advantages and drawbacks in the employment of these DNA regions are also described, with a particular stress to the statistical viewpoint. A further subject concerns possible applications in the study of the biology of wild mammals.

The second chapter takes into account the first study case. A set of polymorphic microsatellite loci was used to investigate the structure and the social behaviour of a wolf population. After a review of the known aspects of wolf biology, attention is paid to the local features of the study population, making collecting samples from free ranging individuals difficult. Thus, the adopted non-invasive sampling design is described and results are discussed. Relationships among packs are considered, pointing out the local genetic differentiation over a small geographic scale, due to constraints to gene flow.

A second study case is described in chapter 3. The studied species is in this case fallow deer. *Dama dama* is one of the most variable ungulate species, respect to mating behaviour. The evolution of lekking behaviour in fallow deer is of special interest, because of the possibility the males have to choose, as alternative, every other known mating strategy. The use of highly polymorphic molecular markers, in a genetically impoverished species, enables to collect information to confirm or reject the hypotheses of kin selection and heterozygote preference, verified in other species. Evidences from microsatellites are presented and results discussed.

In chapter 4, some conclusions are reported, focusing on the main results of the two studies and highlighting the boost genetic investigations may give to the knowledge of inter-individual relationships within wild populations of large mammals.

- Chapter 1 -

Introduction

1.1 Molecular markers: an overview

From the beginning of the 'DNA era', the attention of many scientists was addressed to the discovery of molecular tools to employ in several fields going from human health to farming improvement, from genome mapping to the conservation of biological diversity. Since then, different generations of molecular markers were discovered and used for a high number of aims.

The intrinsic requirement making each of them useful for several purposes is a sufficient level of variation. In other words, for a given molecular marker is necessary to show among taxa, populations or individuals, at least some detectable difference. The origin of diversity is mostly due to mutation, occurring at different rate in different genomes, or within them in different DNA regions.

The first generation of molecular markers considered variation in the amino acidic composition of functional proteins. The source of this variation is represented by mutations in the sequence of the respective coding genes. The effect is, instead, a structural change, modifying the electrophoretic mobility of the protein. This kind of molecular markers are know as *allozymes*. Their main limit is represented by the fact that mutations at coding DNA regions are under selective pressure, because they may influence directly the biological functions of the organism. Hence, allozyme variation in nature can only represent the 'surviving' component of the overall variation arising from mutational events. On the other hand, the use of such markers is simple and cost-effective, small amounts of tissue are required, alleles exhibit simple Mendelian inheritance and are usually expressed as codominant. These positive attributes make allozymes still currently used to detect diversity at the population and phylogeographic level.

In the 1980s the application of *mitochondrial DNA* (mtDNA) analyses caused a revolution, especially for evolutionary biology. Systematic problems and identification of taxa were faced basing on direct or indirect estimate of nucleotide variation. A single molecule of DNA is present in every mitochondrion and thus the number of copies per cell is often in the order of 10^3. Restriction fragment analysis and direct sequencing of this DNA molecule contained in the mitochondrion are the most used approach to investigate mtDNA variation. RFLP (restriction fragment length polymorphism) analysis is a method to indirectly collect information on mutations occurring at specific short regions (restriction sites). In this case, the molecule is not continuously checked for variation, but only a subset of its nucleotide composition is taken into account. The most informative method to analyze mtDNA is direct sequencing. After the introduction of the polymerase chain reaction (PCR), sequencing protocols included the specific amplification by PCR

of the region, going to be sequenced. This improvement avoids the laborious cloning of the target fragments.

Cellular organelles in mammals are maternally inherited and thus also mtDNA is transmitted by the female lineage. This feature makes the phylogenetic transmission of molecular traits more linear in mtDNA respect to nuclear DNA. This is the main reason why evolutionary biologists recur preferentially to mtDNA analysis. The same feature, representing a large advantage for phylogenetic purposes, represents a limitation to mtDNA variation, as it limits the type of mutational processes involving the mitochondrial genome. The absence of trans-molecular recombination at mtDNA, indeed, limits to deletions, insertions and single nucleotide mutations the possible source of variation. Again coding regions (like the cytochrome B gene) are less interested by sequence variation, as they are under selection. On the contrary, non-coding regions accumulate mutations and most of the diversity is concentrated there (e.g. control region).

In the following years, new systems able to detect more genetic variation were developed. VNTR (variable number tandem repeat) are highly polymorphic regions of the nuclear genomes consisting of multiple tandemly repeated copies of a DNA sequence. They include both minisatellites and microsatellites, differing only in the length of the repeat unit and in the number of repetitions. *Minisatellites* are typically formed by a long sequence (10-100 bp) repeated in tandem up to hundreds folds (Jeffreys et al. 1985). This difference in the overall size of the two VNTR classes entails them in different assay methods.

Minisatellites are detected using the traditional methods of endonuclease digestion, agarose gel electrophoresis, Southern blotting and the hybridization of DNA fragments with a specific marked probe. Using core sequence as probe, several loci in the genome are detected simultaneously. Many alleles are thus identified in the range 1000-20000 bp. As consequence of the high degree of polymorphism found at these loci, the derived 'bar-code' pattern is usually individually distinct and therefore is called '*multilocus DNA fingerprint*'. The inheritance of minisatellite alleles follows Mendelian laws. For this reason minisatellites became the markers of choice for many biologists engaged in pedigree or parentage analysis. The main drawback for minisatellite analysis is to be a labor-intensive procedure, requiring large amounts of good quality genomic DNA. Even the high mutation rate found at VNTRs represents a limit when looking for applications in population genetics, as the effect of mutation may not be ignored in quantifying population divergence, unlike it is commonly done for the estimate of many statistics (Burke et al. 1996). The second point is the total lack of information on the fragments one goes to detect, for which multilocus fingerprinting was defined in the past a sort of 'black box' (Lewin 1989).

After the development of PCR, other DNA-fingerprinting methods, based on the amplification of target sequences, were introduced. The basic feature of such molecular makers is the lack of knowledge about the nature of the amplified DNA sequences. The first method is known under the name of *RAPD* (random amplified polymorphic DNA). It employs a single primer (a 10-mer) for a random amplification under specific PCR conditions. The number of amplified fragments depends on the distribution and number of annealing sites throughout the genome. In fact, amplification takes place only when primers anneal on each strand at sites not more distant than 3-4 kb. PCR random products are then detected easily on an agarose gel and the resulting banding pattern represents the DNA-fingerprint (Williams et al. 1990). In comparison with other genetic markers, RAPD provide a more arbitrary sample of the genome and can detect an unlimited number of loci, simply changing the base combination in the used oligomer. The most limiting property of RAPD markers is probably the dominant expression of alleles, making difficult the interpretation of multilocus patterns. Even problems of amplification reproducibility were raised in the past. Moreover, like minisatellites, the ignorance about the resulting fragments and the possibility of linkage among them reduce the potential applications of the method. Nevertheless, RAPDs have been widely employed for studies on taxon identification, hybridization, reproductive behaviour and population genetic structure (Fritsch & Rieseberg 1996). Most studies have been carried out on plants, less on animals.

A variant to RAPD is represented by arbitrarily primed PCR (AP-PCR). It is based on the amplification with a larger primer (15-25 bases) and differs in cycling design. A first phase of few cycles at low stringency is followed by more stringent cycles, generating a pattern of 10-20 bands (Welsh & McClelland 1990).

A different procedure combines enzymatic digestion with PCR. Amplified fragment length polymorphisms (AFLPs) are the result of a digestion with restriction endonucleases, a ligation of specifically designed oligonucleotide adaptors to the ends of each fragment, and a PCR amplification using primers complementary to the adaptor sequence, to which an extended sequence of few bases is added, in order to reduce the number of amplified fragments (Vos et al. 1995). Banding patterns obtained in this way revealed useful for a series of applications like population genetics, systematics and kinship analysis. Their usually difficult interpretation and the problematic use of proper statistics represent, however, strong limitations.

Other less common molecular markers are represented by anonymous single-copy nuclear DNA (ascnDNA, Karl & Avise 1993), DNA amplification fingerprinting (DAF – Caetano-Anolles et al. 1991) and PCR-based single-stranded conformation polymorphism (PCR-SSCP – Orita et al. 1989).

A book edited by Smith and Wayne reviewed the use of several molecular markers in the field of conservation genetics (Smith & Wayne 1996).

In principle, two of the most important features of a molecular marker are their level of variability and the 'detectability' of their polymorphism. VNTRs are considered the most variable regions of eukaryotic genomes so far known. However, the analysis of minisatellites is labor-intensive, time-consuming and costly. The smaller size of microsatellite regions enable them to be easily detected by PCR amplification and electrophoretic sizing. This may explain the current large use of microsatellite analysis for a wide variety of studies. Moreover, differing from minisatellites, microsatellites are usually mapped regions of the genome, identified by a repetitive sequence (e.g. CA) and defined by the flanking sequences, over which a specific primer pair is designed. A high number of loci are available for every species to researchers, thus having the possibility to compensate for the possible lack of overall variability with the number of polymorphic loci analyzed.

1.2 Microsatellites: molecular structure, function, and the origin of polymorphism

Microsatellites or simple sequence repeats (SSRs), or even short tandem repeats (STRs), have been discovered in every genome so far analysed, being abundant in eukaryotes and less frequent in prokaryotes (Tautz & Renz 1984, Tautz et al. 1986, Tautzt 1989). They are represented by a 1-6 bp motif iterated in tandem a number of times. The number of repetitions seldom overcomes 70 units. Microsatellites are grouped in families, formed by regions having in common the same repeat motif (e.g. CA). It was estimated that the most common motif (GT/AC) occurs on average every 30 kb in mammal genomes (Schlötterer 1998). The frequency of microsatellites is higher than expected purely on the basis of nucleotide composition (Tautz & Renz 1984).

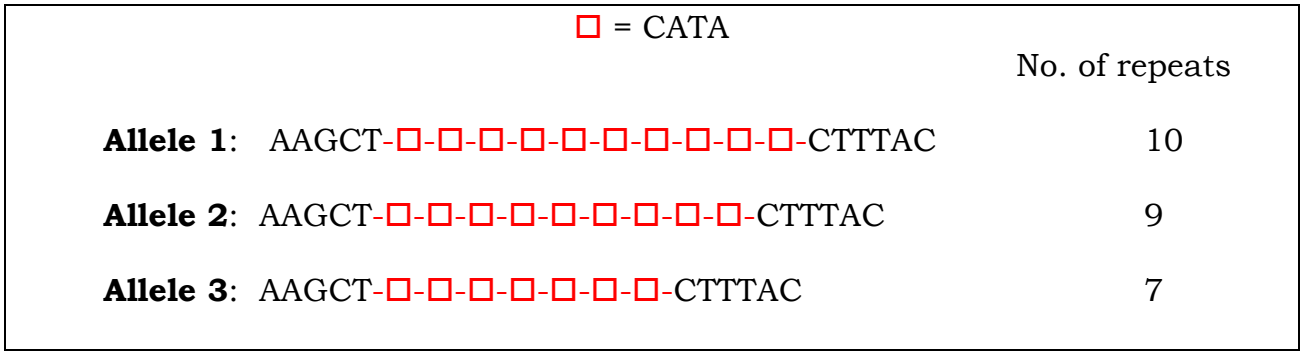

FIGURE 1 – Structure of a microsatellite. Each box represents one repetition of the simple unit (CATA in the example).

These regions are among the most variable in the nuclear DNA molecules. Their polymorphism derives from changes in repeat numbers, caused by mutation mechanisms called 'DNA slippage'. The simplest mutation involves a change of a single repeat unit, producing a difference in the overall length of the region (e.g. 2 bp in dinucleotide microsatellites)

Main features of microsatellites

- interspersed throughout the genome
- usually not expressed
- grouped in families (e.g. the CA family)
- small size (usually < 300 bp)
- biparentally inherited
- codominant markers
- high mutation rate (10^{-3}-10^{-6})

But mutations may be generated by different mechanisms, according to a series of theories about the evolution of microsatellites, described in the next paragraph.

Basing on their composition, microsatellites are 'perfect' or 'compound'. In the former case, the region is represented only by repetitions of the simple repeat motif (for instance $(CA)_{14}$).

'Compound' microsatellites have instead combination of simple units (e.g. $(TA)_5(TG)_{14}$) or non-repetitive sequence interspersed among tandem repeats (e.g. $(GA)_4TCC(GA)_{10}$). This latter type is also named 'interrupted microsatellite'.

The majority of microsatellites are dinucleotides. Only in primates the most frequent class is represented by mononucleotides (Toth et al. 2000). Less abundant are tri- and tetranucleotide SSRs. Microsatellites represent a large fraction of noncoding DNA and are relatively rare in protein coding regions. GT repeats are more frequent in euchromatin rather than heterochromatin, and their location is well conserved in mammal species (Stallings et al. 1991). Dinucleotides were found in the 3' and 5' untranslated ends of nuclear genes, and in introns (Li et al. 2002). This may be due to selection against frameshift mutations in coding regions (Metzgar et al. 2000). The selective pressure seems to be stronger in longer than in shorter microsatellites. Thus variability at tetranucleotide SSRs is expected to be limited, respect to dinucleotides. Schlötterer and Tautz (1993) showed that both repeat length and base composition seem to affect the mutation rate.

Microsatellites are largely used under the assumption of selective neutrality, indeed it is usually thought that they represent neutral molecular markers. However, in many cases their biological function has been proved. Li and others (2002) reviewed the possible functions associated to SSRs. They mentioned: i) chromatin organization, ii) DNA structure (forming secondary structure like loops), iii) centromere formation, iv) hotspots for recombination, v) control of DNA replication and cell cycle, vi) modulation of mutation rate, vii) control of transcription and gene expression,viii) binding of regulatory protein and ix) inhibition of translation. In cancer research, the instability at some SSRs was associated to genetic disorders at the origin of cancer.

Even if neutral, microsatellites may be linked to selected loci, thus suffering of their status.

The effects of selection could confound results, by overcoming the other forces influencing allele frequencies (i.e migration, mutation, and genetic drift). On this way, the genetic structure of a population may be wrongly estimated.

Therefore, this topic should be more taken into consideration in the future, and many of the currently accepted results may be invalidated by further studies, demonstrating a somehow selective pressure on particular patterns.

Most of the microsatellites employed in the analysis of wildlife populations are unknown about their location and their selective status (neutral or under selection). This represents a weak point of such studies.

1.3 Mutation models and the theory of microsatellite evolution

Besides their abundance and putative function, microsatellites represent DNA regions with a high mutation rate, as compared to what found for coding genes. It was estimated in 10^{-2}-10^{-6} mutations per locus per generation. Mutations are basically manifested as changes in the number of repeats. Two main causes were attributed to this source of variation: the first is DNA slippage during DNA replication; the second involves recombination between DNA strands. Various factors may affect the rate of mutation at SSR loci, including allele size, chromosome location and GC content at the flanking regions.

Slippage occurs during DNA replication. Changes in repeat number, due to slip-strand mispairing errors, are frequent during the process duplicating the DNA double strand (Schlötterer & Tautz 1993). Most of this errors are corrected by the proofreading and exonucleolytic activity of the involved enzymes and by the mismatch DNA repair; but in some cases these control systems could fail and these errors become mutations. It is usually assumed that replication slippage is the main source of mutation in SSRs.

Ellegren (2000) suggested that microsatellite length tends to be balanced by biased mutation processes, from one side, and point mutations violating the continuity of repetitive DNA, from the other.

Recombination is the other possible cause of mutation. It may change SSR length by unequal crossing over or by gene conversion. This could happen both during meiosis or mithosis. Depending on the motif, unequal exchange may generate unidirectional (either contraction or expansion) or bidirectional changes (both contraction and expansion).

In heteroduplex DNA formations (e.g. in the Halliday structure in recombinating homologous chromosomes), slippage and recombination may interact, affecting SSR stability.

Mutation rates may vary respect to repeat types, base composition of repeats, microsatellite type (perfect, compound or interrupted), length of the region, chromosome position and nature of the flanking sequences. Differences are also found among taxonomic groups (Balloux & Lugon-Moulin 2002).

Several theories were developed to explain microsatellite evolution. Most estimators of genetic distance and population substructure are dependent on the type of mutation model adopted. The first simple model is named ***Infinite Allele Model*** (IAM) and consider every new mutation giving rise to a new allele. This model fit well with allozymes and Nei (1984) demonstrated it was satisfactory in explaining the observed allozyme variation.

The IAM represents a particular case of a more general model (KAM, K-Allele Model), stating that each mutation may produce one of several (K) alleles at random. Thus the IAM is just the extreme case of K equal to infinite

The ***Stepwise Mutation Model*** (SMM) was created to fit the features of microsatellite loci. In the model, alleles can only mutate by gain or loss of a single repeat unit. Therefore, the passage from an allele to the other presupposes passing through every possible intermediate status. The adoption of SSM as model of microsatellite evolution implies that the difference in size between two alleles is thus informative: the larger the difference, the higher the number of mutation events (thus time) expected to have occurred since common ancestry. In other words, present alleles have a sort of 'memory' of past mutations.

Like KAM for IAM, even SSM is included in a more general model (GSM, Generalized Stepwise Model), considering mutations modifying allele size by every possible number of units. SSM would thus represent the extreme case of mutations involving only one unit.

Since most mutations involve the gain or loss of repeat units, homoplasy (i.e. the situation in which two alleles are identical although being originated by different mutation events) is expected to arise in each of the mentioned model.

Simulation studies were carried out to investigate the mutational processes occurring at microsatellite loci. Both IAM and SMM are mutation-drift equilibrium models, assuming neutrality of microsatellites (i.e. no selective constraints on their evolution). Valdes et al. (1993) analyzed allele frequency at 108 loci from human families, founding that their distributions were consistent with a stepwise model in populations with a constant size. Recurring to computer simulations, Shriver et al. (1993) studied the behaviour of three classes of VNTRs, referring to three parameters: number of alleles, allele size range, and number of modes in allele distributions. They found that all microsatellites with a 3-5 bp repeat motif, most 1-2 bp microsatellites and a minority of minisatellites (15-70 bp repeats) matched values obtained by SMM-based simulations. Most minisatellites and a portion of 1-2 bp microsatellites behaved more closely to expectations according to the IAM. The authors concluded that different classes of VNTRs may undergo different mutational processes.

Di Rienzo et al. (1994), studying the well known human population from Sardinia, investigated the variation observed at dinucleotide microsatellites. They found that, although a stepwise mutation model could explain much of the data, a mechanism including a second phase with larger mutational changes enabled expectations to fit data. The model they developed, known as ***Two-Phase Model*** (TPM), is based on coalescence theory and consider each mutation having a probability p to be a single-step mutation and a probability $1-p$ to be a multi-step mutation. Di

Rienzo et al. (1994) also suggested on the basis of their observations that microsatellites do not evolve at the same rate. Their data are consistent with multi-step mutations being caused by unequal crossing-over. An association was noticed between variance in repeat number and mutation rate. As the former increases, the probability of a crossing over event producing an unequal exchange should increase. In this case the mean allele size is expected to remain constant. A linear relationship was found between variance in repeat number and number of alleles (Valdes et al. 1993).

It is now clear that the evolution of VNTRs is a complex mutational process involving different mechanisms. Both repeat motif and the overall size of the microsatellite loci seem to influence their evolution (Webster et al. 2002). Microsatellites with a 3-5 bp repeat unit seem to evolve via the SMM, while mono- and dinucleotides seem to follow the TPM. The prevalence of single-step mutations decreases with the increase of the complexity of microsatellite repeat core.

It was suggested that microsatellites evolving more according to IAM should be more suitable to study population subdivision and genetic relationships (Estoup et al. 1995), because they are expected to show the lowest level of homoplasy.

Limitations to the direction and total number of alleles seem to exist, as consequence of functional constraints. This is supported by evidences showing that some diseases are due to genetic disorders involving alterations in the number of repeat units at microsatellites. For example, chromosomal instabilities at the FMR-1 gene in humans, generating oversize alleles, are responsible for the fragile X syndrome (Fu et al. 1991). Even Bowcock et al. (1994) observed a size limitation on the number of repeats in human microsatellites. The mechanism for limiting the number of repeats is, however, unknown.

An important discovery for the application of microsatellite analysis was that of the existence of the so called '***null alleles***' (Callen et al. 1993). They arise from mutations at regions flanking the microsatellite, coinciding with primer sites. The result is that heterozygous individuals are mystyped as homozygotes. If null alleles are frequent in a population, the overall heterozygosity will be underestimated and this might explain the heterozygote deficiency observed in several populations. The detection of this possible source of error will be further discussed forward in the text.

1.4 From theory to facts: possible applications

Once one decides to employ microsatellites for its study, the first step is to find them. In absence of previous knowledge, the general approach involves cloning random segments of DNA into a plasmid or phage vector, introducing this latter into *Escherichia coli*, plating out colonies, and then screening them with a synthetic labelled oligonucleotide, containing the repeat motif one is looking for. Such probe will hybridize to clones (positives) containing the corresponding microsatellite repeat, which will be sequenced and the regions flanking uninterrupted microsatellites will be used to design reliable primers for the specific amplification of the microsatellite region. This long procedure may be bypassed if microsatellite sequences are already available for the species under investigation. In this case primers can be easily designed starting from the known sequences, using one of the common software developed at this purpose (e.g. OLIGO). In some cases, authors may have made available the sequences of primers used for microsatellites they developed. If none of these information is available, one may rely on species strictly related to that under study. In fact, flanking regions at microsatellites are often well conserved within taxonomic groups.
At present, a number of microsatellite loci have been detected in a vast amount of taxa and primer sequences are collected in world-wide accessible molecular databases. Thus everyone has the possibility to select and provide himself with microsatellite primers, without any laboratory effort.

Since the advent of polymerase chain reaction (PCR) in the 1980s, the use of microsatellites has become extremely widespread in biology. The improvement of the techniques of analysis and the increasing number of discovered loci have produced an exponential increase in the number of microsatellite-based studies.
Microsatellites revealed useful for a number of applications. They have been used as molecular markers for genome mapping, in particular being extensively utilised for linkage analysis, even in the study of genetic disorders associated to human diseases.
In genetic linkage maps of eucaryotic genomes, microsatellite are far the most widely used molecular markers, by virtue of their abundance and widespread distribution along DNA molecules. Map data based on their use are available for a number of species, like for example the dog (Mellersh et al. 1997, Neff et al. 1999).
Moreover, microsatellites are the tool of choice in a lot of fields including population genetics. The main advantages offered by SSRs are the abundance of loci (1 every 10-20 Kb for trimeric and tetrameric repeats, Edwards et al. 1991), the high degree of polymorphism, their presumed or demonstrated selectively neutrality (compatible with most assumptions used in population

estimates), the overall size of most loci, which enable to amplify alleles via PCR. This latter feature allow the use of microsatellites to be extended to slight and degraded DNA samples, otherwise impossible to get analyzed.

For all the above mentioned reasons, microsatellites have proved useful in kinship analysis and in paternity tests (Queller et al. 1993). Due to their high variability, an important use of such molecular markers is become individual identification (genetic typing). Both these applications made nowadays microsatellites commonly used in forensic investigations and in livestock breeding (e.g. Biondo et al. 2001, Williams et al. 1997).

Several researches have been addressed to the determination of phylogeographical patterns, considering, for example, the effect of genetic drift (e.g. Hedrick et al. 2001, Clegg et al. 2002) or of migration as vehicle of gene flow (e.g. Estoup et al. 1996, Rassmann et al. 1997, Waits et al. 2000, Van Hooft et al. 2000, Eizirik et al. 2001).

The high polymorphism at microsatellite loci allows to investigate the population substructure, with a power not previously reached by alternative molecular markers. A number of studies deals with the temporal and spatial distribution of DNA diversity within natural populations (e.g. Paetkau and Strobeck 1994, Girman et al. 2001, Coltman et al. 2003), verifying, for instance, the effects of a demographic decline (e.g. Taylor et al. 1994, Maudet et al. 2002). Behavioural traits have also been investigated recurring to such genetic approach. The knowledge of social structure (e.g. Amos et al. 1993, Morin et al. 1994a) and mating behaviour (e.g. Coltman et al. 1999, Apollonio et al. 2000, Garnier et al. 2001) has particularly benefited from the use of SSRs. The possibility to estimate on a genetic basis the actual reproductive success in polygamous species was particularly important. The attribution is reached by sampling adult individuals (both actual or potential parents) and a number of offspring in a population. The method proceeds by the exclusion of adult DNA profiles not sharing any allele with the offspring, at least at one locus (e.g. Morin et al. 1994b, Coltman et al. 1998, Moore & Ball 2002).

A crucial advantage offered by microsatellites is the fact that primers developed for a particular species are often applicable across a wide range of related taxa. This is particularly important in conservation genetics, as rare species may be investigated by the use of cross-specific microsatellites. It is the case of baleen whales (Schlötterer et al. 1991) or Ethiopian wolf (Gottelli et al. 1994). On this subject, nuclear markers are employed to study hybridization cases: microsatellite loci revealed a powerful tool in resolving suspected cases of cross-breeding within the same species or between related taxa (e.g. Roy et al. 1994, Gottelli et al. 1994, Beaumont et al. 2001).

1.5 Microsatellites to investigate the structure of natural populations

Several studies focused on the genetic sub-structuring within populations, addressing specific questions in evolutionary and conservation biology. Population structuring can be put in relation with social structure and mating behaviour of the considered species, or with the existence of physical barriers to gene flow.

Population geneticists have developed different tools to establish the degree of subdivision. The start point is the analysis of DNA variation using polymorphic molecular markers. Then a statistical support is necessary to evaluate which significance may be associated to observed data.

The choice of both molecular markers and statistical parameters is crucial for the efficacy of the study. A strong relationship then joins the two variables, as not all statistics fit the model assumed for every given marker.

The first generation of population structure estimators dates back to 1951, and goes under the name of 'Wright's F-statistics'. This fundamental contribution was developed by Wright (1951, 1965) at a time in which only protein variation was in use. F-statistics take into account the correlation of alleles within individuals and describe their non-random association among individuals within a subpopulation (F_{IS}), among subpopulations (F_{ST}) and within the whole population (F_{IT}). The effect of inbreeding, caused by non-random mating within a subpopulation, is expressed by F_{IS} and is consequence of mating between relatives. The result of inbreeding is a high proportion of homozygous individuals in the subpopulation (lower heterozygosity than expected), due to the fact that individuals have many coinciding alleles inherited by a common ancestor (identical by descent), which they transmit to their offspring. A similar effect, known as Wahlund's effect, can be observed measuring variation in a natural population. It consists of an observed deficit of heterozygosity, due to pooling together diverging subpopulations (Hartl & Clark 1989). More divergent the subpopulations (stronger the effect of genetic drift and mutation), lower is the observed heterozygosity. The parameter F_{ST} measures the difference between observed and expected allele frequencies, and therefore is to be considered also as an index of genetic distance. F_{IT} (i.e. the overall correlation of alleles in the population) results from the sum of the other two estimators.

As shown by Nei (1977), Wright's F-statistics is related to gene diversity, expressed as the heterozygosity expected under Hardy-Weinberg equilibrium (Nei 1973), and he proposed the coefficient of gene differentiation (G_{ST}) as estimator of population subdivision.

Weir and Cockerham (1984) introduced other estimators of F-statistics taking into consideration the three components of variance in allele frequencies: between gametes within individuals, between

individuals within subpopulations, and between subpopulations. These estimators were named *F* (corresponding to F_{IT}), θ (F_{ST}) and *f* (F_{IS}) and the authors proposed a weighting procedure when extending the computation over several loci, suggesting to estimate sample variances by the jackknife procedure.

The above reported theoretical methods of measuring population structure are based on selectively neutral markers evolving under the IAM, not considering homoplasy. The introduction of microsatellites forced towards a revision of such methods.

In fact, a basic point in the use of microsatellites concerns the allele status. Alleles at one locus could be compared on two ways: identity/nonidentity and size difference. Two alleles having the same size may derive from a single common mutation event (identical by descent) or different mutation events (identical in state). One has no mean to discriminate between these two situations, and this may lead to erroneous estimates based on allele comparisons.

IAM-based estimators treat alleles of equal size as identical by descent and do not distinguish between alleles differing of a single repeat unit and those differing of several repeats.

Slatkin (1995) described an approach for measuring population subdivision based on the generalized stepwise model of mutation at microsatellite loci. This method takes into account allele size, assuming that size differences are expression of the number of mutation events and thus of the coalescence time (i.e. the time in the past at which alleles diverged). The parameter R_{ST} is calculated from the square of differences in allele size within and between subpopulations.

Hence, Slatkin's R_{ST} is the equivalent of Wright's F_{ST} and of Weir and Cockerham's θ, but more appropriate for microsatellites, as it incorporates the mutational history of the alleles under the SMM (or GSM). At microsatellites, the occurrence of homoplasy leads to a predictable underestimation of F_{ST} respect to R_{ST}. On the other hand, departures from a strict SMM at microsatellite loci will lead R_{ST} and F_{ST} to converge. Balloux and Goudet (2002) noticed that while R_{ST} better reflects true population differentiation in presence of low gene flow, F_{ST} gives better estimates in case of a high gene exchange among subpopulations. A detailed review of these statistics and their applications to microsatellite data is given by Balloux and Lugon-Moulin (2002).

An analogue of the R_{ST} statistics is the analysis of molecular variance (AMOVA) proposed by Michalakis and Excoffier (1996) and considering the number of mutations between molecular haplotypes. It is a hierarchical analysis partitioning the total variance into covariance components due to intra-individual, inter-individual and inter-population differences.

According to the above mentioned procedures for estimating population structure, the amount of gene flow between subpopulations can be indirectly estimated by expressions derived from F_{ST} or F_{ST}-analogous. The migration rate *Nm* (*N* is the population size and *m* is the proportion of migrants

in the population) is calculated as a function of F_{ST}, θ or R_{ST}, depending on the assumed mutation model. Under the island model of migration, F_{ST} decreases as a function of $N(m+\mu)$, μ being the mutation rate. Thus, only if μ is negligible, the relationship between F_{ST} and Nm becomes direct. However, this is not the case of microsatellite loci, where mutations are frequent. Further, the effect of homoplasy is responsible of differences between F_{ST}-based and R_{ST}-based estimations of Nm, with the former being predictably larger than the latter. Rousset (1996) observed that under SMM the relationship between F_{ST} and the number of migrants + mutants no longer holds. Under this model, the R_{ST}-based estimate should perform better, being independent from mutation rate, but its high sampling variance makes F_{ST} often preferable (Gaggiotti et al. 1999).

An alternative method to estimate gene flow is based on private alleles, i.e. alleles exclusively found at one or few subpopulations (Slatkin 1985). In subdivided populations, at demographic equilibrium, both F_{ST} and private-alleles methods provide accurate estimates of Nm, as demonstrated by Slatkin (1989). Private alleles are more dependent on mutation rate than on the mutation model, although under SSM mutation events are less likely to produce novel alleles than under TPM or IAM.

Similarly, even measures of genetic distance were adapted to the case of microsatellite markers. The traditional estimates were based on the infinite allele model. Cavalli-Sforza's chord distance and Nei's standard distance are two of the most widely adopted computations. A further simple procedure was introduced by Bowcock et al. (1994), based on the proportion of shared alleles between individuals or populations. But even this parameter suffers of the effect of allele homoplasy, leading to an underestimation of the actual distance. When divergence is largely dependent on genetic drift, also F_{ST} can provide a good measure of distance (Reynolds et al. 1983).

SSM-based methods of estimating genetic distance were developed by Slatkin (1995) and Goldstein et al. (1995). They proposed the average sum of squares of the difference in allele size as expression of the number of mutational events (thus time) separating divergent alleles. Populations having a large average difference among alleles, according to their model, should have spent much time isolated. Simulations demonstrated that IAM-based estimates of genetic distance are superior when populations have been separated for a short period of time (<300 generations), whereas with longer diverging times the average square distance performs better (Goldstein et al. 1995).

Thus the distance value of choice to construct phylogenetic trees changes in relation to the history of the species, to the type of molecular markers and to mutation rate.

The diversity at microsatellite loci represents a limit in studies involving the calculation of genetic distance values. The high mutation rate at these regions produces so many differences between taxa, that an asymptote is rapidly reached, as much of the possible alleles (repeat numbers) will be

already present. So, higher the divergence between taxa, slighter will be the difference in genetic distance. This constraint makes microsatellites more suitable for comparisons among populations belonging to the same species or among closely related species, where the degree of divergence is expected to be low.

In studying natural populations, it is often the case that evolutionary patterns are explained in terms of genetic relatedness. For instance, the adoption of a particular mating strategy by some individuals is hypothesized as leading an advantage for their relatives, thus increasing their inclusive fitness (kin selection). In other contexts, particular social interactions may be attributed to the genetic relationship between members of the social unit. Furthermore, in threatened species, the level of inbreeding may be under control, and specific management actions can be developed, once known the actual relationships among individuals inhabiting an area.

In all these cases, the researcher needs to evaluate the relatedness between specific individuals or its average level within a social unit (subpopulation, flock, family, etc.). The analysis of genetic markers, like microsatellites, offers this opportunity, but appropriate parameters have to be used. Different relatedness coefficients have been defined, based on the allelic identity by descent. In general, its meaning coincides with that of the coancestry coefficient, i.e. the probability that two alleles, one from each individual, randomly drawn at an autosomal locus are identical by descent. In diploid species, $r_{xy} = 2\Theta_{xy}$, where Θ is the coancestry coefficient and x and y are the compared individuals. Thus relatedness is expressed as 'probability of gene-identity'.

Wang (2002) pointed out that marker-based methods to infer genetic relationship between individuals can be divided into two groups: the first uses a likelihood approach to assign a pair of individuals to a particular type of relationship (such as full-sibs or parent-offspring); the second group uses moment estimators to estimate the true pairwise relatedness between individuals (a continuous probability value). The former methods are particularly useful when one is going to test a work hypothesis in a well-known study population (Blouin et al. 1996, Marshall et al. 1998, Goodnight & Queller 1999). Relatedness estimators are, on the contrary, especially indicated for populations with complex or unknown pedigrees and little information on population structure. They are generally used under the assumption of random mating in the population and neutrality of the selected loci. Moreover, their usefulness is affected by the number of loci, their level of polymorphism and by the mating system adopted (Van de Casteele et al. 2001). Estimators were proposed by Li et al. (1993), Queller and Goodnight (1989), Ritland (1996), Lynch and Ritland (1999) and Wang (2002). The first is a similarity index based on the sharing of alleles between individuals. The second is a regression-based estimator, whereas the others are method-of-moments estimators. Lynch and Ritland (1999), Van de Casteele et al. (2001) and Wang (2002) compared the

performance of some of these estimators under different experimental conditions. Van de Casteele et al. (2001) highlighted the importance of the single-locus weighting procedure in producing multilocus estimates: different weighted averages of the same estimator may produce very different results. Most estimators suffer a large bias due to sampling variance, which can be reduced by weighting loci or increasing the number of studied loci. As remarked by Van de Casteele et al. (2001) and by Wang (2002), in general, the relative performance of these estimators varies in relation to polymorphism and number of loci, allele frequency distribution and population composition (i.e. proportion of relationship categories in the population). Finally, there is no single best-performing estimator, but each of them may behave better under specific conditions.

- Chapter 2 -

Case study 1: Population and social structure in Italian wolves (*Canis lupus*)

PHOTO: E. CENTOFANTI

2.1 Aim of the study

Rationale: the wolf population in Italy recovered after having approached extinction. The status of the species has improved, and several areas now harbour high densities of wolves. Field data, collected on wild populations, have depicted a situation in which relatively small packs occupy close territories, where prey are abundant. Their reproductive success is high and thus also recruitment in a pack is expected to be large. Nevertheless pack size is almost constant, suggesting an early abandonment of the familiar group by young, which would represent the potential for new colonization events. This situation appears to be quite distant from what reported for the best known North-American wolves, which form larger packs ranging over wider distances. This difference leads to expect a different genetic structure of the Italian wolf population, with packs simply composed by a breeding pair with a few offspring and an overall high level of inbreeding.
Traditional methods failed so far to show the actual composition of a wolf pack and the relationships among different packs inhabiting the same area. The use of microsatellite analysis, combined with non-invasive sampling, is considered a promising tool to obtain these information.

Main aim of the study: to evaluate the social system of Italian wolves, verifying whether a pack represents a simple familiar unit (parents + offspring), monitoring pack composition over time and investigating the amount and destiny of individual dispersal.

Secondary aims: to investigate the genetic structure of the population and the relationships among different packs; to estimate the amount of gene flow among different nuclei within the population; to evaluate the effectiveness in characterizing individuals by genetic analysis and in discriminating between wolf and other canids.

2.2 The biology of the species: an overview

Natural history and actual status

The wolf (*Canis lupus* Linnaeus, 1758) is a fascinating species, stimulating human imagination and inducing strong emotions in humans. Its recent history throughout its distribution range is strongly affected by the interactions with people. The fact of representing for humans a direct competitor, threatening flocks and feeding on game animals, was the main source of troubles for the wolf.

The historical range of the wolf covered the whole northern hemisphere. But in the last centuries, as consequence of human persecution, the species disappeared in a large part of its range.

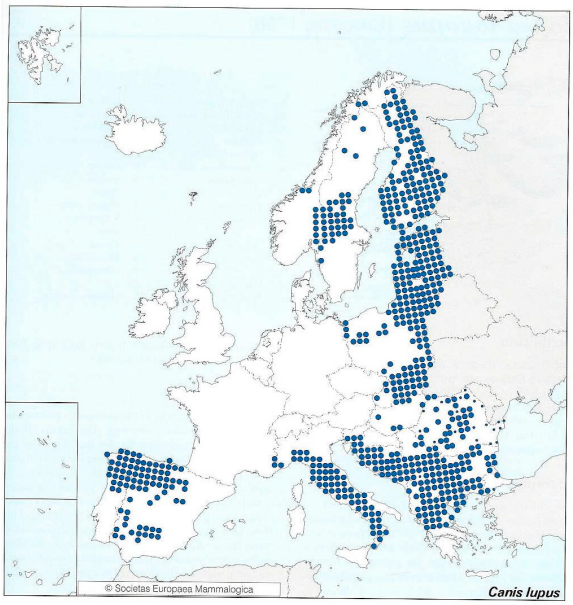

FIGURE 2. Wolf presence in Europe (from Mitchell-Jones et al. 1999)

The conservation of wolf natural populations represents a priority in several European countries, where the species is endangered or was, in the recent past, severely threatened (Promberger and Schröder 1993). The Italian wolf population suffered a strong persecution till 1971, when wolf hunting was suspended and poison baits banned. This change in attitude was completed in 1976 when the species obtained the fully protected status. However, during the period 1950-1970 the number of wolves throughout the peninsula was very low (100 according to Zimen and Boitani, 1975) and their presence was extremely restricted to a few areas of the Apennine mountains. From then on, the wolf population recovered in Italy; its consistence increased and the population range enlarged, leading at the beginning of the 1990s to the recolonization of the Western Alps and to the reappearance of the species in France, where it had been absent for at least one century (Breitenmoser 1998).

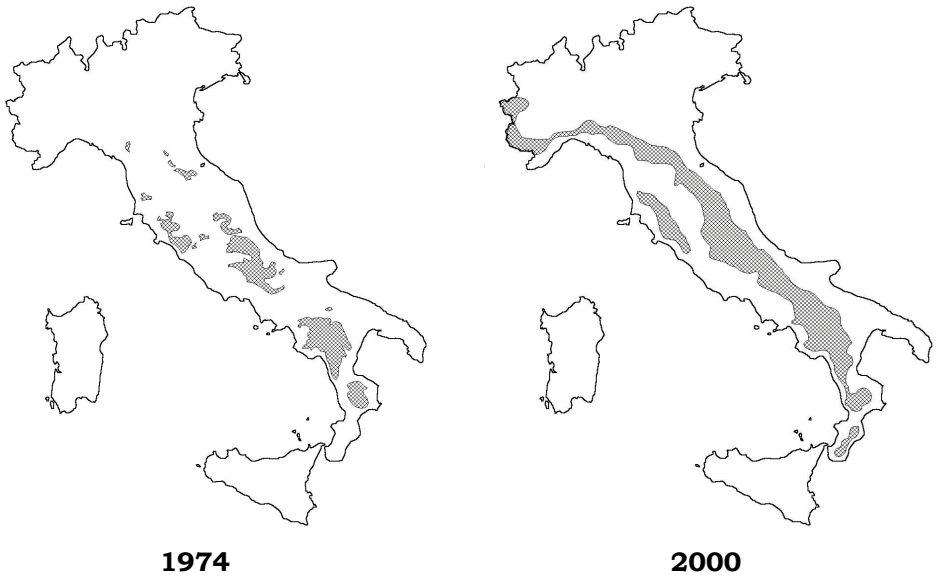

FIGURE 3. Past (from Cagnolaro et al. 1974) and present wolf range in Italy.

As consequence of its history, the Italian wolf shows the effect of a prolonged isolation. Just a single mitochondrial haplotype is shared by all wolves so far analyzed (Wayne et al. 1992, Vilà et al. 1997, Vila et al. 1999, Randi et al. 2000). On the contrary, nuclear markers did not reveal a severe reduction in genetic diversity, testifying that the bottleneck was not so pronounced in terms of effective population size and duration (i.e. number of generations) to produce remarkable effects on heterozygosity (Scandura et al. 2001a).

A factor of risk in declining wolf populations is the possibility of crossbreeding with domestic dogs. When potential partners are scarce, indeed, wild wolves may occasionally accept as partner a feral or stray dog. In case of successful mating, if hybrid pups are raised like wolves and reach the reproductive status, the introgression of canine genes into the wolf population may occur. Such genetic pollution represents a threaten to bottlenecked wolf populations and was recently detected in Eastern Europe (Andersone et al. 2002). In Italy, crossbreeding between wolf and dog was documented by direct observations in the past (Boitani 1982) and more recently by genetic analysis (Randi & Lucchini 2002). Nevertheless the number of proved hybrids is very low, inducing to consider infrequent the actual occurrence of crossbreeding between wolvws and dogs.

Taxonomy

The wolf is a wild canid, considered the ancestor of the dog (Wayne 1993, Vilà et al. 1997).

Taxonomic designation	
Kingdom:	*Animalia*
Phylum:	*Chordata*
Class:	*Mammalia*
Order:	*Carnivora*
Family:	*Canidae*
Genus:	*Canis*
Species:	*Canis lupus* L., 1758

By virtue of its divergence from the other wolf populations, expressed by its smaller body size and peculiar genetic features, the Italian wolf was proposed to do not belong to *Canis lupus lupus*, commonly found in the rest of Europe, but to represent a separate subspecies (Altobello 1921).

After a long controversy, the classification of the Italian wolf as *Canis lupus italicus* was recently validated by Nowak and Federoff (2002). This systematic status gives to the peninsular population a great importance from the conservation viewpoint.

Morphology

Wolf size follows a latitudinal gradient, with a maximum body mass in northern regions (60-80 kg in Alaska, Canada and Siberia) and a minimum in desert areas (20-30 kg). Italian wolves have a medium size, rarely exceeding 40 kg. A sexual dimorphism exists relatively to body mass. Males are usually bigger than same-aged females. Body length approximates 110-150 cm, about 30-35 cm of which represented by the tail. The height may reach 70 cm. On the whole, the body of a wolf is well adapted to trotting, more than running. Like many members of the dog family, it walks on its toes and not on the entire foot. Each toe has a soft pad and a non-retracting claw. This kind of foot presents the same advantages of the hoof of many agile ungulates, enabling wolves to move rapidly on several different substrates (e.g. rock, snow, etc.). Being a meat-eater, the wolf has a head adapted to catching and eating prey. The skull is large and long, with robust jaws, and the dentition (42 specialized teeth) includes four large canines, useful to hold the prey. The colour of wolf pelage is very variable throughout its range in North America and Eurasia. Mech (1981) described coat colour ranging ‘from white through cream-coloured, buff, tawny, reddish, and grey to black’. Nevertheless the common pattern is considered a grey pelage (from which the name ‘grey wolf’ commonly used for the species). The occurrence of black-coated wolves in Italy is not a rarity, and many black wolves were also observed in my study area (Fig. 4). Some author considered this event

as a sign of hybridization with dogs. But no genetic evidence support this hypothesis (Apollonio et al. *in press*).

FIGURE 4. A black wolf in the study area (photo: G. Tortelli).

Behaviour

The wolf is a social mammal, hunting in group. The basic unit of the wolf society is the pack, defined by Mech (1981) as "a group of individuals travelling, hunting, feeding, and resting together in a loose association, with bonds of attachment among all animals". This definition for itself do not consider kin nor hierarchical relationships among pack members. Nevertheless the common belief is that a pack generally corresponds to a familiar unit, represented by a mating pair and its offspring, which remain in the pack for two or more years and help parents to rear future offspring. Pack size is typically comprised between 2 and 8, but it changes a lot throughout the wolf range. At high latitudes wolves may form large packs (up to 30 individuals), whereas in southern regions packs rarely associate more than 8 wolves. Pack size is the result of the trade-off between dispersal and mortality, from one side, and recruitment and fecundity, from the other, and seems to be influenced by size, availability and distribution of prey (Apollonio et al. 2004).

A marked hierarchy is established within wolf packs. Two separate (male and female) dominance order are present. Both are linear, with a few equivalents (Mech 1981). The two individuals at the top of their own sex hierarchy, i.e. the dominant ones, represent the so called 'alpha pair' (Mech 1999). The social status is often established very early in a wolf's life (e.g. play fighting by pups), but order of dominance may change during pack's life. Dominance is usually expressed by two ways: leadership and privilege (Mech 1981). Dominant individuals control and govern the behaviour of other pack members, directing movements and activities (hunting, travelling, resting, etc.) of the group. Privilege is mainly referred to the access to food. Dominant wolves are the first

members of a pack to feed on a kill, and, once they are sated, other wolves in turn may have access to it. Social interactions among pack members are often ritualized, and aggressive behaviours inhibited by the hierarchy. Within-sex aggressivity increases in winter before and during the heat, and a rearrangement of the rank order may result.

Pups usually join their natal pack until the second year of life. During that time, young wolves make practise of hunting, territory defence, and attending at newborns, all precious knowledge for the formation of an own family. After this period, an individual may choose between remaining with the natal pack or leave it and disperse, in search of a mate and of an area where to establish its own territory. Several ecological factors, like abundance of prey, wolf density, availability of free territories and human disturbance, certainly involved in this decisional step.

Monogamy is the rule in wolves (Harrington et al. 1982) and, although exceptions were reported (Van Ballenberghe 1983a), only the alpha pair do normally mate in a pack. Sexual maturity is reached as wolves are approximately twenty-two months old. The breeding season occurs in winter, from late January through April, depending on the latitude (January-February in Italy, Ciucci & Boitani 1998). Oestrus lasts 5-7 days and occurs once per year. The gestation period is reported to be 63 days long. As early as 3 weeks before parturition, the pregnant female digs the den up. Den may also be represented by an existing hole (natural or dug by other animals) arranged in a way to comfortably accommodate a litter. Usually between 4 and 8 pups are born. A high early mortality is common in wild wolves, and litter size in late summer often averages only 3 pups (Mech 1977, Jedrzejewska et al. 1996). Adults, if disturbed, may move pups from one den to another during the same season. Pups spend approximately the first two to three months at the den. Afterwards they are moved to temporary rendezvous sites, where they are attended by pack members, which contribute to their feeding. If undisturbed, wolf packs may use the same den and rendezvous sites for several years (Mech 1981, Apollonio et al. 2004). Alloparental care is typical of highly social mammals. Their adaptive significance in wolves was treated by Harrington et al. (1983).

Wolves are territorial. At the stage of pair formation (i.e. as a pair bond is established), mates settle a new territory. As the pack enlarges, the area it is able to defend increases. Territory defence is active, and includes preventive behaviours like inspecting, scent-marking and howling, and aggressive behaviours like growling, chasing and biting. Territory size is highly variable: from 80-km^2 to over 2000-km^2 territories are found in North America, corresponding to different latitudes and ecological conditions. In Italy, basing on very few studies, territory size was estimated in 75-300 km^2 (Ciucci & Boitani 1998). Depending on prey abundance and behaviour, wolves may cease to keep a territory and follow prey herds. This migratory behaviour is observed in the large North American plains, where wolf packs prey upon caribou (Ballard et al. 1997). Movements within a

territory change in relation to the season, climatic conditions and prey distribution. In summer, pack movements reduce, as wolves come periodically back to the den or to the rendezvous site, to feed the alpha female and her pups. In winter, pack behaviour is more nomadic and tends to follow prey distribution. Territories are not clumped together, but usually are spaced by areas (the so-called 'buffer zones', Mech 1994) occupied by solitary or peripheral wolves. Dispersal is an important factor in wolf population structure. Genetic studies enabled to estimate a dispersal frequency as high as 25% (Lehman et al. 1992, Forbes & Boyd 1997). The species is capable of dispersing over long distances (as high as 800 km), but dispersal distances usually average 8-354 km (Gese & Mech 1991). This information lacks for Italian wolves, but the recent colonization process of the western Alps supports the idea of wolves dispersing over long distances (Breitenmoser 1998, Valière et al. 2003).

Natural wolf preys are large herbivores. Nevertheless, many studies throughout the world have demonstrated the high feeding plasticity of the species, which enables them to survive to long periods of prey unavailability. Alternative food sources may be represented by small mammals, fruits and garbage. Wild ungulates are commonly preferred by wolves and, where a complex ungulate community is present, one species usually more selected than all others (Okarma 1995, Meriggi & Lovari 1996). Food habits were extensively studied in Italian wolves: a high variability was observed, with cases of a diet mostly based on human activity (garbage or livestock) and cases in which it relies almost exclusively on wild ungulates (Boitani 1982, Mattioli et al. 1995, Meriggi et al. 1996). In these latter situations, the wolf population structure and dynamics is largely influenced by the wolf-prey interaction.

Genetic variation

The first extensive studies on the genetic variability of wild *Canis lupus* population was conducted by Kennedy and Kennedy (1991), using protein polymorphisms. In Canadian wolves they detected an intermediate level of heterozygosity, compared to natural populations of other members of the Carnivora order. Similar levels of variability were obtained on 38 Italian wolves in a parallel study carried out in Italy using 40 allozymic loci (Randi et al. 1993). Variation at the mitochondrial DNA sequence was largely investigated throughout the wolf geographic range in the world. Wayne et al. (1992) analysed specimens belonging to 26 different populations of the northern hemisphere. Eighteen haplotypes were found, of which 7 deriving from hybridization with coyotes in North America. In this study, all 14 samples from the Italian population showed the same restriction pattern (i.e. haplotype), not shared by any other wolf population. A further study, concerning 22 wolf samples, detected no difference among patterns generated at mtDNA by 20 restriction

enzymes (Randi et al. 1995). Finally, two exhaustive studies used sequence comparisons at mtDNA control region to detect diversity among wolf populations. The first of them (Vilà et al. 1999) interested 30 localities worldwide (n = 259 samples) and showed a limited partitioning of haplotypes on continental scale. Of the 33 detected haplotypes, one was shown exclusively by the Italian wolf population (including specimens from France), like obtained for two other cases only (Israeli and Mexican wolf). The monomorphy of the Italian wolf was confirmed by the second study (Randi et al. 2000), analysing 101 wolves without detecting differences at any of the 546 screened nucleotides.

The new frontier of the analysis of genetic diversity has become the use of nuclear hypervariable loci, like microsatellites. Studies based on such markers modified the vision of an Italian wolf population largely affected by almost one century of isolation and numerical decline. To the absolute mtDNA monomorphy corresponded a moderate variation at microsatellite loci (Scandura et al. 2001a, Randi & Lucchini 2002). Scandura et al. (2001a), screening 5 loci previously used in other studies, found a slightly lower value of gene diversity in a sample of 38 Italian wolves respect to North American wolf population (Roy et al. 1994, Forbes & Boyd 1997). Moreover, mean heterozigosity is higher than observed in a bottlenecked population of a related species, the Ethiopian wolf (Gottelli et al. 1994). Lower values of microsatellite variation are reported for the Italian population by Randi & Lucchini (2002), which remarked the difference with the high level of polymorphism of domestic dogs.

Population structure

A wolf population is usually composed by a number of territorial packs and by a number of dispersing or solitary-living individuals. The proportion of 'non-pack' individuals (i.e. individuals not belonging to a pack) within the population is difficult to estimate, changes seasonally and depends on the overall wolf density and prey abundance. Fuller (1989) estimated this may amount to 7-20% of the overall consistence in North America. No data is reported for the Italian population.

Moreover, a single pack may sometimes split into two or more smaller packs or, on the contrary, it may temporarily join an adjacent pack, generating a larger social unit (Mech 1981).

Apollonio et al. (2004) outlined two figures for wolf populations. In northern regions, where moose and caribou are major preys, wolves gather in large packs (on average 4-10 members), defending wide territories and their density is low (0.1-2.0 per 100 km^2), or alternatively are nomadic. On the contrary, at lower latitudes, where wolves prey mostly upon deer and wild pigs, they live in small packs (on average 3-6 members), occupy smaller territories and may reach high values of density

(2.0-6.0 per 100 km^2), in dependence of prey availability and level of harvesting. In a complex predator-prey system, the Casentinesi Forests (central Apennines), they reported an average pack size of 4.4 wolves and a density of 4.7 individuals per 100 km^2. In such figure, wolf density is mainly determined by the number of packs rather than pack size. Therefore, some factors should limit pack size, inducing wolves to form new packs rather than remaining within their natal pack. The presence of vacant territories, as in newly protected or recovering populations, and high densities of prey induce wolves to disperse early in their life (as pups or yearlings). An early abandonment of the natal pack is expected to limit the size of existing packs and promote the formation of new social units. Hence, with pack size restricted to low values (usually < 7 individuals), one should expect to find packs being simply composed of a breeding pair and their offspring. This was depicted by Mech (1981) as the typical structure of a wolf pack and confirmed by some genetic investigations on wolves inhabiting different regions of North America (Lehman et al. 1992). The breeding pair commonly consists of unrelated individuals and incest is avoided (Smith et al. 1997). Though all wolves in a pack, except the breeding pair, are closely related (parent-offspring or siblings), deviations from this common situation were documented, where the presence of unrelated non-breeding pack members was proved (Lehman et al. 1992). These individuals may achieve some advantages from joining an existing non-natal pack: they may gain in experience (i.e. in hunting), obtain food, or take over one of the breeding adults. Nevertheless, the replacement of one of both parents by reproductively mature offspring would result in sib-sib or parent-offspring matings. This incestuous pairings is expected to result in a high level of inbreeding within wolf populations. On the contrary, a study of relatedness within breeding pairs in two North American areas (Alaska and Minnesota) revealed an opposite trend, that is pairs were mostly made up of unrelated or low-related individuals (Smith et al. 1997). Incest avoidance would imply that offspring have alternative advantages in joining the natal pack, other than replacing their parents. Thus the gain in experience seems to be, to young wolves, the most advantageous investment of remaining in the natal pack, at least during an early stage. As the alternative behaviour is dispersal, inbreeding avoidance might represent the primary motivation to disperse, and the risk of dispersing might be a sufficient cause for mature wolves to remain in the pack where they are born.

Evidences of pack structure in Italy were so far reported only for colonizing wolves in the Alps (Lucchini et al. 2002). In this area, packs are also mainly composed of closely related individuals, although a significant proportion of unrelated individuals (3 out of 14) were detected in the territory of two packs.

FIGURE 5. A wolf pack in the Foreste Casentinesi National Park (photo: E. Centofanti).

Regards to the effects of dispersal on gene flow within wolf populations, the high mobility of the species is expected to favour genetic exchange among even distant populations, thus reducing the level of genetic differentiation. Indeed, difference in the genetic composition of wolf populations are often correlated to their geographic distance over a very wide geographic scale (Forbes & Boyd 1997). However, the presence of topographic barriers can influence the pattern of gene flow over a smaller scale (Carmichael et al. 2001). Even prey specialization may affect the structure of wolf populations, as it influences the spatial behaviour of this carnivore (Carmichael et al. 2001).

High level of genetic variation in colonizing wolves of North America were interpreted as resulting from population structure (Forbes & Boyd 1996). Breeding groups confined to intermountain valleys may be in semi-isolation, despite of their geographic distance, and thus locally diverge by the effect of genetic drift. Unbiased Nei's genetic distances among Rocky Mountains wolf populations were in the range 0.005-0.223 (Forbes & Boyd 1996), whereas the same parameter estimated among populations spread throughout the whole continent ranged between 0.182 and 0.418 (Roy et al. 1994). Values of F_{ST} referred to North-American populations, obtained by microsatellite data, were in the range 0.074-0.168 (Roy et al. 1994, Forbes & Boyd 1996).

Human-caused mortality has a high impact on wolf populations. In Italy, based on the amount of recovered wolf carcasses, a minimum estimate of mortality amounted to 15% of the actual population size (Boitani & Ciucci 1993). However, only a slight number of dead wolves are discovered and the proportion of them resulting from human actions (shooting, poisoning, driving) is overrepresented. Actually, mortality rate in a population is largely underestimated and certainly it may represent a strong constraint to the successful dispersal of young wolves. The high productivity of wolf packs could balance the suspected high mortality, maintaining constant the number of packs/individuals within a given area (Apollonio et al. 2004). Mortality can influence the structure of wolf populations in two ways: 1) the death of pack members (especially dominant individuals)

enhances the pack turn-over; 2) the death of dispersing individuals limits the genetic exchange among packs in a population. In the Italian population a high proportion of dead wolves was found out of the model-defined potential range of the species (Corsi et al. 1999). Moreover, young (≤ 2 years old) individuals, thus at the age of major dispersal (Gese & Mech 1991), suffer a higher mortality than adults (Ciucci & Boitani 1998). Such evidences could be interpreted as a high mortality rate involving dispersing animals, potentially carriers of gene flow. Consequently, the protection/exploitation level of a wolf population is expected to strongly influence its genetic structure.

2.3 Study area and population

Study area

The study was conducted in central Italy within a 3200 km^2 provincial district (Arezzo province, Fig. 6). The area includes mountains belonging to the Apennine chain. Altitude ranges from 300 to 1500 m a.s.l.. Forests are abundant and spaced by meadows and pastures, particularly along valleys. Two main rivers flow through the study area: Arno and Tevere. The Arno river, in its first portion, forms a valley, known as Casentino, then running northward toward Florence. Instead the Tevere river flows southward, forming the Tevere Valley, and continues in direction of Rome.

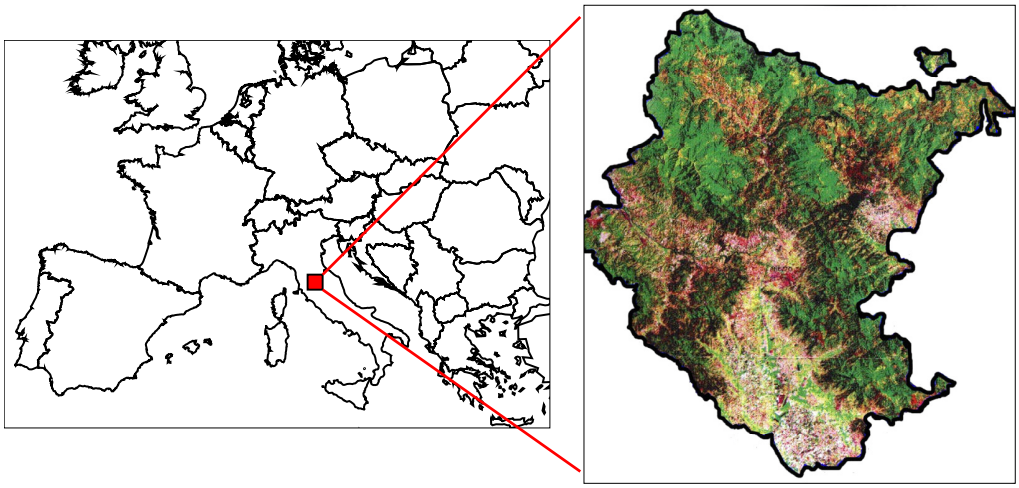

FIGURE 6. The study area in the Arezzo province.

Villages are concentrated at low elevations, mainly along the main valleys and in the plain. Resident population density averages 100 inhabitants per km^2, but human presence in wolf areas is limited, although increasing during the summer because of tourism. Climate is mild, with occasional snowfalls from November to April in mountain areas. Rarely snow lasts on the ground for more than one week, except for the highest elevations (on average 56 days per winter at 1100 m a.s.l., of which only 28 days with a snow depth $\geq$ 10 cm). The study was mostly concentrated within protected areas, represented by the Foreste Casentinesi National Park and five Natural Reserves (Pratomagno, Alpe di Catenaia, Alto Tevere, Monte Modina, and Alpe della Luna) and their direct surroundings, for on the whole 1600 km^2 covered by the investigation.

An abundant and diversified ungulate community is present. Wild boar (*Sus scrofa*) and roe deer (*Capreolus capreolus*) are ubiquitous; a reintroduced population of red deer (*Cervus elaphus*) is spreading out in the National Park, whereas fallow deer (*Dama dama*) is restricted to some portions of the area. All these species are protected within National Park and reserves, but outside they are

hunted in the period comprised between August and January. For wild boar, collective hunting is widely practised, whereas roe, fallow and, only since 2000, red deer are under programmed culling. The study area was divided into four parts (Fig. 7), corresponding to the main mountain districts of the province: Pratomagno (abbreviated as PM), National Park (PN), Alpe di Catenaia (AC) and Alpe della Luna (LU). They are separated by the two main rivers (Arno and Tevere). This subdivision was considered to investigate genetic differentiation.

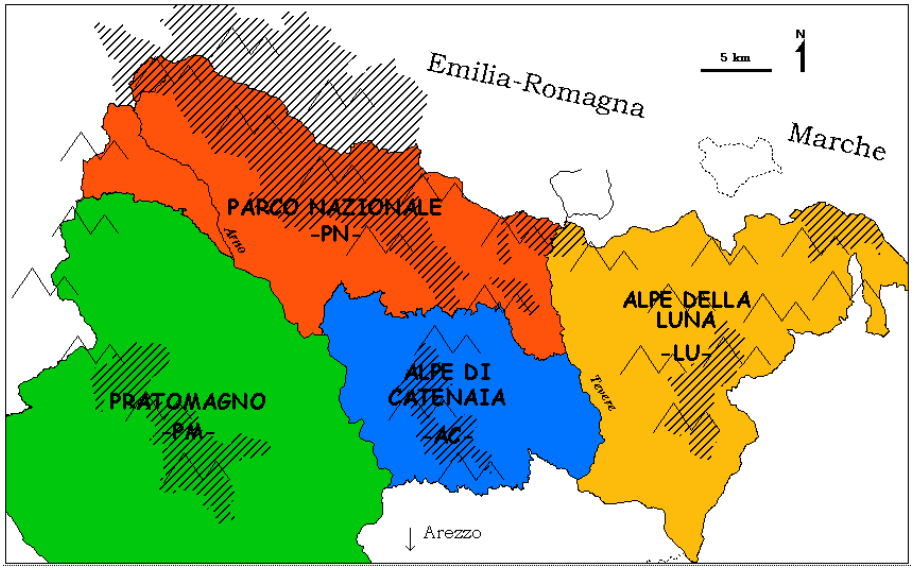

FIGURE 7. Geographic areas considered in the study (protected areas are shaded).

Population history

In this part of the Apennines, wolves declined progressively during the first half of the last century. During the historical minimum of the Italian wolf population (1950-1970), their presence in these area was debated. Some authors (Cagnolaro et al. 1974) reported a few individuals or packs occasionally recorded in the area, while others (Zimen & Boitani 1975) considered the wolf population restricted to some 'islands', limited to more southern regions. I have recently investigated, recurring to genetic markers, the distribution of wolf microsatellite diversity among different Italian regions (Scandura et al. 2001a). The published results support the hypothesis of the persistence of a local wolf population in these areas, even through the period of decline at a national scale. From 1970 on, like in other parts of the peninsula, wolves increased in number following the increase of their wild prey populations. The first studies in the Arezzo province started at the end of the 1980 years, when most mountain massifs were concerned by the presence of the species.

Field activities

The local wolf population is monitored since 1998. All direct observations of wolves within the study area have been recorded in a specific database. Field activities include summer detection and count of territorial packs and winter tracking of wolves in the snow. All these activities are realized by a skilled group of researchers and managers, specialized on the behavioural and trophic interaction between wolves and wild ungulates, and financed by the Provincial Administration of Arezzo and the Regional Government of Tuscany.

'Wolf-howling' surveys (Harrington & Mech 1982) were carried out in summer, from June to September, to ascertain the presence of wolf packs, their reproductive status (i.e. birth of a litter) and to locate homesites (den or rendezvous sites – Mech 1981). The approach is based on the use of artificial stimuli (playback) to elicit howling by wolves. Replies are recorded and their spectrographic analysis enable to discriminate between adult individuals and pups. Both response rate and discriminative power are higher in summer, when the pack reduces its movements, frequents rendez-vous sites and attends for the pups (Gazzola et al. 2002). During pack counts, simultaneous surveys by several teams were carried out in adjacent areas, to avoid double counting.

In winter, wolves were tracked in presence of fresh snow (24-48 hours after a snowfall). As a wolf trail was found, tracks were followed until the number of individuals travelling along became distinguishable. The major number of wolves travelling together within a considered area was used as an estimate of winter pack size.

Wolf faeces (scats) were collected throughout the study area between 1998 and 2000, and analyzed to determine food preferences by wolves. As result, wild ungulates are far the most used category, being preferred to livestock and to other mammals. Among wild ungulates, variations were detected in different areas, with wild boar and roe deer representing often the majority of food residuals in the scats (Mattioli et al. 2003, Capitani et al. 2004).

Population size and dynamics

The results of monitoring activities during the period of study are reported in Fig. 8 and in Tab. 1. Most packs were detected at summer rendez-vous sites. The presence of pups was used as index of successful reproduction for a given pack. Nine to eleven different packs were yearly counted in the Arezzo province, corresponding to 39-45 wolves (minimum estimate). Most of them were detected within or in close proximity of protected areas. The estimated consistence suffered of methodological limitations: elicited wolf howling is unlikely to enable the detection of solitary or peripheral individuals (Harrington & Mech 1982). Hence, considering the mentioned estimate as

accounting only for wolves in a pack, a 7-20% (Fuller 1989) of uncounted solitary/peripheral heads may be added. Thus, the overall yearly consistency could be approximated to 42-54 wolves.

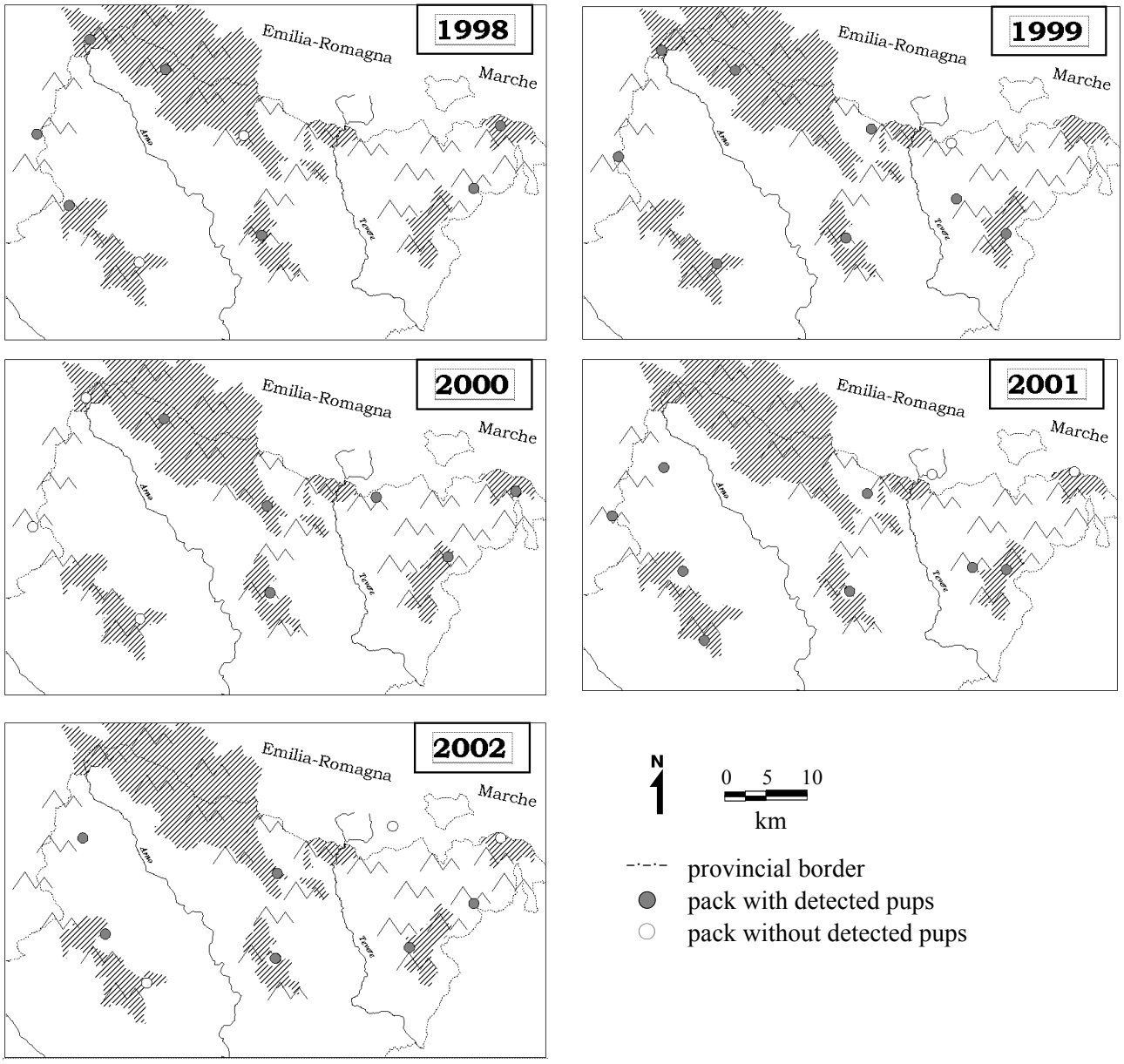

FIGURE 8. Approximate location of wolf packs detected in summer 1998 to 2002. Protected areas (national park or natural reserves) are shaded.

Year	Area (km^2)	No of detected packs	No. of wolves	Summer average pack size	wolves per 100 km^2	km^2 per pack	Average distance between adjacent packs (km)
1998	1568	11	45	3.8	2.9	143	12.7
1999	1568	10	40	4.3	2.6	157	12.4
2000	1568	10	41	4.0	2.6	157	13.7
2001	1338	10	39	4.0	2.9	134	13.6
2002	1338	9	44	4.6	3.3	149	12.3
	Mean	**10**	**42**	**4.1**	**2.8**	**147.7**	**12.9**

TABLE 1. Number of packs, mean pack size and wolf density yearly estimated in the study area in the period 1998-2002. Average inter-pack distances refer to summer locations of rendezvous sites used by adjacent packs.

Minimum pack size was comprised between 2 and 6 and averaged 4.1 wolves.

An overall density of 2.8 wolves per 100 km^2 was estimated. One pack was present on average every approximately 150 km^2. The average distance between adjacent packs was 12.9 km. A high fidelity to summer rendez-vous sites was highlighted during the study (Fig. 9). In the Alpe di Catenaia area, wolf-howling responses were heard every year in August-September in the same small valley (Scandura et al. 2001b, Capitani et al. 2003).

Referring to the different geographic districts in which the study area is divided, in PM and in LU from 2 to 4 wolf packs were counted per year respectively, AC included one pack only, whereas in PN 3 packs were present from 1998 to 2000, as the monitoring was suspended in a large portion of the area; from then on a single pack was counted in the remaining portion.

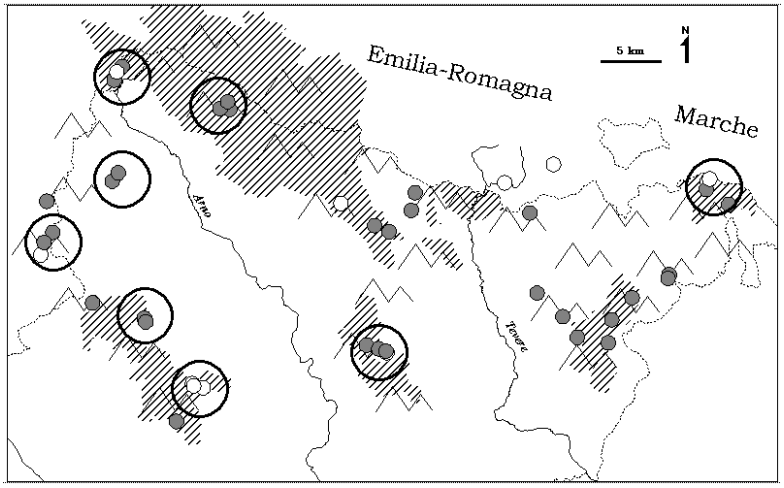

FIGURE 9. Constancy of summer locations of different monitored packs during the study (1998-2002). Circles show overlapping locations of the same pack in different years.

2.4 Methods

Sample collection

Genetic investigations on wild large carnivores, like wolves, are affected by the difficulty of collecting samples. This is a consequence of the small number of free-ranging individuals, usually present at low densities, and of their elusive behaviour. Where populations are large and not severely threatened, like in most regions of North America, wolves are legally harvested. In such cases, the simplest way to obtain samples for genetic analysis is from killed individuals. In several regions, studies are conducted involving the capture and radio-collaring of free-ranging wolves; protocols usually include the collection of blood samples from every specimen for laboratory analysis. Nevertheless, in many countries, wolves are or have recently been severely endangered and conservation efforts are addressed to recover their range and consistency. In Italy, wolf harvesting is not permitted, and conservation concerns have so far had priority respect to the knowledge of its biology. Thus, research activities based on the capture of wild individuals (i.e. radio-tracking) were limited to very few individuals (Ciucci et al. 1997) and most studies are based on indirect methods (e.g. snow-tracking). Unfortunately, although illegal, wolf killing (by poison, snare or shot) still represents one of the main cause of mortality in several Italian regions.

As soon as innovative technologies enabled alternative DNA sources to be employed for genetic analysis, not previously considered sample typologies became useful for this purpose. Hairs, feathers, faeces, and urine have become reliable samples for researches on wild animals, having the advantage of being collected without an impact on the population under study (Morin and Woodruff 1996). Some recent studies on natural wolf population recurred to this 'non-invasive' approach, using mostly scats collected in the field (Lucchini et al. 2002, Valiere et al. 2003).

In this study, except for a blood sample extracted from a wounded wolf, samples were obtained by two ways. Seventeen specimens were sampled from wolf carcasses. Muscle or hair samples were collected from dead animals found by provincial or forestry guards within the study area. Cause of death was in most cases related to humans (shot, poison, car accident). Such samples were stored dry (only hairs), in freezer at –20°C or in absolute ethanol. The second class of samples was provided by non-invasive collection. During field research activities in mountainous areas, three kind of samples were collected. Scats were found along pre-defined transects throughout the year, and along tracks on the snow in winter (Fig. 10). Shed hairs were found on the ground or entangled in barbed wire or in bushes (Fig. 10). They could occur individually or grouped together; in this second case the kind of grouping was decisive in establish whether to consider them a single sample or not. The possible estimated number of wolves travelling on a trail was helpful to this decision.

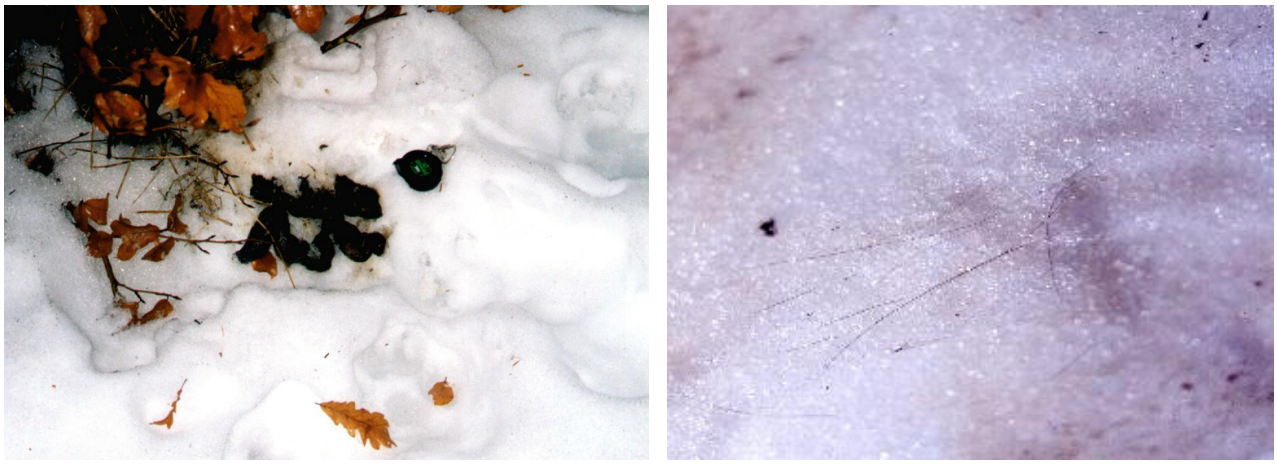

FIGURE 10. Scats (left) and shed hairs (right), used for molecular genotyping, were collected mostly on snow in winter (photos: C, Capitani and E. Avanzinelli).

Most scats and hairs were found in winter while tracking wolves in snow. These samples were the most suitable for genetic analysis, as regards freshness and degree of conservation. Moreover, samples collected along travel routes in the snow are likely to belong indifferently to pack members, whereas summer scats, deviating from a random distribution, are mostly resulting from the marking behaviour of dominant individuals (Peters & Mech 1975, Vilà et al. 1994).

A third kind of sample was limited to a snowy substrate: it is represented by blood spots found along trails of tracks. They may derive from haematic losses of free-ranging individuals, due to body injuries or to incipient estrus. Vaginal seepage during pro-estrus is typical of canid species (Mech 1981). However, blood spots along wolf tracks may also derive from shreds of prey they carry during their movements. The reliability of the analysis of this latter type of samples was demonstrated in a specific article submitted to Conservation Genetics. Urine samples were not employed as they were shown to encounter dilution and contamination problems (Valiere & Taberlet 2000).

Hairs were collected in plastic tubes or bags and stored in refrigerator (+4°) or in freezer (-20°C). Different preservation methods were tested with faecal samples, including storage in lysis buffer (SLP), silica powder and absolute (>90%) ethanol. This latter resulted the most valuable method, and thus for most scats, at the moment of their collection or after few hours, a portion (variable from one tenth to one third) was put into a plastic tube and filled up with ethanol. Ethanol had been found to preserve faeces better than alternative methods also in other studies (Frantzen et al. 1998, Murphy et al. 2002). All tubes were then stored at –20°C.

All considered samples, at the moment of their collection, were attributed to the wolf on the basis of their aspect or of their association with wolf signs. This evaluation is subjective and error-prone, and depends on the skill and experience of the researcher. Thus it needs to be confirmed by the genetic analysis itself.
On the whole, 329 samples from non-invasive collection were analysed; of them 174 were scats, 124 shed hairs and 31 blood spots.

Non-invasive sampling

In the last years the number of studies involving non-invasive genetic tagging has vastly increased. Several of these researches had been conducted on wild large carnivores, including bears (Taberlet & Bouvet 1992, Höss et al. 1992, Taberlet & Bouvet 1994, Kohn et al. 1995, Taberlet et al. 1997, Wasser et al. 1997, Woods et al. 1999) coyotes (Kohn et al. 1999, Fedriani & Kohn 2001), mountain lions (Ernest et al. 2000, 2002), Iberian lynx (Palomares et al. 2002) and wolves (Lucchini et al. 2002, Creel et al. 2003, Valiere et al. 2003). However, caution in the use of very small or degraded samples derives from the fact that low quality and quantity of DNA in them strongly affect the outcome of the analysis (Kohn & Wayne 1997, Gagneux et al. 1997, Goossens et al. 1998, Taberlet et al. 1999). Templates are present in low numbers and are degraded. This feature exposes the analysis to stochastic events in pipetting and to the production of PCR artefacts. Only short regions have a reasonable probability of being amplified. Two kind of scoring errors are associated to microsatellite genotyping from shed hairs and faeces: allelic dropout (failure of amplification of one allele at a heterozygous locus) and false alleles (occurrence of erroneous alleles in the genotype). To avoid such errors leads to mistyping of individuals, a special experimental design is required, thus multiplying time and cost of the analysis (Taberlet et al. 1996, Taberlet & Luikart 1999, Waits & Leberg 2000). Different authors adopted the so called ‘multiple tubes approach’, introduced for the first time by Navidi et al (1992) in analysing humans. It consists on the contemporary or successive analysis of more aliquots of the same DNA sample. To minimize the number of repetitions, a two-step strategy was proposed (Taberlet et al. 1996): a first round of amplifications involves only three repetitions and is conclusive only if their results are coincident; in the opposite case of contradictory profiles, four further repetitions are carried out and the prevalent result is considered the actual one and attributed to that sample. Taberlet et al. (1996) observed that following this procedure a confidence level of 99% is reached for single genotypes. Gagneux et al. (1997), facing limited sample quantities, reduced the number of repetitions on the basis of the obtained results. They applied a formula to calculate the critical number of needed amplifications, given a pre-determined error frequency.

For hairs, the number of follicles was critical (Goossens et al. 1998). Single hairs give poor results, as allelic dropout was estimated as high as 14% for plucked and 31% for shed hairs (Gagneux et al. 1997, Goossens et al. 1998). Increasing the number of hair bulbs, the error rate decreases rapidly down to < 1% for 10 plucked hairs.

Morin et al. (2001) described an efficient approach for microsatellite genotyping, based on a preliminary screening of DNA extracts, sorting them for quality and regulating the number of repetitions per sample (less for high-DNA-content and more for low-DNA-content samples). Accurate DNA quantification is at the basis of sample quality judgement. Less than 100 pg of DNA per reaction is considered the critical point.

An alternative approach was recently proposed by Paetkau (2003), who remarked the loss of applicability associated to the multiple tubes approach. He supported the use of a quality control method, by which multilocus genotypes are accurately checked and a systematic evaluation process is adopted to exclude poor samples, producing inconsistent genotypes. Repetitions are contemplated just in the case of poor amplifications for a minority of loci in a sample, or in case of close pairwise similarity (1-2 mismatches) between profiles. This method was widely adopted in genetic inventories of bear populations in North America (Woods et al. 1999).

DNA isolation

Different extraction protocols were adopted depending on the used DNA source. Muscle and whole blood samples were extracted using the QIAamp Blood kit (QIAGEN), according to manufacturers' instruction. Two elution steps of 200 µL were used for tissues, a single step for blood samples.

DNA was isolated from hair follicles using the resin Chelex-100 (BIORAD) with a quick protocol, avoiding the use of proteinases (Walsh et al. 1991). Final volume was regulated on the basis of the number of follicles in the sample. In the best case of no limitation in their number, five to eight hairs were used in a volume of 200 µL. When few hairs were in a sample, volume was proportionally reduced, until a minimum of 80 µL for a single hair.

Diluted blood samples were used for DNA isolation by the QIAamp Blood kit. Two hundreds microliters of melted blood-snow mixture were used to obtain up to 60 µL of final DNA solution, on the basis of the initial dilution (red tone). Care was taken to avoid any risk of cross-contamination among samples.

Apart from very slight samples, DNA concentrations were measured by a GeneQuant spectrophotometer (Pharmacia Biotech).

With regard to faecal samples, the efficacy of all downstream applications requires a particular attention to the isolation of DNA. Indeed, a scat represents a mixture of several biotic and abiotic

substances, overabundant respect to the few epithelial cells containing wolf DNA. Soil, bacteria, fungi, prey residuals, and several other components enter into their composition. This variety makes difficult to completely isolate a component from all the others. Moreover, a quantity of inhibitors of enzymatic activity (like the polymerase) is present and has to be accurately removed before further use of the extracted DNA (Gerloff et al. 1995, Morin & Woodruff 1996). Specific PCR amplifications enable researcher to detect a target DNA region within this mixture. In this way, a wolf microsatellite may be selectively amplified. Being the selectivity of the analysis entrusted to the PCR, no need exists to completely isolate wolf DNA from exogenous nucleic acids.

Upstream attempts were made in order to obtain good amplifications from faecal DNA. Extraction and purification were conducted with a series of different protocols, including phenol-chloroform, and a series of extraction kits: Wizard (Promega), GeneClean for Ancient DNA (BIO101) and QIAamp Stool (QIAGEN). Yields differed among them, referring both to overall recovered DNA and degree of purity. The trade-off between number of isolated target molecules and number of active inhibitors in the sample was critical for a successful PCR.

Preliminary experiments revealed that GeneClean and QIAamp kits performed better, and they became the kits of use for the extraction of DNA from the collected scat samples.

Microsatellite selection and amplification

Both the aim of the study and the use of non-invasive sampling imply individual genotyping of free-ranging wild wolves. High level of polymorphism are required to obtain unique genotypes in the population. Loci were chosen among published data, deposited in the online GeneBank database. Many microsatellites had been previously developed in domestic dog and for them primer sequences are available in the literature or in GeneBank.

Ten autosomal microsatellites, including five dinucleotides (cxx.109, cxx.123, cxx.204, cxx.250 from Ostrander et al. 1993, and cxx.377 from Ostrander et al. 1995), and five tetranucleotides (FH2004, FH2054, FH2137, FH2158 and FH2175 from Francisco et al. 1996), derived for domestic dog, were selected for wolf genotyping. Apart from cxx.250, all of them were included in linkage studies for mapping the canine genome and assigned to different linkage groups (Mellersh et al. 1997, Neff et al. 1999, DogMap Project). By virtue of this, independence among loci may be assumed. Locus information including linkage group, repeat motif, and primer sequences are reported in Table 2.

Locus	Linkage group a	b	c	Repeat	Ta (°C)	Primer sequence (5'-3')	Reference
CXX109	-	-	-	$(AC)_{15}$	58	AACTTTAAGCCACACTTCTGCA ACTTGCCTCTGGCTTTTAAGC	Ostrander et al. 1993
CXX123	L2	L6	CFA23	$(AC)_{21}$	58	AACTGGCCAAACATAAACACG TTCATTAACCCTTTGCCCTG	Ostrander et al. 1993
CXX204	-	L24	-	$(AC)_{15}$	58	CGAGAGCAACATAGGCATGA CAAAGTGCTGTGGCAGGTC	Ostrander et al. 1993
CXX250	L18	CFA9	CFA9	$(AC)_{18}A_2(TC)_4$	58	TTAGTTAACCCAGCTCCCCA TCACCCTGTTAGCTGCTCAA	Ostrander et al. 1993
CXX377	L19	CFA5	CFA5	$(AC)_{12}$	55	ACGTGTTGATGTACATTCCTGC CCACCCAGTCACACAATCAG	Ostrander et al. 1995
FH2004	L17	-	CFA11	$(GAAA)_{13}$	58	CTAAGTGGGGAGCCTCCTCT ACTGTGACCTACTGAGGTTGCA	Francisco et al. 1996
FH2054	L6	L9	CFA12	$(GATA)_{16}$	58	GCCTTATTCATTGCAGTTAGGG ATGCTGAGTTTTGAACTTTCCC	Francisco et al. 1996
FH2137	L3	L4	CFA3	$(GAAA)_{21}$	58	GCAGTCCCTTATTCCAACATG CCCCAAGTTTTGCATCTGTT	Francisco et al. 1996
FH2158	L22	CFA20	CFA20	$(GAAA)_{44}$	58	ATGGCCACATCACCCTAGTC CTCTCTCTGCATCTCTCATGAA	Francisco et al. 1996
FH2175	L13	L12	CFA16	$(GAAA)_{18}$	58	TTCATTGATTTCTCCATTGGC AGGACTCTAAAAACTTGCCTCC	Francisco et al. 1996

a - Mellersh at al. 1997
b - Neff et al. 1999 (Table 1 at http://www.biostat.jhsph.edu/~kbroman/data/dogs/table1.html)
c - DogMap Project at http://www-recomgen.univ-rennes1.fr

TABLE 2. Microsatellite loci employed in the study. Linkage group, repeat motif, annealing temperature and primer sequences are reported.

All loci were polymorphic in dogs and the former five had revealed also variable in North American wolves (Forbes & Boyd 1996, 1997). Six loci (cxx.109, cxx.123, cxx.204, cxx.250, cxx.377 and FH2158) were already used for a previous screening of the Italian wolf population (Scandura et al. 2001a). In that study, the average expected heterozygosity was close to 0.6, both for the overall Italian population and for wolves in the Arezzo province only (represented there by 13 dead specimens). Such level of polymorphism guarantees a sufficient resolution in individual genotyping, as probability of identity among multilocus genotypes is expected to be sufficiently low (below the threshold of 1%).

For every DNA extract, amplifications started with the most robust (i.e. best-performing) markers, in order to maximize the probability of success. In case they did not amplify properly, samples were discarded.

Amplifications were carried out in 10 μL reaction volume containing 3 μL of DNA solution, 0.5 units of Red Taq DNA polymerase (Sigma-Aldrich), 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mg/mL BSA, 100 μM of each dNTP and 2 pmol of each primer. The PCR profile was set up with an initial step of denaturation at 95 °C for 3 min, followed by 35 cycles with 92 °C for 40 sec,

40 sec at the established annealing temperature (55-58 °C), and 72 °C for 30 sec. A final extension step of 72 °C for 10 min was added. One PCR blank was included in each amplification, for contamination checking. One oligonucleotide of each locus-specific primer pair was labelled at its 3'-end with a fluorophore molecule detectable by ABI PRISM systems (Applied Biosystems). As dyes, HEX (cxx.123, FH2004, FH2137), 6-FAM (cxx.204, cxx.377, FH2054, FH2175), TET (cxx.109, cxx.250), and NED (FH2158) were used. PCR products were combined, in relation to the admissible dye combinations, mixed into a 0.5-mL PCR tube, to which a ROX-labelled size standard was added. Amplified fragments were analysed by capillary electrophoresis using an ABI PRISM 310 automatic sequencer (Applied Biosystems), and alleles were sized using the GENESCAN and GENOTYPER software (Applied Biosystems).

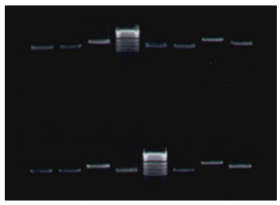

FIGURE 11. PCR products on agarose gel.

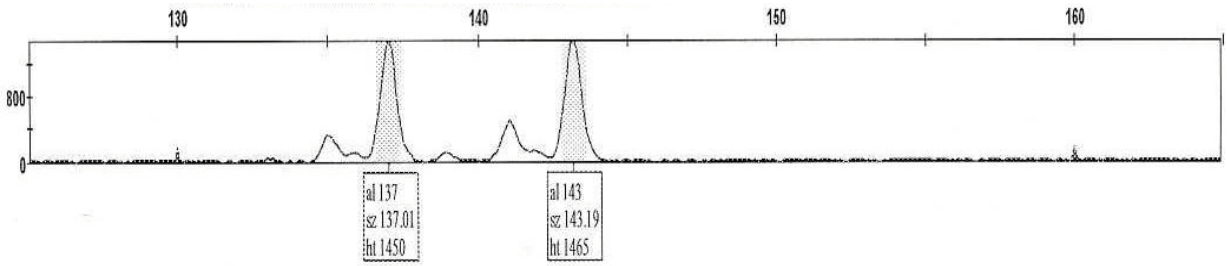

FIGURE 12. Peak detection on ABI PRISM 310 automatic sequencer.

Molecular sexing

As for microsatellites, even gender determination of non-invasively sampled individuals, was based on the PCR technique. In this case, the region to amplify is located in the Y chromosome and is part of the SRY gene. In the PCR, a primer pair was employed, proved to amplify a target 147 bp sequence in several mammal species (5'-CATTGTGTGGTCTCGTGATC-3' and 5'-AGTCTCTGTGCCTCCTCGAA-3', Richard et al. 1993). The amplification protocol was the same as for microsatellites, except for $MgCl_2$ (2.5mM) and primer (0.16 μM) concentrations in the reaction mix and for annealing conditions (1 min at 55 °C). PCR products were run on 2% agarose gel, containing ethidium bromide, for 20 minutes at 60V.

Hence, the amplification was not species-specific. Being the Y chromosome exclusive of male cells, the successful amplification of the target sequence is considered diagnostic of the sex. In case of amplification failure (no SRY product amplified), gender attribution may not be immediately diagnosed. In fact, troubles in the PCR, other than lack of template, can produce a negative result. Being the size of the SRY-product in the average range of microsatellite alleles, I would expect that DNA samples well-performing with microsatellite loci are likely to perform as much with the sex-determination system. Moreover, in order to have an internal amplification control, a portion of the mtDNA cytochrome b gene was co-amplified using universal primers L14841 and H15149 (Kocher et al. 1989). In so doing, Y-negative samples, which did amplify the mtDNA fragment, were identified as females.

A similar approach was reliable for most samples, apart from faecal DNA. Indeed, wolf scats contain a lot of exogenous DNA, comprising mammal DNA (prey). This means that SRY-primers can find several possible templates, belonging to different species. The non-specific amplification of all SRY regions in the sample may confound results, producing false positives. For this reason, I did not consider faecal samples for sex determination. Thus sex was determined only for genotypes resulting from the analysis of at least one non-faecal sample.

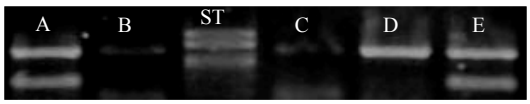

FIGURE 13. Sex-specific amplification. SRY (higher band) and mtDNA (higher band) were simultaneously amplified in a multiplex. PCR products are detected on 2% agarose gel. A and E are ♂♂ (both SRY and mtDNA regions were amplified); B, C, and D are ♀♀ (only the mtDNA region was amplified); ST is a DNA size standard.

Quality check and genotype selection

Non-invasive genotyping from low-quality samples is an error-prone procedure. Above the drawbacks of non-invasive sampling were mentioned and the possible source of typing errors examined. Different authors, following suggestions given by Taberlet and Luikart (1999), recurred to a 'multiple tubes approach' to avoid scoring errors associated to low DNA concentrations. A similar approach, although circumventing scoring errors, requires a large quantity of sample per locus amplified. If extensively used, it would limit enormously the use of some samples (e.g. shed hairs), as the total volume of DNA solution will be entirely consumed to analyse a small number of loci, even if the overall amount of DNA would be sufficient to completely type a large set of polymorphic markers. For this reason, such approach has found a wide application only in studies

based on the analysis of faecal samples. These latter are indeed large enough to enable multiple DNA extractions, giving larger volumes of DNA solutions. Moreover, this procedure, although theoretically robust, is in practice costly and time-consuming and thus rarely applicable to extensive studies on natural populations.

The alternative approach, proposed by Paetkau (2003), seems to be in practise more valuable, as conciliating the time and financial aspects to the care and rigour of a scientific procedure.

In this study a similar approach was adopted, establishing a quality control procedure which include the attribution of a score to every single-locus genotype for each stage of the analysis. Single-locus scores are then combined over loci, producing a multilocus score, and a threshold is established under which genotypes (and relative samples) are culled. Scores referred to: a) amplification quality (PCR score), b) allele sizing quality (SEQ score), c) number of typed loci (LOC score). To evaluate the quality of amplifications, band intensity and sharpness, and the absence of non-specific products on a post-PCR agarose gel were taken into consideration (Fig 14). The second phase controlled for quality was allele designation after the run at the automatic sequencer. Allele profiles were evaluated considering shape and intensity of allele and shadow peaks (Fig. 15).

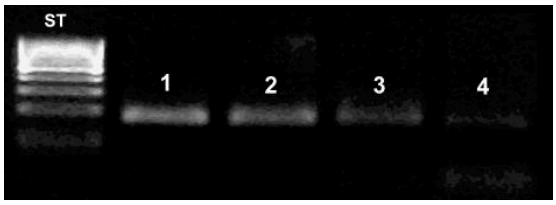

FIGURE 14. PCR scores attributed to different band patterns on 2% agarose gel. The same quantity of PCR product (5 μL) was loaded into each slot of the gel. Number correspond to PCR scores attributed to each single amplification.

A score was also referred to the number of analysed loci, in order to penalize samples only partially typed (score 0 for complete genotypes; 0.2 was added for every lacking locus). Score decreases with the better quality of the genotype: both PCR and SEQ scores ranged between 1 and 4, whereas LOC score ranged between 0 and 2 (in order to have a lower weight on the overall score). Scores are combined over loci, by averaging single-locus values and then summing the obtained values for each score type. Thus an overall score comprised between 2 and 10 is obtained for each sample. Thresholds are fixed at: PCR score = 2, SEQ score = 2 and LOC score = 1. Acceptance of sample profiles was thus subordinate to the satisfaction of all the following criteria: PCR score $\leq$ 2, SEQ score $\leq$ 2 and LOC score $\leq$ 1. Samples violating these criteria were excluded from data processing.

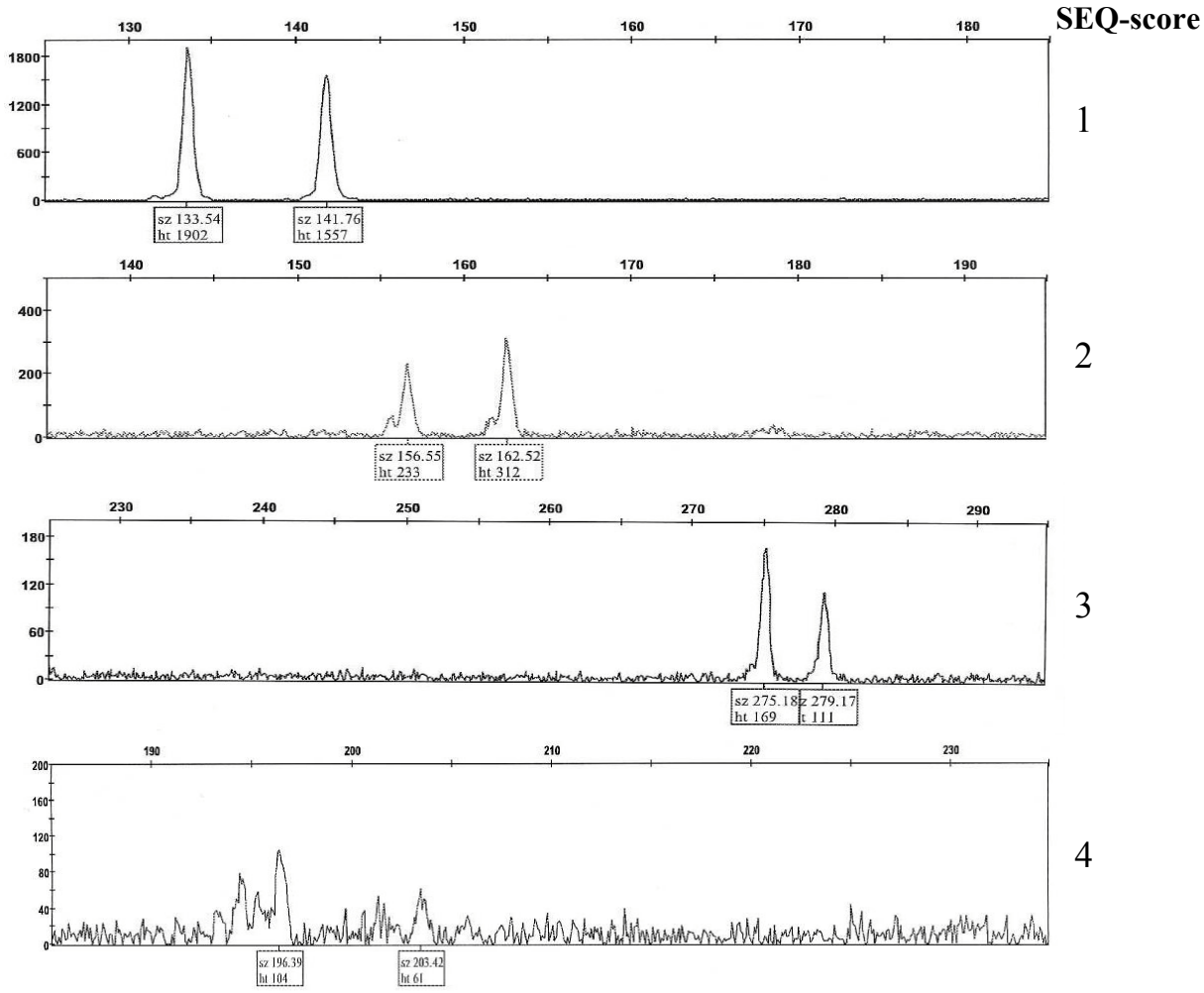

FIGURA 15. SEQ scores attributed on the basis of peak patterns (shape, intensity, etc.), obtained by capillary electrophoresis on ABI PRISM 310 (Applied Biosystems). Numbers on the right correspond to SEQ scores attributed to single runs of PCR products.

Therefore, the analysis proceeded as follows: a DNA sample was amplified at every locus; if no product was detectable at one locus, PCR was repeated; multilocus profiles were checked for quality (see above); in case of good quality profile, the corresponding genotype was considered acceptable and included into a data processing file; in case of low quality profile, either a sample was completely culled, or bad amplifications were repeated in order to improve the quality score (Fig. 16).

Dropout rate and false allele frequency were verified for each category of samples, by 2-6 PCR repetitions of selected DNA samples. All microsatellite loci were included in such experiments.

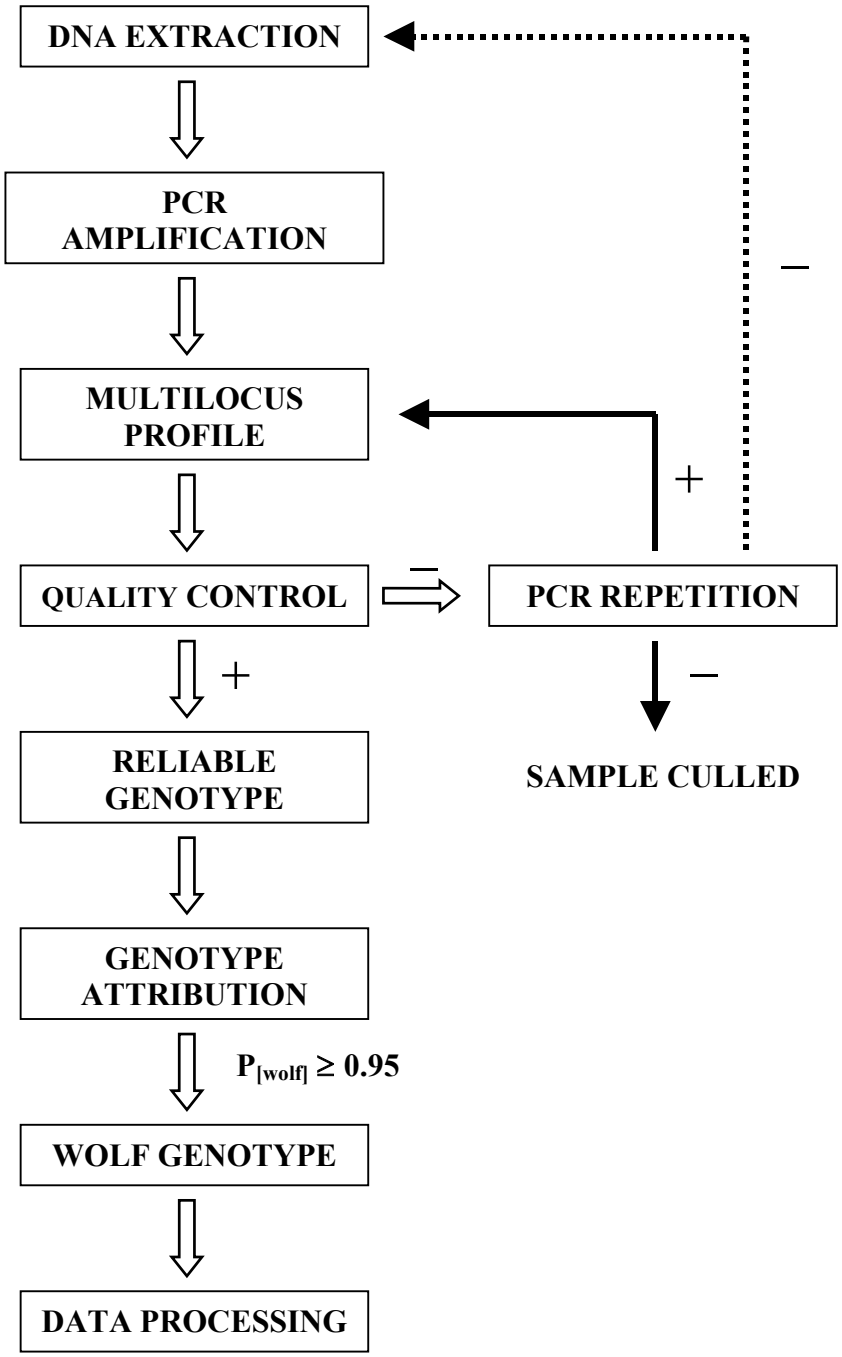

FIGURE 16. Flow chart of the analysis strategy, including decisional steps determining the acceptance or the culling of a microsatellite profile.

Genotype assignation

Scats, hairs and blood drops were not easily to assign to wolves at the moment of collection. In fact, neither sample features (shape, composition, smell, etc.), nor information associated to the sample in the field (footprints, kills, etc.) may unequivocally prove its origin.

As consequence, genotypes deriving from non-invasive samples have to be checked for wolf attribution. This is possible on a genetic basis, referring to differences in allele distribution at microsatellite loci. In order to discriminate among wolf, dog and fox (*Vulpes vulpes*), uncertain genotypes were compared to 22 wolves (dead during last 10 years within the study area), 19 dogs of different breeds, and 4 foxes. The statistical approach for the correct attribution of an uncertain genotype relies on a bayesian clustering method, implemented in the software STRUCTURE version 2.0 (Pritchard et al. 2000). For every individual genotype a probability (*q*) was estimated to be assigned to each cluster inferred by the program. Cluster attribution is based on the genetic closeness to reference samples. As wolves and dogs may crossbreed in nature (Vilà & Wayne 1999), the presence of hybrids in a wolf population should be contemplated, especially if it experienced a numerical decline. By the way, the software may take into account the possibility of hybridization events in the ancestry of a given individual, adopting an admixture model included in the algorithm. Thus genotypes were probabilistically assigned to one population or jointed by two of them in case of admixed ancestry.

The elaboration was carried out using population information just for reference genotypes, whereas no previous information was used by the program for uncertain samples. The run consisted of 20,000 and 100,000 Monte Carlo Markov Chain (MCMC) replications for burn-in period and data collection, respectively. In this calculation, genotypes were attributed to the wolf population if the respective probability ($P_{[wolf]}$) was ≥ 0.95.

Match probability

The non-invasive approach implies that a single wolf specimen may go sampled several times during the study. Hence, one should ascertain that identical genotypes in the population correspond to a single individual. In other words, the probability that two different individuals within the population share the same genotype should be sufficiently low. Woods et al. (1999) proposed to calculate matching probabilities, representing the conditional probability of a matching genotype for a given pair of individuals, considered the allele and single-locus genotype frequencies observed in the population. Such probabilities change in relation to the level of genetic relatedness between the selected individuals. The authors considered three different formulae, referred to random individuals, parent-offspring and siblings. As the probability increase with the relatedness among individuals, the estimate will reach its maximum values when calculated for pair of siblings.

In order to univocally assign a genotype to one individual in the population, I obtained a case-by-case estimate, calculating the conditional probability for sibs ($P_{m[sib]}$) according to Woods et al. (1999), and setting at 0.05 the threshold under which a match was referred to a single individual.

Statistical analysis

All wolf genotypes, which had passed the quality control, were included into a data processing file in Microsoft Excel format. The genotype database was converted by the EXCEL MICROSATELLITE TOOLKIT (Stephen Park, University of Dublin), a Visual Basic compiled macros available at http://oscar.gen.tcd.ie/~sdepark/ms-toolkit/, into multiple formats requested by the most used statistical softwares.

Population parameters were calculated, including allele frequencies, number of alleles per locus (A), expected (H_e) and observed heterozygosity (H_o), with the software CERVUS version 1.0 (Marshall et al. 1998). This program was also used to estimate the polymorphism information content (PIC, Botstein et al. 1980) of each microsatellite marker, and the expected frequency of null alleles, according to Summers and Amos (1997). Probabilities of identity were estimated using GIMLET version 1.0.3 (Valière 2002), referring to a random population with the observed allele frequencies and to a theoretical population of all siblings, following Paetkau and Strobeck (1994) and Waits et al. (2001), respectively.

Deviations from Hardy-Weinberg equilibrium (HWE) were evaluated for the overall population and for each subpopulation (samples within geographic sub-areas) using the Markov chain method proposed by Guo and Thompson (1992) and implemented in the software GENEPOP version 3.2a (Raymond & Rousset 1995). The algorithm used 1000 dememorizations, 200 batches and 5000 iterations per batch. Probability values were interpreted using the standard Bonferroni's correction for multiple tests (Rice 1989). Single-locus probabilities were combined over loci by the Fisher's method. Lack or excess of heterozygosity were evaluated by two ways: by specific tests implemented in GENEPOP or by the calculation of F_{IS} values (inbreeding coefficient) with the software GENETIX version 4.03 (Belkhir et al. 2001). Deviations from linkage equilibrium, despite of the marker physical location, were tested for significance recurring to the software FSTAT version 2.9.3 (Goudet 2001), using 4500 permutations and correcting α-values by the Bonferroni's method for multiple tests.

In order to evaluate levels of heterogeneity among samples across the four geographic areas , F-statistics was computed using Weir and Cockerham's estimators of F_{ST}, F_{IS} and F_{IT} (Weir & Cockerham 1984). Calculations were performed using the program GENETIX, which enabled also to define 95% confidence intervals by 1000 bootstraps over loci. Significance of F-values was assessed by comparison with the distribution obtained in 1000 permutations of individual genotypes. When more than 95% of permutations produced values higher or lower than the actual one, this latter was considered significant. The level of population substructure was evaluated

referring to overall F_{ST} rather than R_{ST} (Rousset 1996). In fact, as remarked by Balloux and Goudet (2002), in presence of medium-high levels of gene flow and a limited number of analysed loci, F_{ST} performs better, especially if mutation model is unknown for the considered loci. Moreover, whereas, for the small population size and the limited time scale, genetic drift is expected to contribute more than mutation in producing allelic divergence, F_{ST} should be preferable over R_{ST} (Balloux & Lugon-Moulin 2002) . Anyway, for comparison, the overall value of R_{ST} was calculated as well, with the program FSTAT. Two different approaches were used to estimate the level of differentiation among areas with GENEPOP: a Fisher exact test was performed to test the homogeneity of allelic distributions across populations (Raymond & Rousset 1995), whereas a log-likelihood (G) based exact test (1000 dememorizations, 100 batches, 1000 iterations per batch) was used for genotypic differentiation (Goudet et al. 1996). The significance level was always established using Bonferroni's criterion for multiple tests. The degree of differentiation was also evaluated graphically using the correspondence factorial analysis (CFA) implemented in GENETIX, and plotting genotypes over a two-dimensional space. To evaluate whether the arbitrary geographic subdivision was consistent with the genetic differentiation among samples, a bayesian clustering analysis was carried out using STRUCTURE (Pritchard et al 2000). The program was let estimate the log likelihood value $Pr\ (X|K)$, i.e. the probability of the data associated to a certain number of subpopulations (K). Values of K comprised between 2 and 10 were tested, considering that 10 was the average number of detected packs in the area during the study. Convergence of this value was obtained over 100,000 replications following a burn-in period of 10,000 iterations. Ten runs were performed for each value of K to verify if results were consistent. The higher log likelihood was associated to the most probable situation, in terms of number of subpopulations. Individual probability were calculated for every genotype to be assigned to each of the K clusters. The correspondence between inferred clusters and geographic affiliation of genotypes was evaluated. Groupings of individuals assigned to the same cluster with a probability ≥ 0.90 were classified as 'core clusters', as they are expected to represent local groups of closely related individuals (packs?). Pairwise genetic distances among geographic areas were calculated with MICROSAT version 1.0 (Minch et al. 1995). Distance measures included Nei's unbiased genetic distance (D, Nei 1978) and pairwise F_{ST}. A neighbour-joining (NJ) tree was drawn using the matrix of $F_{ST}/(1-F_{ST})$ pairwise values with the NEIGHBOUR algorithm included in the PHYLIP 3.5c package (Felsenstein 1993) and visualized in TREEVIEW (Page 1996). The amount of gene flow among subpopulations was estimated as $Nm =(1- F_{ST})/4F_{ST}$.

Further, population subdivision and dispersal were studied by performing assignment tests to individual genotypes. The performance of different assignment methods was evaluated. The

software GENECLASS version 1.0.02 (Cornuet et al. 1999) enabled to test the bayesian method (Rannala & Mountain 1997) and a distance method based on the shared allele distance (Chakraborty and Jin 1993). A third approach, the 'log-likelihood method' (Paetkau et al 1995), was implemented in the software ARLEQUIN version 2.0 (Schneider et al. 1999). For a given genotype, the area for which it obtained the highest assignment value was considered, and the congruence of this affiliation across different methods was evaluated.

The correlation between genetic relatedness and spatial distance was calculated with the software SPAGEDI version 1.1 (Hardy & Vekemans 2002). As concerns parameter choice, the Lynch and Ritland's relatedness coefficient (R_{LR}, Lynch & Ritland 1999) was adopted, as it was depicted performing better and having a lower variance than other similar coefficients (Van de Casteele 2001). To obtain a spatial distance matrix, the geographic coordinates of each sample location were considered. As to a multi-sampled genotype corresponded several locations, in this case the harmonic mean of their coordinates was used. In order to avoid bias in the data, in presence of correlated locations (e.g. a single genotype found more times along the same trail), only one of them was used in the computation. The following distance intervals were chosen: 0-5 km, 5-15 km, 15-30 km and >30 km. Mean relatedness was also estimated within and among geographic areas and within core clusters resulting from the clustering analysis.

Parentage was studied within areas by evaluating parental compatibility for every pair of individuals. A possible parent should have at least one allele in common at each locus with the putative offspring. All pairs of genotypes were thoroughly screened for such allelic compatibility. Hypothetical parent-offspring dyads were then examined by a log-likelihood method implemented in CERVUS, in order to rank candidate parents on the basis of their log-likelihood of parentage (LOD score). The obtained values were used to critically look for the most likely combination of one male and one female, both compatible with the parentage of each given genotyped individual.

Finally, pack structure was determined, wherever possible, combining different kind of data: 1) genetic relatedness, 2) parentage compatibility, 3) sample location, 4) association of genotypes along winter trails.

2.5 Results

Three hundreds forty seven samples were analysed, of which 329 were from non-invasive sampling, 17 belonged to dead wolves and one to an alive wolf. On the whole, 218 of them (62%) produced successfully amplifications, but only 110 multilocus profiles passed the quality control (Tab. 3). With regard to non-invasive samples, overall yields amounted to 65% for blood remains, 35% for hairs and 18% for scats.

Sample type	No. of processed samples	No. of successfully amplified samples	Quality score (mean ± sd)	No. of microsatellite profiles	Yield
ALIVE WOLVES					
Blood	1	1	3.0	1	100%
DEAD WOLVES					
Muscle	8	8	2.9 ± 0.9	7	88%
Plucked hairs	9	9	3.2 ± 0.8	7	78%
NON-INVASIVE					
Blood spot	31	21	2.7 ± 0.6	20	65%
Shed hairs	124	85	3.7 ± 1.3	43	35%
Scat	174	94	4.3 ± 1.1	32	18%
Total	347	218	3.8 ± 1.2	110	32%

TABLE 3. Analysed samples and amplification success.

Multilocus profiles corresponded to 60 different genotypes. Forty five of them derived exclusively from non-invasive sampling, and for them the correct attribution was verified. The discrimination power of the clustering analysis realized by the software STRUCTURE was high, as reference wolves, domestic dogs and foxes were assigned to different clusters with probabilities comprised between 0.963 and 0.999 (Tab. 4).

		INFERRED CLUSTERS			
		I	II	III	n
REFERENCE SAMPLES	WOLF	**0.963**	0.033	0.004	22
	DOG	0.013	**0.987**	0.001	19
	RED FOX	0.000	0.001	**0.999**	4
UNCERTAIN GENOTYPES		0.815	0.115	0.070	45

TABLE 4. Genetic discrimination among wolves, dogs and foxes. The attribution to inferred clusters was based on a bayesian approach.

Uncertain genotypes (i.e. genotypes derived exclusively from non-invasively collected samples) were attributed to the wolf in 37 cases with $0.943 < P_{[wolf]} < 0.996$ (Fig. 17). Five genotypes were attributed to dogs ($0.979 < P_{[dog]} < 0.994$) and 3 to foxes ($0.979 < P_{[fox]} < 0.996$). They were represented by 3 hair samples and 5 scats, mainly collected in peripheral areas respect to known pack territories.

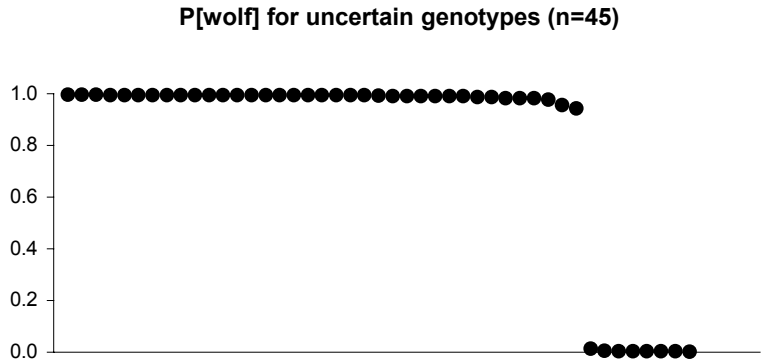

FIGURE 17. Genotype attribution. The probability of assignation to the inferred wolf cluster is reported for 45 uncertain genotypes.

Therefore, the overall number of different wolf genotypes was 52. They are listed in Tab. 5, where their temporal distribution is also reported. Match probabilities averaged 0.0059 ± 0.0099. All genotypes but one had $P_{m[sib]}$ lower than the threshold of 0.05. This latter exception was represented by a partially genotyped individual, different from all others, and sampled just once. As consequence, every individual genotype was assumed to be unique in the population and identical genotypes, resulting from different samples, were attributed to the same wolf.

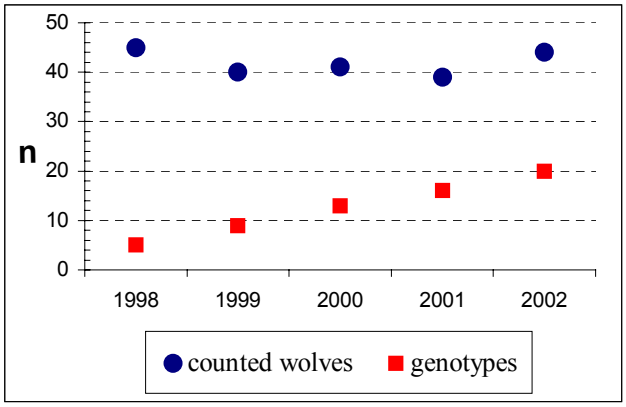

FIGURE 18. Number of genotypes obtained per year and estimated number of wolves in the study area.

The number of genotyped wolves during the study period represents only a portion of the estimated number of wolves yearly present in the population (Fig. 18). The maximum proportion is reached in 2002, as it approximates 45%.

	Sex	N	P_{match} (sibs)
PM5	M	4	0.0025
PM9	M	1	0.0012
PM12	M	1	0.0002
PM14	-	1	0.0138
PM16	-	1	0.0015
PM17	F	5	0.0008
PM18	-	1	0.0005
PM19	M	3	0.0005
PM20	-	2	0.0002
PM22	M	1	0.0107
PM23	M	1	0.0002
PM24	-	1	0.0140
AC8	M	1	0.0115
AC1	M	8	0.0005
AC9	-	3	0.0025
AC3	F	2	0.0009
AC4	F	1	0.0008
AC10	F	1	0.0052
AC11	-	1	0.0087
AC12	F	3	0.0014
AC13	M	2	0.0035
AC15	-	1	0.0016
AC16	M	11	0.0018
AC17	M	5	0.0011
AC18	F	1	0.0038
AC19	M	1	0.0009

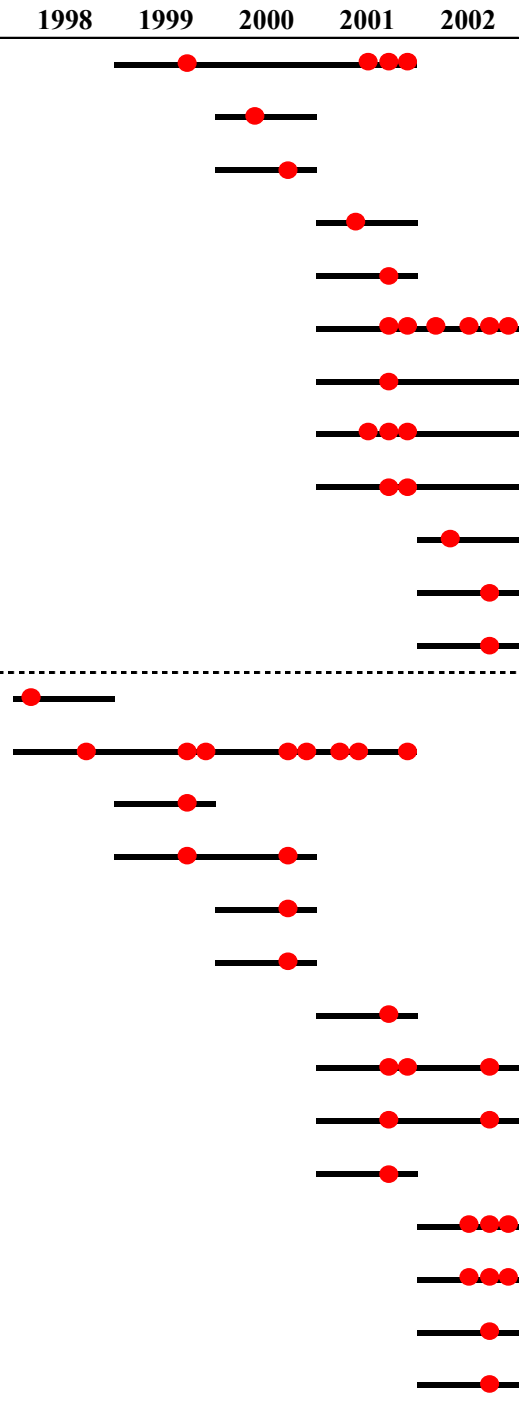

TABLE 5. (continue)

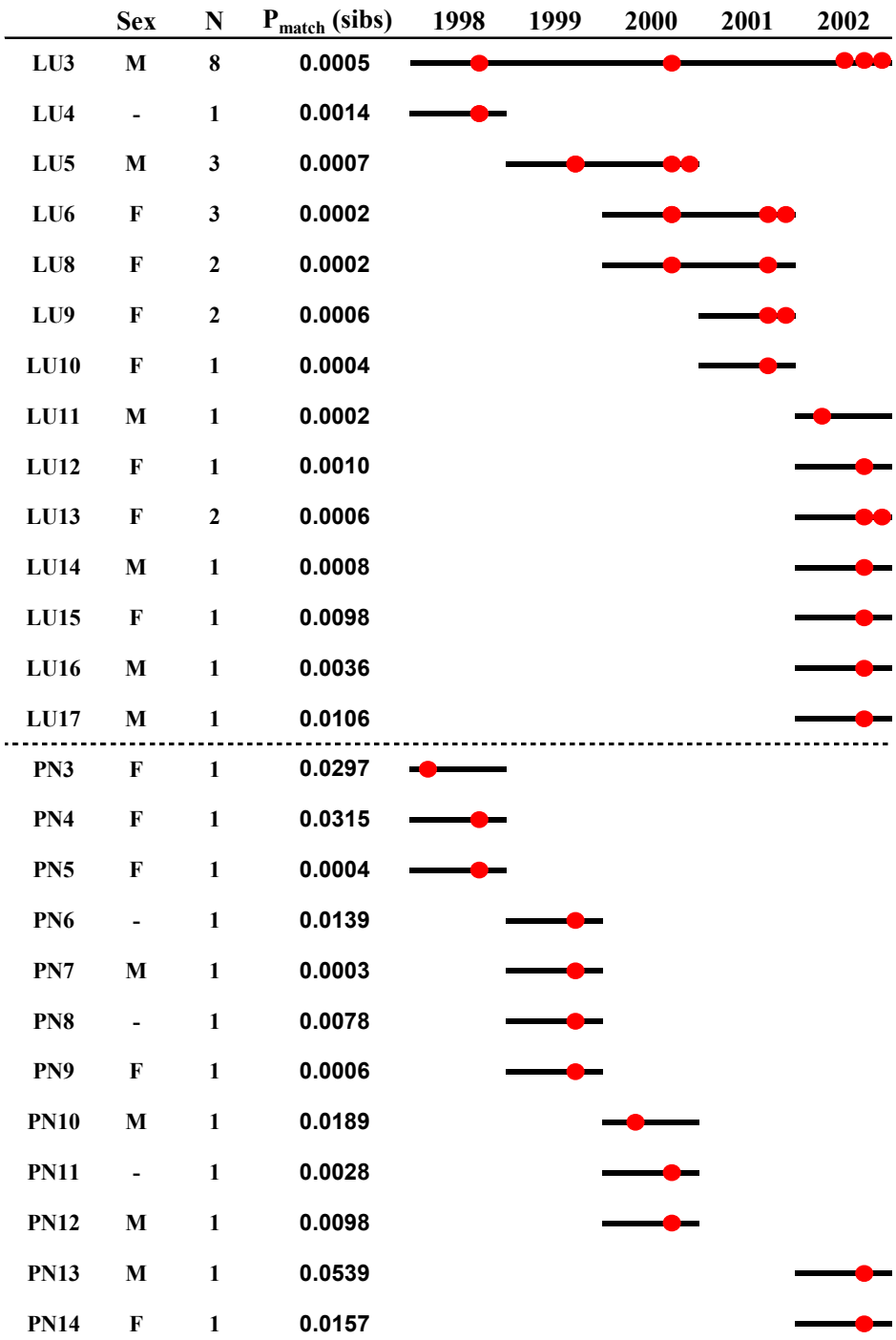

TABLE 5. List of individual genotypes. N refers to the number of samplings. Solid lines indicates the presence of the genotype during the corresponding year. Every red dot represent one or more analyzed sample producing that genotype.

Sampling was spatially unequal, limited to areas intensively used by packs, and 5-6 packs out of 10 were more represented in the data set. Thus, considering such limiting factor, the probability to fully type all members of a pack was scarce.

Genotypes represented almost evenly geographic areas (PM n=12, AC n=14, LU n=14, PN n=12). Their distribution in the study area is shown in Fig. 19.

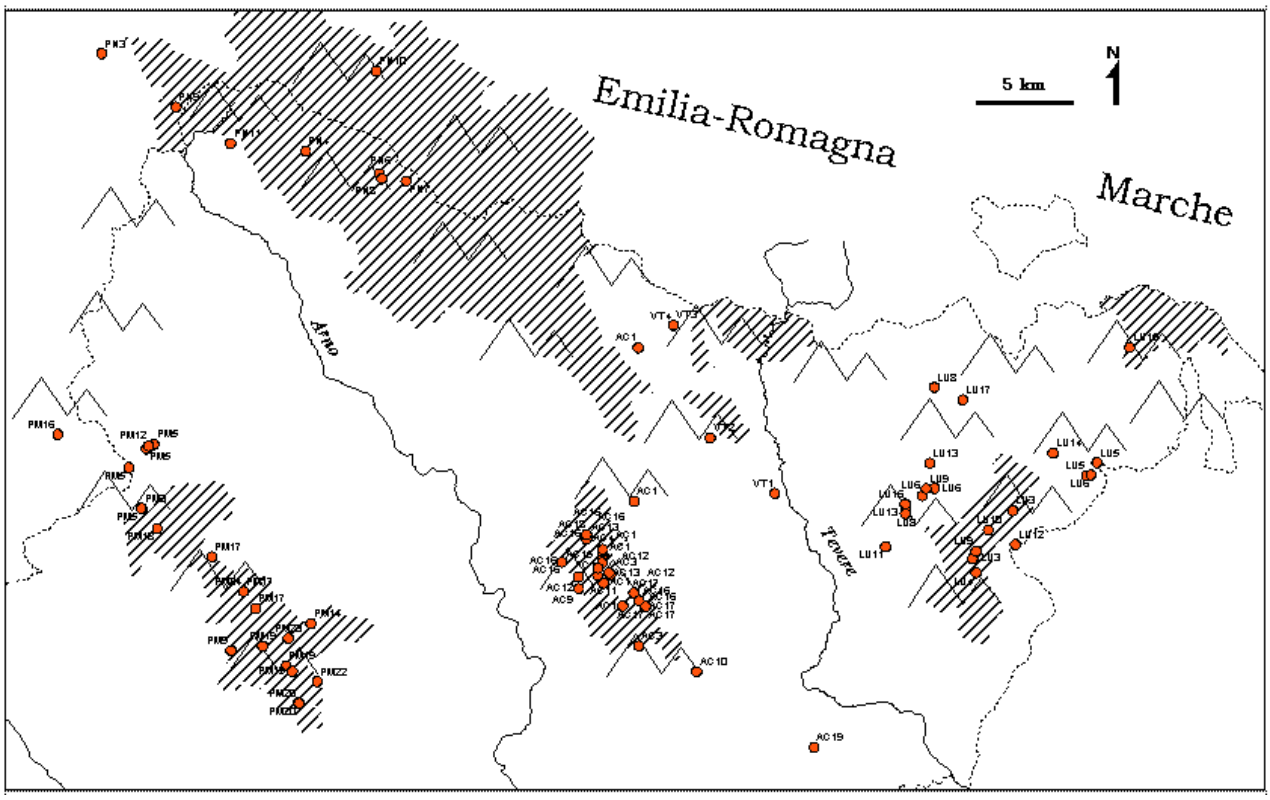

FIGURE 19. Spatial distribution of wolf genotypes in the study area.

Resampling rate

Individual genotypes resulted from one up to eleven different samples, averaging 1.9 samples per single genotype. Excluding samples from dead wolves, the mean resampling rate was 2.2. Samples collected along the same trail in the snow were most likely to belong to the same individuals (not independent). Thirty six genotypes (69%) resulted only from one sample.

Genotypes were resampled on average over a 2-years period. Maximum permanence of genotypes referred to AC1 and LU3 lasting respectively 4 and 5 years (Tab. 5).

Excluding genotypes obtained from dead wolves, 67% of all typed individuals were not resampled in the years following their first sampling.

Genetic diversity, Hardy-Weinberg and linkage equilibrium

Polymorphism varied a lot among loci. PIC ranged between 0.373 (cxx.204) and 0.793 (FH2054) and averaged 0.577 across loci. On the whole, 60 alleles were detected, ranging in number between

3 (cxx.204) and 9 (FH2137) per locus. On the basis of this level of variability, the overall probability of identity (for sibs) was equal to 6.6×10^{-4}.

Expected heterozygosity and number of alleles per locus averaged 0.632 and 6.00, respectively, for the overall population (Tab. 6). Observed heterozygosity was lower than expected for most loci and for the overall population across loci (0.595).

Microsatellite	A	H_e	H_o	PIC	$P_{id(random)}$	$P_{id(sib)}$
CXX109	6	0.554	0.529	0.487	0.266	0.542
CXX123	6	0.741	0.730	0.693	0.111	0.411
CXX204	3	0.483	0.440	0.373	0.377	0.605
CXX250	5	0.703	0.633	0.640	0.149	0.439
CXX377	6	0.520	0.510	0.478	0.272	0.561
FH2004	6	0.594	0.683	0.529	0.229	0.514
FH2054	7	0.830	0.848	0.793	0.058	0.356
FH2137	9	0.788	0.804	0.749	0.079	0.380
FH2158	6	0.480	0.310	0.441	0.310	0.590
FH2175	6	0.632	0.458	0.590	0.1742	0.484
Overall loci	6	0.632	0.595	0.577	2.6×10^{-08}	6.6×10^{-4}

TABLE 6. Genetic variability of the wolf population at 10 microsatellite loci. A is the number of different alleles per locus. Other abbreviations are explained through the text.

H_e was similar among areas and varied between 0.544 (PM) and 0.600 (PN). The Arezzo province wolf population deviated significantly from HWE (Fisher's method, $\chi^2 = 48.3$; df = 20; P = 0.0004). Two loci (cxx.123 and FH2158) violated significantly the equilibrium after Bonferroni's correction. Globally, a deficit of heterozygotes was observed. As possible explanation, the presence of null alleles was only suspected at one locus (FH2158), for which an expected frequency of null alleles of 0.218 was estimated. However, no sample during the study was noticed to do not amplify at one locus, whereas amplifying at other loci, as expected in case of null homozygotes. Even F_{IS} values demonstrated a deviation from HWE. Overall F_{IS} (0.061) was significant at the 5% level (2.3% of 1000 permutations produced higher values), and two loci out of ten (FH2158 and FH2175) showed significant excess of homozygotes. Considering areas separately, none of them deviated significantly from HWE across loci (Tab. 7). A slight excess of heterozygotes was detectable in LU (P = 0.0317).

Area	No. of genotypes	A	H_e	H_o	HWE (exact test)	H deficit P-val	H excess P-val	F_{is} (W-C)	% val. (1000 perm.) >	% val. (1000 perm.) <
PM	12	3.4	0.564	0.511	0.707	0.131	0.867	0.099	7.8	92.0
AC	14	3.8	0.544	0.592	0.163	0.874	0.127	-0.095	91.5	8.5
LU	14	4.1	0.569	0.611	0.651	0.967	0.032*	-0.077	89.2	10.7
PN	12	3.6	0.600	0.652	0.952	0.931	0.072	-0.092	84.2	15.8
TOTAL	52	6.0	0.632	0.595	0.000**	0.034*	0.956	0.061*	2.3	97.7

TABLE 7. Deviations from Hardy-Weinberg equilibrium for the overall population and for geographic areas, separately. Significance of F_{IS} values is expressed as percentage of values, over 1000 permutations, higher (>) or lower (<) than the observed one.

As mentioned above, chromosome location of the selected markers in the canine genome excluded the existence of physical linkage among most of them. Nevertheless, tests for linkage disequilibrium, based on the association of genotypes across loci, produced in two cases slightly significant results: pairs cxx.123 – cxx.377 and cxx.250 – cxx.377 were significant at the 5% level. As no other combination of loci gave significant results, the independence of selected loci was assumed throughout the study.

Population differentiation

The analysis reported a structured wolf population, with a high overall value of F_{ST} (0.125). This latter resulted significantly high, as all values obtained over 1000 permutations of individual genotypes were lower (Tab. 8).

Locus	F_{IS} (f)	F_{IT} (F)	F_{ST} (θ)	R_{ST}
CXX109	0.0296	0.0492	0.0202	-0.014
CXX123	-0.1574	0.0567	0.1850	0.123
CXX204	-0.0038	0.1168	0.1201	0.055
CXX250	0.0055	0.1277	0.1229	0.411
CXX377	-0.1228	0.0569	0.1600	0.082
FH2004	-0.1965	-0.1382	0.0488	0.041
FH2054	-0.1709	0.0190	0.1622	0.433
FH2137	-0.1421	0.0136	0.1363	0.118
FH2158	0.3410	0.3625	0.0327	0.036
FH2175	0.1453	0.3137	0.1970	0.266
All	-0.0412	0.0893	0.1253	0.1340
Significance over 1000 perm.	P = 0.111	P = 0.000	P = 0.000	
Jackknife (mean ± se)	-0.0428 ± 0.0498	0.0884 ± 0.0421	0.1260 ± 0.0187	

TABLE 8. F-statistics for the Arezzo province wolf population. Jackknife was carried out over the 4 subpopulations. Overall R_{ST} value is weighted over loci according to Rousset 1996.

Being F_{IS} slightly negative (-0.041), although not significant, the level of inbreeding within the population is not such to justify the low number of homozygotes. This was also confirmed by results of tests for allelic and genotypic differentiation. Both produced highly significant levels of heterogeneity (Fisher's method, χ^2=infinity, df = 20, P=0.0000). Further, every pairwise comparison between sampling areas resulted significant at the 1% level.

Even the AFC pointed out graphically the effect of the observed level of genetic differentiation (Fig. 20).

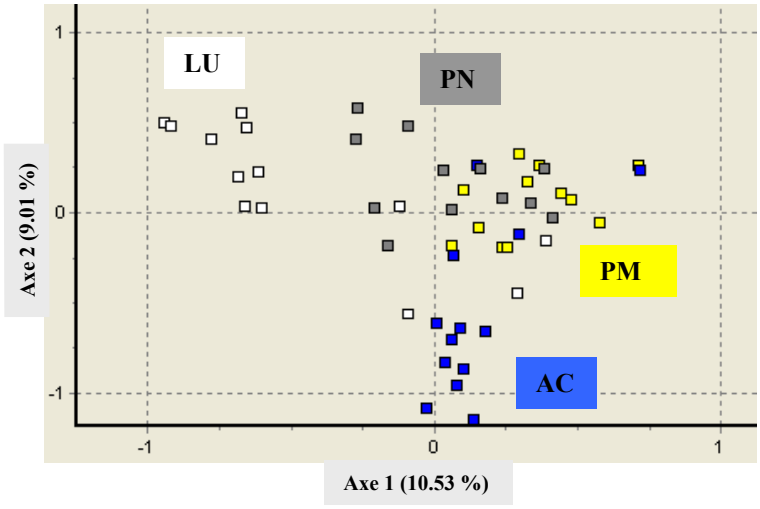

FIGURE 20. Factorial analysis of correspondences for wolf genotypes.

Pairwise F_{ST} and D values, shown in Tab. 9, are in agreement. The NJ tree in Fig. 21 shows values assumed by a measure of genetic distance usually correlated with geographic distance ($F_{ST}/(1-F_{ST})$). Estimated gene flow (Nm) assumed values comprised between 1.26 (PM-LU) and 3.74 (PM-PN).

Area	PM	AC	LU	PN
PM	*	0.129	0.312	0.101
AC	0.086	*	0.218	0.214
LU	0.166	0.139	*	0.235
PN	0.063	0.135	0.136	*

TABLE 9. Matrix of genetic distances between geographic areas. The upper triangular matrix contains values of D (Nei 1978), whereas lower triangular matrix contains pairwise F_{ST} values.

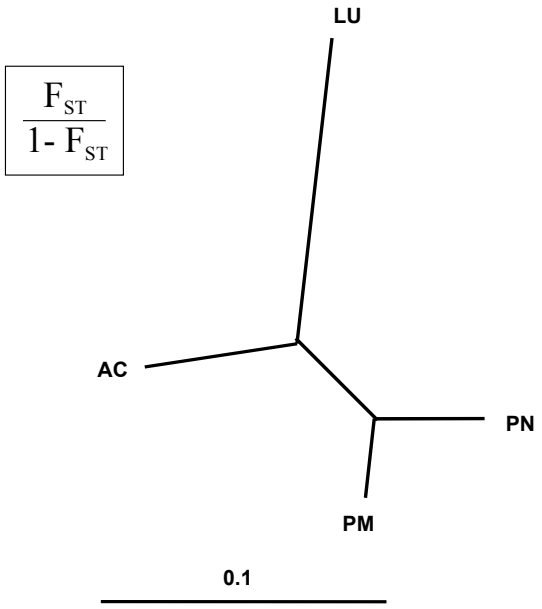

FIGURE 21. Neighbour-joining tree based on the genetic distance estimator ($F_{ST}/(1-F_{ST})$).

The bayesian cluster analysis, including all 52 individuals, contributed to characterize the population substructuring. The highest log likelihood values (Fig. 22) were obtained for *K* = 4 (mean LnP = -940). The low standard deviation associated to this value over 10 runs (sd = 5.6) confirmed the reliability of the estimate.

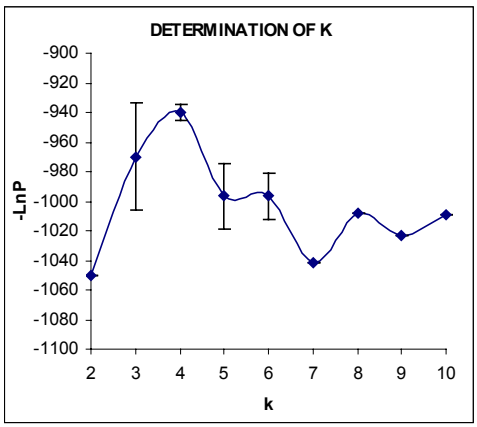

FIGURE 22. Probability associated to different values of *K* (number of subpopulation).

Looking at the correspondence between inferred clusters and geographic areas, PM, AC and LU were consistent to be identified with clusters II, III and IV respectively (Tab. 10). The identification of the area PN with cluster I is on the contrary less consistent. Considering individual probability of

assignation to each cluster, AC and LU were represented by 9 and 8 individuals, having a high (>0.90) probability to be assigned to clusters III and IV, respectively (Fig. 23).

		INFERRED CLUSTERS			
	n	I	II	III	IV
PM	12	0.198	0.724	0.066	0.012
AC	14	0.173	0.178	0.637	0.012
LU	14	0.156	0.112	0.131	0.601
PN	12	0.428	0.367	0.050	0.156

TABLE 10. Inferred clusters by bayesian analysis (STRUCTURE) and geographic areas.

These groupings, named 'core clusters', were further considered in order to test the hypothesis of representing familiar groups characterizing genetically the respective geographic area.
Three individuals showed a significant association to a cluster not corresponding to the geographic area in which they were found. Thus they are strongly suspected to represent immigrants .

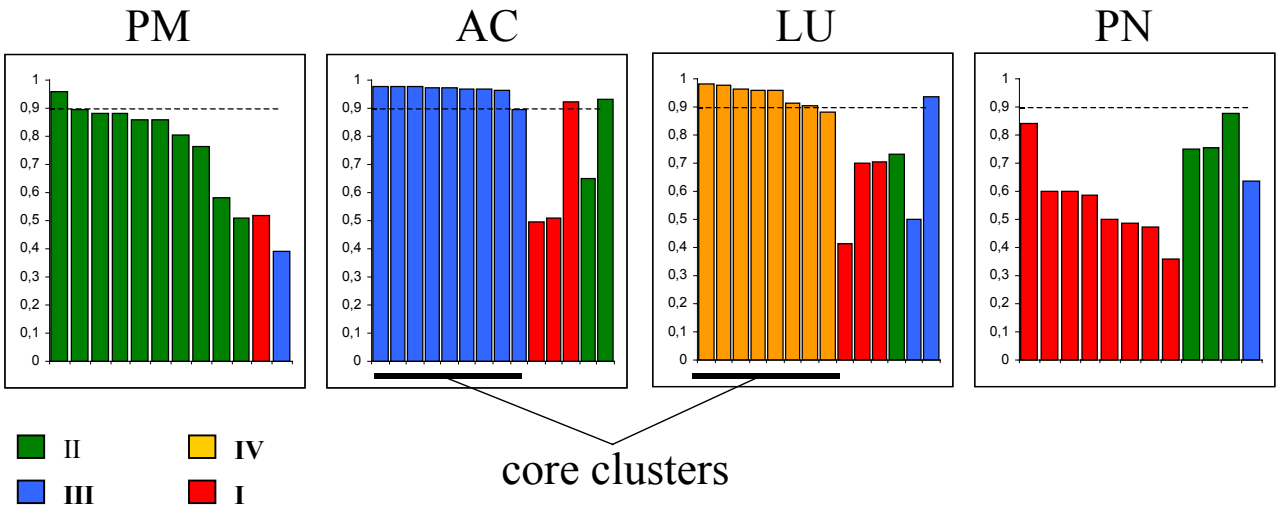

FIGURE 23. Genotype assignation to inferred clusters. Only clusters (colours) with the highest probability of assignment are reported for each genotype. Genotypes are grouped into four areas on the basis of sample location. A significance threshold of 0.90 is assumed.

The compared assignment tests produced a high proportion of 'correct' assignments, varying from 62% (genetic distance method) to 87% (allelic frequencies method). The 'validity' of the method was referred to the geographic location of genotypes. The best-performing test enabled to discriminate well among sampling areas by plotting log-likelihood values on a bi-dimensional space

(Fig. 24). Dispersing individuals (i.e. individuals moving from the natal territory to a new different area) are represented by genotypes plotting together with wolves from a different geographic area.

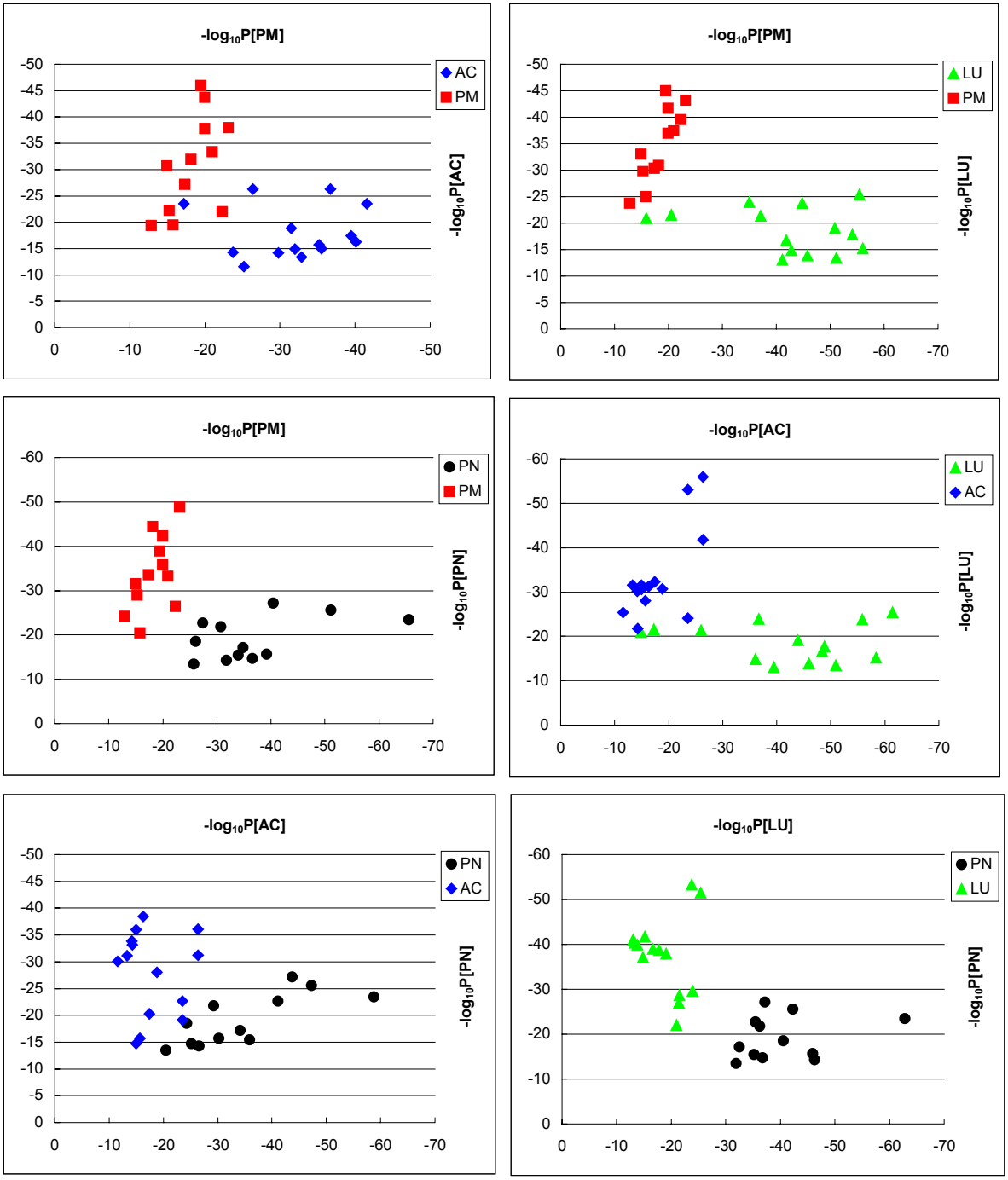

FIGURE 24. Log-likelihood plots. Geographic assignation based on genetic data enables to evaluate the degree of genetic similarity among individuals of different areas.

Relatedness and pack structure

Patterns of relatedness reflect geographic partition of samples. Calculations were based on 1326 pairwise values. R_{LR}-values averaged 0.126 ± 0.306 within populations and –0.066 ± 0.214 between populations, both deviating significantly from zero ($p < 0.001$). Average pairwise distances were 7.5 km within and 28.5 km between areas. A clear inverse correlation between relatedness and geographic distance was observed ($r^2 = 0.0953$, $p < 0.01$; Fig. 25). R_{LR}-values were positive for up to 15 km of distance between samples. Pairs within this range (0-15 km) showed significantly high levels of relatedness ($P_{[expected > observed]} = 0.000$, based on 10,000 permutations). No sex bias was detectable in the data set. The decline of relatedness over space was similar for both sexes (Fig. 25).

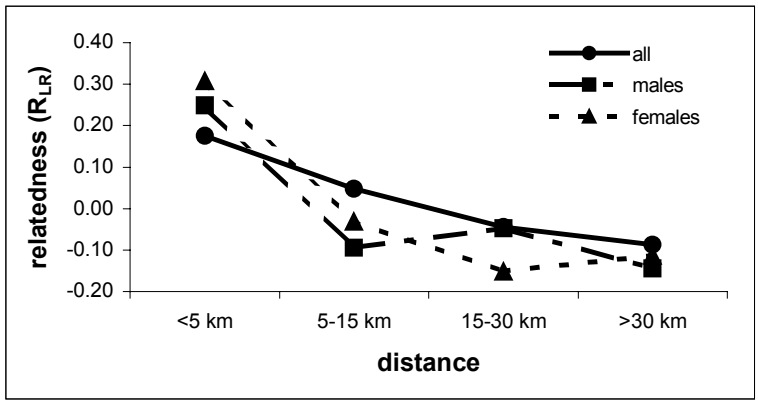

FIGURE 25. Genetic relatedness and linear distance between individuals.

In AC and LU the average R-value considering all sampled individuals was close to the expected value for first cousins (0.155 and 0.125 respectively).

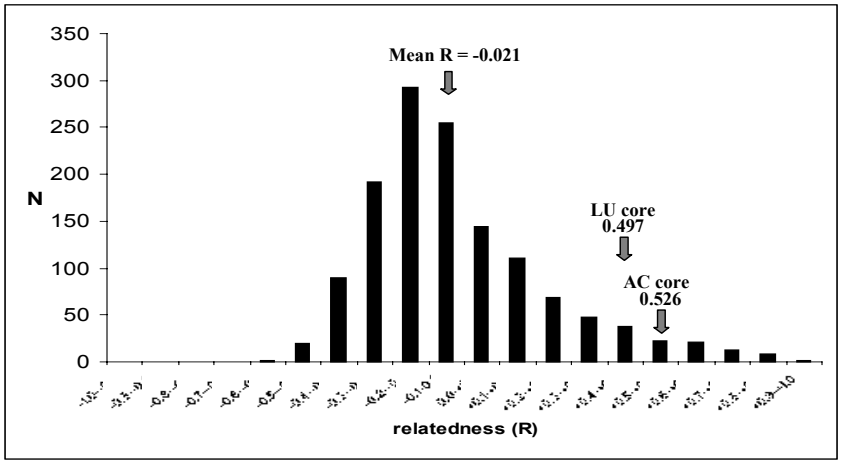

FIGURE 26. Distribution of pairwise relatedness values. Average values for AC e LU core clusters are reported.

Restricting the computation to the so called 'core clusters', a far higher relatedness was found (Fig. 26). Indeed, AC-core wolves had a coefficient of relatedness averaging 0.526, whereas core individuals in LU were slightly less related (mean R = 0.497). In both cases R-values approximated the expected estimates for parent-offspring and full-sibling pairs. Considering parental compatibility based on allelic transmission, space-temporal sample locations, genotype association in the field, relatedness measures and LOD scores, three possible familiar lines may be identified in different areas.

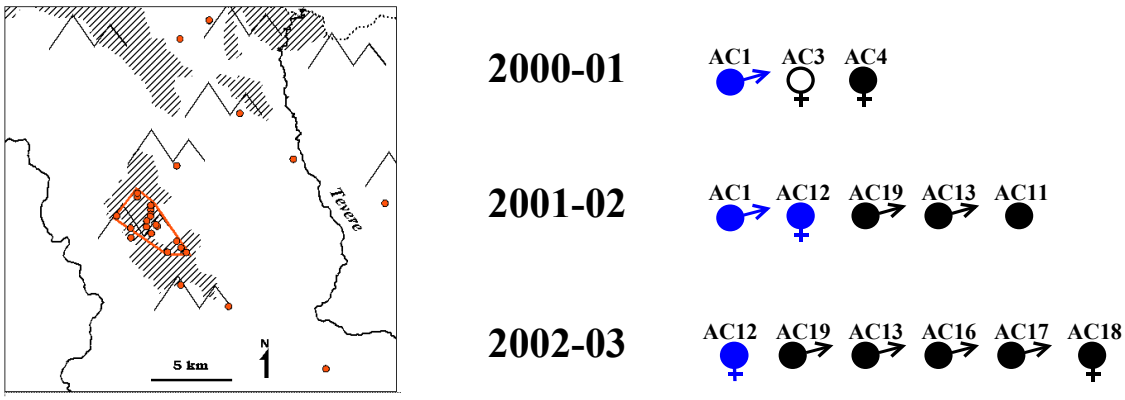

FIGURE 27. AC pack composition in winters 2000-01, 2001-02 and 2002-03 and area interested by locations of related wolves (red polygon). Individuals in blue are likely to represent the alpha pair. Solid symbols represent related ('core') wolves. Empty symbols represent unrelated wolves.

Among genotypes within the AC area, nine (core) were attributable to the same familiar group. All respective samples were collected over 4 years within a 10-km^2 area (Fig. 27). Their possible relationships were studied and a possible pedigree resulted (Fig 28), justifying the parentage of six individuals in the last two years from the same supposed breeding pair (male AC1- female AC12). These supposed mates were not strictly related (R = 0.097), but they were related to all the other 'core' wolves sampled in the area.

Male AC1 was detected through four consecutive years in this area, thus suggesting a dominant position in the pack. In winter 2000-01, a trail in the snow revealed the association of AC1 with two other individuals. One of them (AC4) was closely related to AC1 (R = 0.631), whereas the other was unrelated (AC3, R = -0.041). Male AC19 was not sampled until winter 2002, when he was found dead approximately 10 km away from the area. He was 20 months old (i.e. born in spring 2001) and autopsy revealed he was shot and transferred afterwards to the final location. Thus, his belonging to the AC pack was attributed just on a genetic basis.

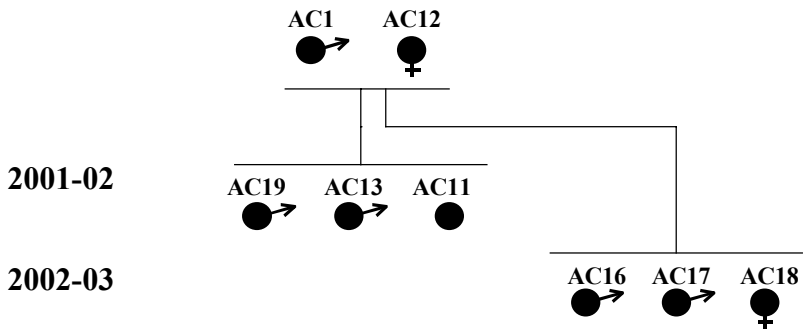

FIGURE 28. Proposed relationship among AC 'core' wolves. Parentage is based on visual analysis of allele sharing and on pairwise LOD scores.

Two dead individuals (male AC8 and female PN12) were likely to be the parental pair of wolf AC3, sampled for two consecutive years (1999 and 2000). AC8 and PN12 were both adult (more than 3 years old) as they were killed, respectively in May and December 1998, and were only slightly related (R = 0.109). The exact death site is unknown, because both carcasses were brought into villages and there abandoned by poachers. However such villages are close to the Alpe di Catenaia massif, where AC3 was sampled. Assuming AC3 was born in spring 1998 or before, it was compatible with having generated individuals from summer 2000 on. On the basis of allele sharing, PN10, AC15, PN14 and PN15 represented possible offspring (Fig. 29). No other possible parent was among the typed individuals. PN10 was found dead more than 20 km north-west respect to AC3. He was a young and was not related to the closest genotypes sampled within the National Park. Wolf AC15 was sampled in 2001 in the AC area, close to the sampling sites of several 'core' genotypes, but did not resulted related to any of them. PN14 and PN15 were sampled in winter 2002-03 along a trail travelled by 4 wolves in the snow. They were in a valley north to the AC area, within the territory of a different pack, identified by wolf-howling in summer.

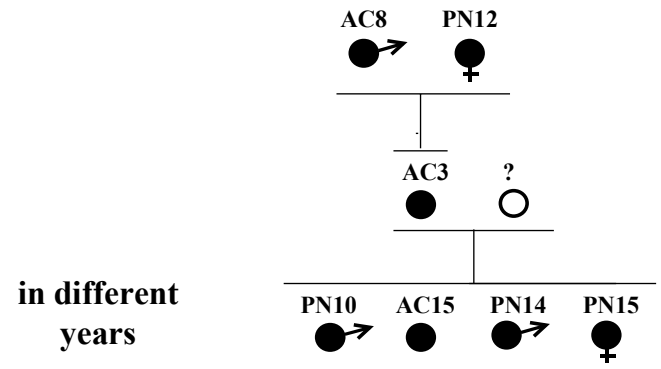

FIGURE 29. Proposed relationship among wolves sampled in AC and PN areas. Parentage is based on visual analysis of allele sharing and on pairwise LOD scores.

The same evaluation approach enabled to formulate a possible figure for the LU area. Here the situation is complicated by the fact that more than one pack was present. The eight 'core' wolves were sampled within a 40-km^2 area, in the range of two packs (Fig. 30). Male LU3 was consistent with being the father of all other related wolves. This seems to be enforced by the long permanence of this genotype; it was sampled in 1998, 2000 and 2002, demonstrating it was at least five years old at the end of the study. In the case of LU3 alpha male, no typed female could be indicated as his mate and mother of more than one sampled wolf. All core genotypes were strictly related, minimum at the first cousin level. Three wolves (LU9, LU12 and LU14) were found dead in March 2002 and in winter 2002-03. Their age was estimated < 1 year. They were possible to have common parents, one of which may be represented by LU3.

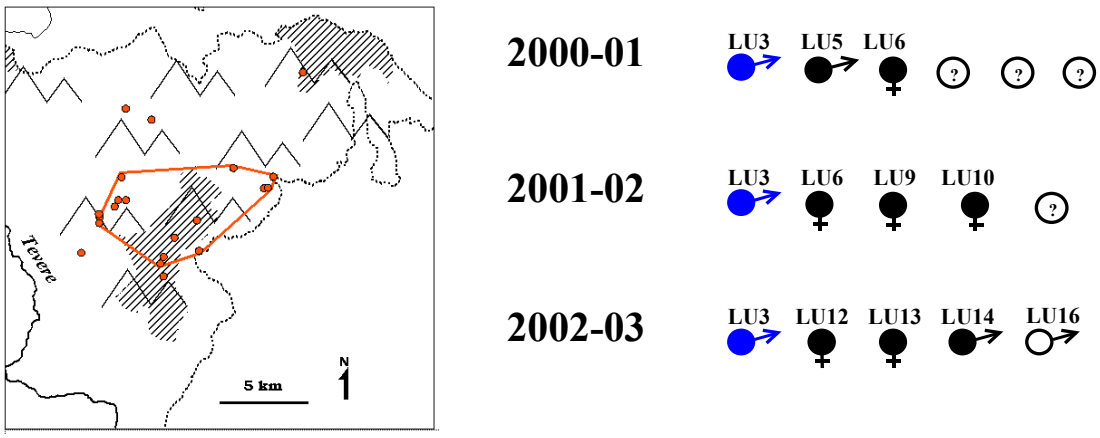

FIGURE 30. LU pack composition in winters 2000-01, 2001-02 and 2002-03 and area interested by locations of related wolves (red polygon). The individual in blue (LU3) is likely to represent a breeding male. Solid symbols represent related ('core') wolves. Empty symbols represent unrelated or not typed (question mark) wolves.

In the other two areas (PM and PN), the assignment test did not detect a group of individuals strongly characterizing these areas themselves. In relation to this, overall levels of relatedness were lower among typed individuals.

Two wolves were not related to any other genotypes in the data set. A wolf carcass (AC10), found in the periphery of the AC area, showed traces of a past hybridization with the domestic dog. The second corresponded to an other dead wolf (PN3) recovered at the north-west extreme of the study area, outside of the National Park.

Dispersal and spatial behaviour

The large majority of genotypes (90%) was sampled in the same area over a period of only 1-2 years. Just two individuals were sampled during a period longer than 3 years. They are males AC1 and LU3, which probably represented the breeding males of the AC and LU packs respectively.

One out of 10 individuals resampled in different years was found in two different geographic areas (Fig. 31). Male AC1, indeed, was in AC for four years consecutively, but in November 1999 he was sampled in a close zone (PN); nevertheless his permanence in this area was limited, as one week later he was resampled in the AC area. On the contrary most genotypes were constantly found in the same area and mean distance among individual locations was 3.8 km (maximum 13 km), corresponding to an average sampling interval of 10 months. Moreover, 77% of all pairwise distances were lower than 5 km.

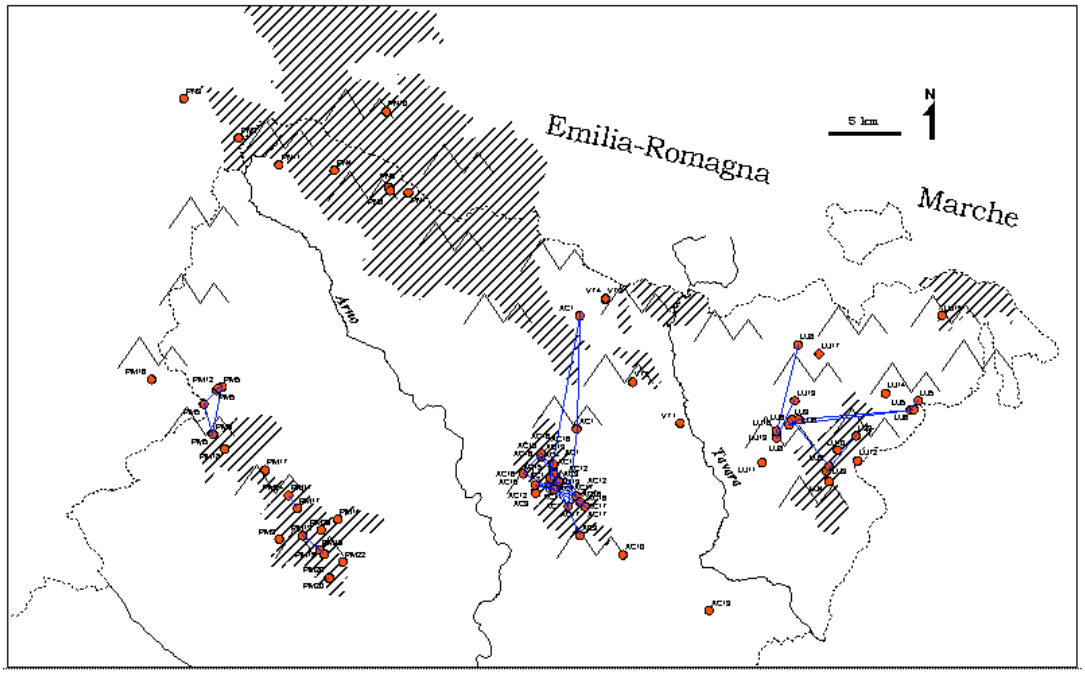

FIGURE 31. Movements of resampled wolves (in blue), as resulting from genotype locations.

Mortality rate is not evaluated in the study area. Recovered carcasses are expected to represent a small proportion of all dead wolves. Among genotypes 14 derived from wolf carcasses. For 12 of them an estimate of the approximate age was obtained and 9 were young, less than two years old. Their location was mostly peripheral to pack territories.

Assuming 'core' genotypes are members of the same pack, their locations in the same year can represent an useful information on the pack spatial behaviour. In AC yearly (mainly winter) locations of related individuals fell within a range of 5 km, both in 2001 and 2002. For LU, where individuals were suspected to belong to two adjacent packs, the same estimate increased to 9 km.

2.6 Discussion

The genetic structure of a wild wolf population was investigated by microsatellite analysis. The use of a non-invasive sampling design and the employment of a panel of ten polymorphic loci provided sufficient samples and genetic information to trace the microsatellite profiles of 52 individuals over a period of 5 years. The obtained data set was used to verify some common beliefs, based on the knowledge of the species in other ecological contexts. Results are summarized in Table 11.
The following working hypotheses were considered:

1. wolf packs are formed by a breeding pair of unrelated individuals and their offspring;
2. offspring disperse early in their life, dispersal is substantial and mostly successful;
3. the population has low genetic structure, due to high gene flow;
4. territories are relatively small.

1) The first point should be faced by full sampling of several wolf packs. This was not possible in the present study. Nevertheless, the best results were obtained in Alpe di Catenaia, where pack structure was derived in 2000, 2001 and 2002. In all cases no more than one pair of unrelated individuals was detected. The obtained genotypes were consistent for at least two winters (2001-02 and 2002-03) with the figure of a pack formed by a pair of unrelated breeding individuals (AC1 and AC12) and their offspring (Fig. 28). In spring 2001, an additional unrelated individual (AC15) was sampled in the area, and this was interpreted as a temporary crossing of the AC pack territory by a dispersing wolf. The lack of signs of association with other local genotypes and the proposed origin from AC3 support this interpretation.
In Alpe di Catenaia, the most consistent breeding pair (AC1-AC12) was formed by unrelated wolves, whereas the other tentatively identified pair (AC8-PN12) was slightly related.
Although the definition of wolf pack given by Mech (1981) does not take into account relatedness among its members, the common statement is that packs are formed by related individuals (Bekoff et al. 1984) and basically represent one-family units (Mech 1981, Peterson 1977, Potvin 1987). Although some cases of 'non-family' packs have been reported (Mech & Nelson 1990), in the few cases in which intra-pack relatedness was genetically verified, results confirmed the common belief. The first attempt to study the composition of free-living packs was conducted by Lehman et al. (1992) using mtDNA and multilocus fingerprinting analysis. They analyzed wolves belonging to 35 packs inhabiting three different regions of North America (Minnesota, Inuvik and Denali) and measured genetic similarity among individuals within each cluster of packs. In this study animals were live-captured and for most of them pack affiliation, age, social rank and reproductive status

were known. Most packs resulted composed by closely related individuals, but, interestingly, in several packs the presence of unrelated non-breeding individuals was detected. In a recent study on the Italian Alps, a colonizing wolf population was investigated by non-invasive molecular methods employing microsatellites (Lucchini et al. 2002). Individual genotypes were obtained from collected wolf scats and their respective relationships were indirectly reconstructed by combining sample location and pairwise relatedness between individuals. Wolves clustered into two pack-like units, spatially separated. The two inferred breeding pairs were formed by first-order relatives and pack composition resulted different from a simple family (parents + offspring). Additional related individuals were present in association with breeding adults and their putative offspring. Furthermore, three unrelated genotypes were sampled in the areas frequented by packs.

These examples provide evidences that, though a familiar unit is likely to represent the base element of a pack, complex patterns, deviating from the 'pack as a family' situation, are likely to occur in natural populations. As occurring in other canid species (e.g. African wild dog *Lycaon pictus*, Girman et al. 1997), packs may represent extended family units, consisting of parents, their offspring and their adult siblings, which may occasionally be joined by unrelated individuals. This can explain the finding of only two consistent clusters of closely related individuals in my data set.

2) Pack composition and size is a function of mortality and recruitment, as well as time of dispersal by pack members. Dispersal patterns in natural wolf populations are influenced by a series of factors, including wolf density, prey abundance, protection/exploitation level, and availability of potential territories (Van Ballenberghe 1983b, Gese & Mech 1991, Boyd & Pletscher 1999). Responding to some of these variables, wolves may disperse early in their life. This strategy was, for instance, adopted by young wolves in a newly protected population in Minnesota (Fritts & Mech 1981), where their dispersal peaked at 11-12 and at 17-19 months of age (Gese & Mech 1991). Actually, the minimal physiological requirement for wolves to disperse seems to be puberty, they reach at approximately 10 months of age, as remarked by Mech (1987).

If early dispersal is a common behaviour among young wolves, they could be sampled in their natal territory only during the first 1-2 years of their life. Then, in case of successful dispersal, they will be sampled in a different area, where they have established a new pack. Alternatively, if the search of a mate and a new territory fails, these individuals will roam and are more likely to go killed (at the present level of induced mortality). In such latter scenario, genotypes will disappear from the natal area and will never be resampled.

During the study, a slight proportion of individuals was sampled over a time longer than one year. Considering only such genotypes, a high spatial fidelity was remarked. In very few cases, the genotyped individual was resampled in different areas. The most remarkable case concerns male AC1, who was always located within the AC area, apart from an 'excursion' into the adjacent territory of the Valle Santa pack (approximately 13 km apart). All other wolves in AC and PM were steadily found within the same area, even over a period of three years. A more complex situation was found in LU, where number of packs and territory definition changed from year to year. Here most genotypes were resampled over longer distances (up to 8 km), over a period of 2-5 years (Fig. 31). These movements may be associated to joining a new pack or to territory changes by their own pack. The fact that a single genotype (LU3) was compatible with being the father of all 'core' wolves and that these were sampled all throughout the area suggests the existence of two strictly related packs or, alternatively, of an unique pack, temporarily split into two groups.

These data seem to deny high levels of successful dispersal in the wolf population, at least over short distances. In fact, the limited geographic scale of the study is expected to do not detect the effects of possible long-range dispersal. The evidence that most genotypes were sampled just over one year, may be either indicative of a high proportion of unsuccessful dispersal or of a substantial dispersal towards far non-monitored areas. The high proportion of pups and yearlings among dead wolves (60% – Capitani et al. 2003) indicates that high human-caused mortality can severely prejudice the success of early dispersal.

If high levels of long-range dispersal exist, and this is bi-directional, a number of immigrants coming from distant areas are expected to appear each year in the population. In this case, a high proportion of wolves in the study area should have no genetic relatedness with any other sampled wolf. This was not the case in the present study as only two genotypes (4%) matched this condition. Hence, long-range dispersal does not seem to strongly affect the composition of the studied population. But dispersal may also be directional, if wolves leaving the study area are more abundant than incoming immigrants. In this case, the detectable effect on the population would be similar to that of unsuccessful dispersal.

The wolf is considered one of the carnivore species able to disperse at highest rates and over longest distances (Mech 1995). In North America wolves are documented to move up to 670 km from their natal territory (Van Camp & Gluckie 1979), and pups were reported to cover longer distances respect to yearlings and adults (Gese & Mech 1991). Average dispersal distances were 40 km in a forested lowland of Quebec (Potvin 1987) and 77 km in Minnesota (Gese & Mech 1991). For the recovering Italian population, Boitani (1992) reported dispersal distances of 50-80 km for 18-24 months old wolves. Upon dispersal a continuum of possibilities are described. A disperser might

find a mate and an area with sufficient prey at the border of its natal territory, next to it, or in neither of these places and be forced to search for them far away (Mech 1987). If the population is locally saturated, no vacancy will be found in proximity of the natal pack, even in presence of potential mates. In such a case wolves move over long distances. Two patterns are associated to this strategy: directional dispersal or nomadism. In the former case, dispersers move along an almost straight line in one general direction. This behaviour could be adaptive as in such a way they reach quickly the border of the population, where the chance to find a vacant territory is generally higher. On the other hand, wolves may wander over wide areas (> 4,000 km^2, Mech 1987) checking for a mate and an available territory. In all cases, when they fail in one or both these purposes, they can at any time go back to the natal pack or try to join another existing pack.

The study population shows the features of a local saturation. Packs are close to each other and most of them successfully reproduce every year. Consequently, it is likely that dispersing wolves are in the condition to move far away in search of unattended territories, where to establish a new pack. The high local density would, on the other hand, prevent the immigration of dispersing wolves from other areas of the Italian peninsula. By virtue of this scenario, the population under study is likely to behave as a *source*, from which wolves disperse toward *sink* areas. This issue is of crucial interest for the wolf conservation in Italy, where, although a continuous distribution of the species throughout the peninsula, patchy conditions are present as concerning favourable habitats, prey availability and human impact.

3) Social structure and dispersal pattern strongly influence the genetic structure of a population. In wolf, from moderate to high level of genetic differentiation were detected throughout North America (Roy et al. 1994, Forbes & Boyd 1997). Weir and Cockerham's F_{ST} values ranged between 0.009 and 0.188 between different regions in northern Canada, in presence of significant physical barriers (Carmichael et al. 2001). These studies were carried out on a wide geographic scale, where the effect of local population dynamics are diluted.

In the present study a high level of population structure was found within the Arezzo wolf population. Despite of the small geographic scale, a significant level of genetic differentiation was observed among areas harbouring different wolf packs. The significant value of F_{ST} (0.125) could be attributed to a Wahlund effect, since the overall heterozygote deficiency could depend on the combination of locally differentiated gene pools. This interpretation is supported by the fact that, unlike the overall population, each of the four considered subpopulations was in Hardy-Weinberg equilibrium. All tests for genotypic differentiation produced significant values, and the Bayesian analysis assuming different number of subpopulations confirmed the goodness of the geographic

subdivision considered *a priori* in the study (maximum likelihood for K = 4). Gene flow was limited among areas, especially in the eastern part of the province. No apparent topographical barriers to gene flow are present in the study area. The two main rivers (Arno and Tevere) are close to their springs and therefore have a limited water load. More importance could have human settlements and human activities concentrated along the valleys, formed by these rivers. However, wolves in Italy, like elsewhere, are documented to tolerate even high levels of human interference (Boitani 1992).

The detected level of differentiation may be affected by the misleading effect of unequal sampling. In other words, the detected structure may result from single familiar units representing subpopulations; in this case, the measured divergence would simply reflect the random differences in gene frequencies among few wolf lineages. In order to understand the weakness of this interpretation some aspects should be remarked.

Wolf packs were not evenly distributed among the four areas and their location was largely influenced by topographical features. Anyway, each year every area, but AC, contained more than one pack. Moreover, as already mentioned, even monitoring and sampling efforts were not homogeneous through the study area, but territories of at least seven packs were interested by intensive sampling. Nevertheless, the Bayesian assignment test and coefficients of relatedness enabled to detect two lineages, respectively in AC and in LU areas. Besides these two cases, no other clear lineage was reconstructed, as consequence of very partial sampling and of a relevant turn-over in many packs in the study area. Further, the effect of time is to be considered. Samples were collected over 5 years (1998-2003), and therefore the genotypes data set includes different overlapping generations. This entails that in one or more cases an existing lineage could have been replaced by a new social unit, genetically different from the previous one. Hence, it is quite difficult to imagine that in my data set, each sampled area may correspond to a single or few related familiar units.

More likely, the local differentiation may arise from the overall stability of the population, due to the saturation of suitable habitats. The high fidelity to rendezvous sites shown by packs in summer (Fig. 9) and the low variance in the average inter-pack distances (Tab. 1) suggest that pack territories are quite stable, as expected in a saturated population. As expressed above, in this situation low-range dispersal is unlikely to occur, as well as immigration from distant areas. The reduced genetic exchange over the study area would lead to the observed local genetic divergence.

4) Finally, genetic data may give some indications even on the extension of wolf territories. Territory sizes may range from 78 km^2 in areas where wolves prey primarily on deer (Fuller 1989)

to 2,541 km^2 where moose and caribou are the main prey (Ballard et al. 1987). In Italy, territories comprised between 75 and 300 km^2 are reported (Ciucci & Boitani 1998).
The measured average distance between packs in summer (12.9 km) indicates that they are confined to restricted areas, even if the amount of territory shared by contiguous packs is not known. Assuming such inter-pack distance as the diameter of the average hypothetical territory, and assuming no overlap among adjacent territories an upper limit of 170 km^2 is obtained. This information and the limited size of monitored packs would suggest a figure in which small packs defend small territories, as already reported in a previous long-term study in the area (Apollonio et al. 2004). The constancy of genotype locations, even in different years, supports this figure. Moreover, calculations referred to 'core' genotypes testify that related wolves were sampled through the same winter within a range of 9 km. Even in this case, the sampling effort, major in areas where a maximal frequentation by wolves was observed, can strongly affect such result. Nevertheless, even where a mountain chain was continuously monitored (e.g. PM area), individual locations were extremely concentrated. A similar result was obtained by Lucchini et al. (2002 – figure 1) in the western Alps, where individuals of two packs were monitored by molecular tracking through 15 months. Most individuals were fixed along one or two adjacent valleys, within a range of 10-12 km.

Considering all evidences here reported, I can conclude that Italian wolves, in mostly protected mountain areas and in presence of abundant prey, may reach saturation even if subject to high levels of induced mortality. Under these circumstances, such areas become sources from which dispersing wolves are likely to prime new colonization events. This process limits local gene flow, producing unsuspected levels of genetic divergence among wolf nuclei.

HYPOTHESIS	EXPECTATION	EVIDENCES	RESULT
❶ pack composed by an unrelated breeding pair and their offspring	high relatedness among all individuals inhabiting the same area, except for a single pair (breeding adults)	in AC area all sampled individuals in 2001-2003 were highly related apart from a pair (AC1-AC12), consistent with representing the breeding pair	***partially supported***
	possible unrelated individuals temporarily present in the area occupied by a pack or peripheral to it (dispersing or satellites)	one unrelated individual sampled once in the AC area in 2002 was not associated to member of the local pack and was assigned to a different pack	
❷ high early dispersal	high proportion of individuals present only for 1-2 years in their natal pack	90% of genotypes sampled over a period of 1-2 years in the same area	***supported***
		the only two individuals sampled in the same area over >3 years (AC1 and LU3) were probably breeding males	
- successful over short distances	many individuals are found at different times in different geographic areas	no evidence	***not supported***
	few or no individuals unrelated to any other individual in the population (immigrants)	no evidence	
	high gene flow and low level of genetic differentiation over small geographic scale	no evidence	
- successful over long distances	many individuals suddenly disappear	67% of genotypes, after first sampling year, were not resampled in the following years	***partially supported***
	many individuals unrelated to any other individual in the population (immigrants)	no evidence	
	moderate gene flow and low level of genetic differentiation over small geographic scale	no evidence	
- unsuccessful	many individuals suddenly disappear	67% of genotypes, after first sampling year, were not resampled in the following years	***supported***
	high proportion of dead wolves represented by dispersing young (< 2 years old)	9 out of 12 aged dead wolves were ≤ 2 years old and were generally recovered in peripheral areas respect to their natal pack	
	few or no individuals are found at different times in different geographic areas	one out of 10 individuals resampled in different years was found in two different geographic areas (temporary excursion)	
	few or no individuals unrelated to any other individual in the population (immigrants)	only two genotypes did not show any significant relatedness with any other individual sampled in the study areas	
	low gene flow and high level of genetic differentiation over small geographic scale	limited gene flow, highly significant F_{ST}, Wahlund's effect, significant inverse correlation between relatedness and geographic distance	
❸ small territories	low distance among packs	distance between adjacent packs averages 13 km in summer	***partially supported***
	individual locations are extremely confined	average distance between individual locations over 5 years equals 3.8 km	
	strictly related individuals frequent the same restricted area	'core' genotypes of the same area were sampled in winter within a range of 9 km	

TABLE 11. Hypotheses verification on the basis of evidences provided by the present study

- Chapter 3 -

Case study 2: Genetic relatedness and heterozygosity in a lekking fallow deer population

PHOTO: G. CALEO

3.1 Aim of the study

Rationale: fallow deer shows high intra-specific variation in mating behaviour. It may adopt a wide range of mating strategies, including the lek. Several studies attempted to clarify the mechanism behind lek formation and maintenance, and to give evidences in support to the different models proposed for the evolution of leks. One of the most recent models invokes kin selection, which would operate by the inclusive fitness of lekking males. On the basis of this model, females prefer large aggregations, in which they choose attractive males (carrying 'good genes') for mating; non-preferred males take part to the lek without a direct benefit (low or no chance to copulate), but they enhance the indirect component of fitness by joining related attractive males. Their joining the lek makes it larger and so more attractive to females, with a positive outcome, in terms of fitness, for all males in the lek. Alternatively, males may gain access to the lek and become territorial on it, simply because they are stronger and healthier than others. In such a case, their body condition may be related to their level of heterozygosity, as resulted in other species. Female choice for these mates would represent the selective force ensuring the conservation of the lek and maintaining the overall level of genetic diversity within the population, balancing the effect on it of the extremely high degree of polygyny.

Main aim of the study: to verify the hypothesis that males in a fallow deer lek are more related than expected by chance. This hypothesis would explain the formation of a lek by kin selection, where adult males contribute to their fitness and to the fitness of their relatives forming, during the breeding season, an aggregation having the function to attract and concentrate females.

Secondary aims: to evaluate level of heterozygosity of lekking males, in comparison with the overall genetic diversity of the population. High heterozygosity of territorial males in the lek could result into a better body condition, enabling males to defend territory in the lek and to be selected for mating by females. This would play also a role in preserving the overall variability of the fallow deer population.

3.2 The biology of the species: an overview

Natural history and actual status

During last interglacial period fallow deer was widespread distributed in the Eurasian continent, as testified by fossils of that period discovered throughout Europe and the Near East. The most critical period for the species was during last glaciation (Würm), when fallow deer suffered a mass extinction, remaining only in some areas of the Mediterranean basin (Halternorth 1959) Unknown causes led to the further disappearance of fallow deer from all areas apart from present Macedonia and Turkey (Masseti 1996). From these refuges, starting from the X century b.C., fallow deer spread out in the Mediterranean as consequence of translocations operated by humans (Phoenicians, Greeks and Romans). Normans carried the species back to Britain, and several other civilizations contributed to restore a number of nuclei throughout Europe (Chapman & Chapman 1975).

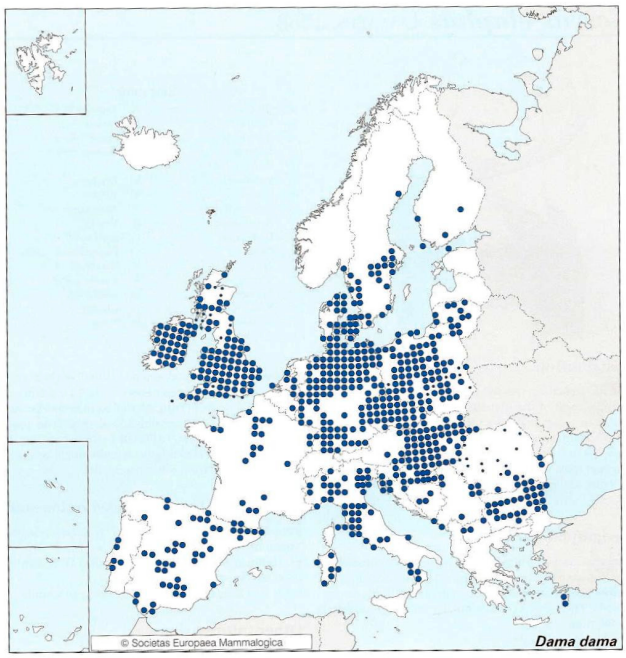

FIGURE 31 - Distribution of fallow deer (*Dama dama*) in Europe (Mitchell-Jones et al. 1999).

Two different sub-species are currently recognized (Wemmer 1998): *Dama dama mesopotamica* and *Dama dama dama*. The first, known as Persian fallow deer, was present in dense woodlands of Iran, Iraq, Israel, Jordan and Lebanon, but declined in the last centuries and is now considered endangered (1996 IUCN Red List), surviving in small number in Iran and in Israel, where it was reintroduced. The second has a worldwide distribution being particularly diffused in Europe and New Zealand, where it is considered the most widespread 'park game'. In Italy the most ancient

nuclei are represented by the enclosed populations of San Rossore and Castelporziano, but the species is present in several regions with a patchy distribution.
Many fallow deer populations are enclosed and may reach high local density.

Taxonomy

An old classification attributed the fallow deer to the genus *Cervus*, for its similarity to other deer species (i.e. red and sika deer). Nevertheless, the current taxonomy refers to the Linnean classification.

Taxonomic designation	
Kingdom:	*Animalia*
Phylum:	*Chordata*
Class:	*Mammalia*
Order:	*Artiodactyla*
Family:	*Cervidae*
Subfamily:	*Cervinae*
Genus:	*Dama*
Species:	*Dama dama* L., 1758

Morphology

The fallow deer is a medium size deer, approaching 100 cm of shoulder height and 100 kg of body weight (in males). The species shows a pronounced sexual dimorphism. The male (buck) is larger in size and carries palmate multi-point antlers on its head. The female (doe), on the contrary, is smaller and does not carry antlers. Buck weight varies a lot during the year, being maximum before the beginning of the breeding season. The "Adam's apple" (larynx) is prominent in males.
Antlers are secondary sexual traits, developing under hormonal control. They undergo annual cycles of casting and regeneration: from April by August the antlers grow, as consequence of the vascularization assured by the covering velvet, then this tissue dies and is lost by the deer, the antler remains uncovered during the rut and further on until next spring, when it separates from the frontal bone and is shed. In the first spring (about 1 year of age), male fawns develop antlers represented by a simple beam. In the next years the morphology of antlers becomes complicated, increasing in size and in the number of points. As bucks are three years old, the typical structure know as ‘palm’ appears, growing year after year until full maturity.

The human manipulation affected fallow deer aspect, particularly referring to coat colour. *Dama dama* have, indeed, the most variable pelage coloration of any species of deer. Typically, the pelage is darker on the dorsal surface of the body and lighter on the ventral surface, chest, and lower legs. Four main colour patterns are reported: 'common' (brown-reddish, white-spotted shoulder and upper flanks, white buttocks), 'menil' (similar to the previous one, but brighter), 'white' (full white but not to be confused with albinism) 'black' (dark grey-black back, flanks and rump, brown-pale grey head, neck and chest). A black stripe runs dorsally along the nape of the neck to the tip of the tail. In San Rossore only common (70-80%) and black (20-30%) patterns are found. The pelage is subject to two moulting per year (in spring and fall): the summer coat is pale, smooth, and thin while their winter coat is darker and rougher with a heavy undercoat.

Age and sex classes

According to Apollonio and Toso (1988), animals in the population were classified into the following age and sex categories:

SEX	AGE	ANTLERS	DENOMINATION
FEMALE	< 1 year	no	fawn
	> 1 year	no	doe
MALE	< 1 year	no	fawn
	1-2 years	beams without branches	yearling buck
	2-4 years	ramifications with small, or without, palm	subadult buck
	> 4 years	ramifications with large palms	adult buck

Behaviour

The fallow deer is a herbivorous animal defined as 'selective grazer' by Hoffman (1978). Its diet changes on the basis of the season and of the environmental features, including grasses, buds, shoots, bark and acorns. This plasticity in feeding allows fallow deer to live under many ecological constraints. The species is indeed found from the Mediterranean coasts up to mountain habitats. Their peak feeding periods are usually at dusk and dawn but they may also forage at intervals throughout the day.

Its social behaviour is typically gregarious. The social organization is influenced by the environment, and differences in type of joining, group size and group composition are observed in

different habitats (Apollonio et al. 1998a). In open lands fallow deer tend to form large herds of mixed sexes.

As for other species, social organization changes during the year. Males and females spend most of the year in separate herds and segregate spatially, selecting different habitats (Apollonio et al. 1998a, San Josè et al. 1999). Just in autumn, when mating occurs, bucks and does share the same areas and may form mixed groups until early winter.

Three grouping types are found in a population: 1) does with their fawns; 2) adult males in bachelor groups 3) mixed groups (individuals of both sexes). The first type is more stable, whereas the second fluctuates in size during the year. The third situation is commonly observed in spring at large pastures. The smallest female herds are found during the fawning season. Females often become secretive and try to find hiding places prior to giving birth. The mother-fawn bond is established immediately after birth when she licks it clean. The mother does not rejoin the herd immediately after birth. She hides the fawn in dense bushes and only returns to nurse it during the day. After 3 to 4 weeks the mother and fawn rejoin a herd of females and their young. Suckling may last as long as 9 months, but soon fawn diet is integrated by vegetables. After approximately one year, the young are independent. Only female fallow deer care young.

Fallow is active in the 24 hours and exhibits peaks of activity at dusk and dawn, but their rhythms may change in relation to human disturbance. They have a good sense of smell and hearing and very good vision. In general, deer are more alert in open areas or in smaller groups; females are usually more alert than males, especially when their fawn are present. Depending on reproductive status and diet quality, fallow deer spend most of their time feeding, resting, and moving.

Dama dama has different types of vocalizations, of which groaning, produced by rutting males, is the most impressive. The common visual communication among fallow deer when disturbed is alerting, where they gain an upright stance with their head held vertically and their body rigid. They may also use different forms of touching, stiff-walking, tail positions, and head positions to communicate.

Genetic variation

Dama dama is one of the less variable ungulate species. Its genetic diversity was investigated recurring to different genetic markers. The first studies, referring to allozyme variation, revealed a low level of biochemical polymorphism in several European populations (Pemberton & Smith 1985, Hartl et al. 1986, Randi & Apollonio 1988, Schreiber & Fakler 1996). Pemberton & Smith (1985) did not detected any electrophoretic variation at 30 loci, screened on 794 samples from England and Wales. Just a single polymorphic locus (CAT - catalase), not used by the previous

authors, was instead found in a German herd (Hartl et al. 1986). Randi and Apollonio (1988) in a study on the San Rossore (Italy) population employing 51 loci, confirmed the variation at the CAT locus and detected a new polymorphism at the POX (perossidase) locus. Finally in Coto Doñana (Spain) the NADH diaphorase locus resulted also variable (Schreiber & Fakler 1996).

Similar results were obtained by RAPD (Random Amplified Polymorphic DNA) analysis carried out on fallow deer samples from a population in Maremma (Scandura et al. 1998). Out of 97 putative loci detected, only 17 (17.5%) were polymorphic, confirming a high homogeneity among specimens.

Finally, the use of nuclear highly polymorphic markers (microsatellites) allowed a major detection of diversity within fallow deer populations, enabling the estimation of reproductive success on a genetic basis (Say et al. 2003). However, even microsatellite loci showed signs of a genetic impoverishment (low heterozigosity, low number of alleles per locus), limiting the studies on this species (Poetsch et al. 2001, Say et al. 2003).

The causes of the low genetic variability in *Dama dama* were attributed to the history of the species and to the adopted mating systems. A long-term reduction in the effective population size may dramatically lower the genetic diversity within a population (Nei et al. 1975). The historical contraction of fallow deer range and its strong management by man may have had a strong effect on allele frequencies (genetic drift), erasing a portion of the species variability. Moreover, in cervids, genetic variability seems to be positively correlated with the degree of polygyny (Apollonio & Hartl 1993, Apollonio 1998). Thus, the high reproductive skew found in many fallow deer populations might be responsible of a further loss of heterozygosity.

Reproduction

Sexual maturity is reached at the second breeding season (16-17 months) by females and at 14-17 months by males. In this latter case, the maturity is only physiological (spermatogenesis occurs), but bucks are usually prevented from breeding by social constraints until the age of four years, unless they live in heavily exploited populations. In male fallow deer copulatory success is, in fact, directly related with age (Apollonio et al. 1989a). The breeding season falls in autumn (September – December), but the highest percent of fertilizations occurs in October, coinciding with the major peak of testosterone level in adult bucks.

The length of the oestrous cycle for females is approximately 24 to 26 days. Females are polyoestrous and may cycle up to seven times in one breeding season, but they usually conceive during their first cycle. It is usually observed a synchronization of female oestrus within the same population. *Dama dama* usually gives birth to one fawn (less than 5% twin births), after a gestation

period of 33 to 35 weeks (approximately 234 days). The majority of fawns in the Northern Hemisphere are born in early June. Their weight at birth ranges generally between 3 and 4 kg.
In summer, bucks may form small bachelor herds of fewer than 6, later groups dissolve and males begin joining the female groups by early autumn. In this period they use to paw the ground to create scrapes, where they may urinate, to thrash and fray vegetation with their antlers, and to produce low-pitched groans and grunts. The rubbing against soil and vegetation is a form of marking behaviour, by which the secretion of sub-orbital and inter-digital glands is spread in the rut areas (Chapman & Chapman 1975). Even the urine in this period contains a strong scent, given by preputial secretions. This scent may represent a chemical stimulus inducing oestrus in females (Kennaugh et al. 1977).
Adult males assume protein-rich food from spring on (Bruno & Apollonio 1991) and may suspend completely foraging during the rut (Apollonio & Di Vittorio *in prep.*).
Matings occur during the rut. Males fight often and violently during the mating season but injuries are rare; their fights involve a ritual antlers display that follows fixed rules (McElligott et al. 1998). When mating, the male approaches the female many times, sniffing and licking her genital areas in order to determine if she is in oestrus. The female responds with a high-pitched whine and moves away. Eventually the female allows the male to mount. Several attempts may be necessary to a male to copulate, and the success is remarked by a sudden and vigorous pelvic shove, coinciding with the ejaculation, common to every species of deer (Clutton-Brock et al. 1982). After mating, the female raises the tail and urinates. Soon she will abandon the male and its territory. Each female may mate only once during the breeding season (Clutton-Brock et al. 1988, Apollonio et al. 1989a).

Mating systems in fallow deer

Dama dama is polygynous, like several mammal species with exclusively female parental care (Clutton-Brock et al. 1988, Apollonio et al. 1992). Like feeding, also mating behaviour shows a considerable plasticity in fallow deer, exhibiting most of the mating systems ever reported in ungulates (Thirgood et al 1999). This variability is not only found in different populations (under different ecological conditions), but several mating strategies may be adopted by members of the same population and by the same individual during its life or during a single rut (Thirgood 1991, Apollonio et al. 1992). Male individual strategies may vary in relation to population density, , body and social conditions, and habitat type (Braza et al. 1986, Clutton-Brock et al. 1988, Apollonio 1989, Langbein & Thirgood 1989, Moore et al. 1995). This latter factor is particularly important, as it determines the female spatial behaviour which influences the male one, ultimately affecting the adopted mating system (Emlen & Oring 1977).

The polygynous mating systems reported in fallow deer produce a big skew in male reproductive success, with a few males monopolizing copulations during the rut (Clutton-Brock et al. 1988, Apollonio et al. 1989a, 1989b, 1990, Say et al. 2003). Langbein and Thirgood (1989), based on the level of territorial behaviour, classified mating systems in 'territorial' and 'non-territorial'.

Territorial mating systems are defined: single stand, multiple stand, lek and lek satellite.

Non-territorial mating systems are: dominance within groups, harem and following.

Single stand or 'rutting stand' is a strategy, considered for long time the only one for the species, in which a male occupies an isolated territory, without visual contact with other males (Chapman & Chapman 1975). The defence of the territory may be permanent during the rut, or be concentrated in some part of the day. In any case, such territories are located near trophic resources, attracting females.

Multiple stand is a sort of aggregation of bordering territories defended by two to three males. The size of each territory is similar to a single stand. Even in this case, the presence of food resources is crucial to attract females for breeding.

A lek was defined by Bradbury & Gibson (1983) as 'an aggregation of males visited by females only for breeding'. Territories in a lek are not associated to trophic resources. Their size is smaller than in single and multiple stands. A full description of this mating system is treated in next section.

A satellite lek is represented by a maximum of three fallow bucks standing close to the main lek, but usually not in visual contact with males in the lek. Satellite leks are located in proximity of female routes toward lek. Moreover males can gain a territory in the lek, when no female is present on the satellite lek.

Among non-territorial, the dominance within groups was firstly observed by Schaal (1987. It consists in herds formed by does and mature bucks, in which a strict hierarchy of dominance/submission is established. Just one male (the dominant buck) can have access to females for reproduction (Gammell 2001).

The harem is by far more common in red deer (Clutton-Brock et al. 1982), but it was reported also for fallow deer (Braza et al. 1986, Langbein & Thirgood 1989). It seems to be associated with a low female density. A male do not defend any territory nor resources but just a group of females.

The last strategy is the following, often used by subadult males. They cannot compete for females with adult bucks using different strategies, and thus they can only follow female herds during their movements. This behaviour is found in low density populations or, on the other hand, when strong social pressure prevents subadults from employing more favourable strategies.

The lek

This peculiar mating system was first described in the XIX century, by a series of observations of mating arenas in bird species. The first meaning of *lek* was an aggregation of males which display close together.

Since then, it was found to be adopted by animal species belonging to several taxa. It is quite common in birds (e.g. Robel & Ballard 1974, Avery 1984, Gibson & Bradbury 1985, McDonald 1989), but was also detected in mammals (e.g. Büchner & Roth 1974, Bradbury 1977, Lazenby-Cohen & Cockburn 1984, Gosling et al. 1987, Fryxell 1987, Schaal & Bradbury 1987, Bartos et al. 1990), amphibians (e.g. Arak 1988, Cherry 1993), reptiles (Trillmich & Trillmich 1984), fishes (e.g. McKaye 1983), and insects (e.g. Sullivan 1981, Lederhouse 1982, Shelly 1987). An extensive review of taxa for which lekking behaviour was described is reported by Höglund and Alatalo (1995). Although its diffusion among animal taxa, lek is far to be considered a common mating systems.

The definition of *lek* was controversial. Höglund & Alatalo (1995) defined a *lek* as 'an aggregated male display that females attend primarily for the purpose of fertilization'. They also remarked the usefulness to define mating systems only in terms of spatiality, pair bonding and parental care. According to them, it should be clear that females visit a lek and are free to choose any male in it, which provide no resource, apart from the sperm.

Features of lekking

- Territoriality (small territories)
- No resource defended
- Free female mate choice
- Costly strategy
- Skew in male reproductive success
- Strong sexual selection
- Exclusively female parental care
- Attractiveness of large clusters

Territoriality is typically associated to lekking systems, but it needs of topographic features and, in absence of them, no fixed territorial behaviour could be observed. It is the case of black grouse (*Tetrao tetrix*) on ice-covered lakes of the northern taiga. Absolute or relative territory location may influence female choice, but even conversely female preference of a particular male might induce other males to defend positions close to him.

The role of sexual selection in lekking species has become a big concern for many studies. The combination of a strong selection on males in a population with the prevailing female preference has led to the expression 'lek paradox'. It seems paradoxical that in a situation where females, from their choice, can only get an indirect benefit (genetic quality of their mates) are more discriminating than females of resource-defending species, in which the benefit associated to mate choice is direct

(i.e. resources) (Reynolds & Gross 1990). In any case, male-male competition and female mate choice are to be considered the main behavioural traits involved in lek formation and maintenance (Höglund & Alatalo 1995).

A particular interest concerns the evolution of lek systems. Many hypotheses were taken into consideration: some are accompanied by a mathematical model, others are verbal arguments only. They are not mutually exclusive and a single hypothesis is difficult to exhaustively account for the lekking behaviour in a population. Here several hypotheses will be considered, limiting to those addressed for lekking in ungulates:

1) The *predation risk* hypothesis refers to the reduced individual risk of predation on both males and females if they aggregate in a lek. It is the same reason explaining the origin of large flocks or herds in animal species. By aggregating in a lek, males may reduce their time expenditure in vigilance and concentrate on display. As for feeding activities, the simultaneous presence of many individuals in the area help to detect early approaching predators and possibly to mob it. This explanation seemed plausible for a minority of case studies (e.g. in tùngara frogs *Physalaemus pustulosus*, Ryan et al. 1981), but it was denied by evidences in other cases (e.g. in Uganda kob *Kobus kob*, Balmford & Turyaho 1992). Moreover some lekking species, like fallow deer, do lek in areas where they live from centuries in complete absence of predators.

2) *Passive attraction* may also explain leks. Females may be more attracted by large aggregations of males, representing a stronger stimulus. Thus males should obtain more benefits by joining other displaying males, rather than betting on individual display. The main drawback of this explanation is that the attracting stimuli are not additive and asymptote quickly, thus cancelling the proportionality between number of females per male and number of lek-forming males (Bradbury 1981).

3) A third hypothesis concerns *habitat limitation*. Höglund & Alatalo (1995) stressed that if this aspect can fully explain clustering and spacing by males in all the available habitat, the result is not to be named lek. However it can partially explain the structuring of a lek, especially in relation to the lekking site. A patchy habitat may influence male aggregation directly, by limiting the availability of suitable sites for clustering, or indirectly, by affecting female distribution and forcing males to cluster where females are abundant. In birds, for instance, feeding and nesting sites, and females with them, have often a patchy distribution. Thus male clustering is usually the ultimate result of this situation. Nevertheless, the importance of habitat constraints in affecting male distribution may change from species to species.

4) A further hypothesis for the formation of leks is the so called *hotspot hypothesis* (Bradbury & Gibson 1983). According to this model, males form aggregations where the probability of

encountering females is maximal. This sites can coincide with areas where females' home ranges overlap (Bradbury et al. 1986). Simulations were carried out to support this idea, demonstrating that local female density may explain well the male clustering. Some evidences in bird species fit the hotspot hypothesis. On the contrary, studies on lekking ungulates seem to contradict the model, as leks occur even in high-density populations or in absence of sites where female density is predictably high. In hotspot-modelled leks, the inter-lek distance is expected to correspond to less than one average female home range, and females are expected to visit more than one lek before breeding (Bradbury & Gibson 1983). Moreover, another indirect evidence for the hotspot model would be the difference in female home range size between lekking and non-lekking populations, with the formers expected to have wider ranges. A final suggested prediction is that the number of settling males would distribute in proportion to the number of overlapping female home ranges (Höglund & Alatalo 1995). In nature, the most likely situation is that both ecological constraints and female home ranges overlapping determine the existence of hotspots for lek settlement.

5) Stillman et al. (1993) suggested a fascinating hypothesis, named the *black hole hypothesis*, attributing the evolution of some leks to the avoidance by females of sexual harassment by young inexperienced males. The underlying assumption is that mating with males without a sufficient skill is risky for females, which can be injured by them. Thus receptive females would approach territories kept by adult males in the lek, just because it appears to them as a shelter to the harassment and not as a source of potential mates. Larger the lek, stronger is the protection assured by adult males. Further, central territories are expected to attract more females than peripheral ones (Clutton-Brock et al. 1992).

6) The *hotshot hypothesis*, so called by Beehler and Foster (1988), was first proposed by Arak (1982), who explained lek formation with the female preference for few attractive males (the 'hotshots'), and the joining to them of other unattractive ones. It would represent a sort of parasitism on preferred males (kleptoparasitism). Experiments realized on great snipe (*Gallinago media*) demonstrated that, while the removal of peripheral males in a lek was followed by their immediate replacement by neighbouring or floating males, the removal of central birds strongly compromised the integrity of the lek (Höglund & Robertson 1990). In this case male-male competition seems to be the main factor affecting the cluster structure, as it determines the dominance of attractive males. Nevertheless, the female mate choice is likewise important, as it determine which males are hotshots.

7) An other hypothesis, proposed by Kokko and Lindström (1996), invokes *kin selection*. The model they developed is useful to clarify situations where young males take part to the lek. In this case, the scarce mating success of low-rank males may be overridden by the indirect component of

fitness, due to the high success of related dominant males. In lekking tetraonids, like black grouse, natal philopatry in males may produce a structuring at the population level, which promote the encounter of related males within a lek. In fact, in black grouse, it was demonstrated that males within a lek are more related than expected by chance (Höglund et al. 1999).

8) Last hypothesis takes into account harassment avoidance by females (Clutton-Brock et al. 1992, 1993). Females in oestrus leave their usual herd to avoid harassment by males and prefer clustered territories because representing safe areas, where they are exposed to a reduced harassment rate. This hypothesis, although convincing in fallow deer (Clutton-Brock et al. 1992), was unlikely to explain lek formation in other ungulate species (Bro-Jørgensen 2003).

Other hypotheses, like information sharing, have not been reported here, because they were considered unlikely to take place (Höglund & Alatalo 1995).

Whatever the mechanism involved in its formation, it should be remarked that a lek is an outcome of a dynamic process, probably entailing more than one of the described models, rather then a strategy simultaneously adopted by several males.

Lekking in fallow deer

Fallow deer was discovered to be one of the few lekking mammals (Schaal & Bradbury 1987, Pemberton & Balmford 1987). In species with alternative mating strategies, lekking was found to be correlated with several factors like population density, group size, sex ratio, female spatial behaviour and habitat structure.

Langbein and Thirgood (1989) compared mating systems adopted by fallow deer in nine enclosed parks in Britain. Deer density appeared to be the main factor influencing the mating behaviour. At low density harem formation prevailed. As density increased, males became more territorial and at the highest level of density, bucks formed leks. The effect of density may be explained considering that a high density corresponds to a high encounter rate of conspecifics and this influences the cost associated to female or territory defence. At low densities it may be worthwhile to defend females (harem strategy), because of the limited number of competing bucks. But as the density raises up, this strategy may become too costly and the trade-off may switch in favour of territory defence.

Langbein and Thirgood (1989) indicated even habitat structure and tree cover as other important variables. Leks were more likely to take place in open habitats, a situation close to that observed for African lekking ungulates, like kob, topi (*Damaliscus korrigoni*), and lechwe (*Kobus leche kafuensis*), for which most observed leks lay in open areas (Büchner & Roth 1974, Schuster 1976, Gosling et al. 1987).

It was also observed that, in fallow deer populations with sex ratio strongly biased towards females, a lek is difficult to occur, because of the scarcity of mature bucks (Clutton-Brock 1988). This is the case of many exploited populations. In protected or enclosed areas, where the species may reach high level of concentration, population density seems to be the main factor determining lek formation (Apollonio 1989). Jagesborg in Denmark (Schaal & Bradbury 1987) and Petworth in United Kingdom (Clutton-Brock et al. 1988) are the best known examples. At high densities, the limitation of aggressive interactions in which dominant males are involved was suspected to play a role in lek formation (Pélabon et al. 1999). In some cases, the density cannot be sufficient to explain the adoption of lekking by adult males in a population. In the enclosed population of San Rossore (Italy), a high local concentration of females, due to an uneven food distribution in the area, was invoked to explain lek formation (Apollonio 1989, Apollonio et al. 1998b). In at least one case (Clutton-Brock et al. 1992), females seem to be motivated to join a lek, to avoid harassment by young bucks.

Female mate choice seems to be influenced by the position of male territories in the lek, central ones appearing often the most successful (Clutton-Brock et al. 1988). On the other hand, where habitat constraints limit female approach to the lek, successful bucks defend territories close to the site used as entrance by females (Apollonio et al. 1989a, 1990). Moreover, Clutton-Brock and McComb (1993) demonstrated that does copy other does' movements, and thus in a lek they are attracted to the more successful males (i.e. those with larger harems). Therefore, a self-enforcing mechanism may derive, by which few bucks monopolize mates in a lek. The success in fighting is not related with the reproductive performance, suggesting that it could not directly influence female choice (Festa-Bianchet et al. 1990). On the contrary, male body condition has certainly a role, as only fit males are able to defend a territory during the rut (Apollonio et al. 1989a).

Given its capacity to switch between several mating strategies, fallow deer represents an ideal species for the study of lek evolution.

3.3 Study population

Study area

The study was conducted in the San Rossore estate, near Pisa, in central Italy (43°N, 10°E). The area is a flat lowland 46 km^2 wide, of which 39 km^2 are accessible to the deer (Fig. 33). It lies comprised between two rivers, Arno and Serchio (south and north respectively), and its western side looks the Tyrrhenian sea. The eastern side is fenced. Climate is submediterranean. Vegetation is dominated by deciduous (oak, *Quercus* spp.) and pine (*Pinus* spp.) forest, but open pastures, marshes and cultivated field are also present. Apart from fallow deer, wild boar (*Sus scrofa*) is the only wild ungulate living in the estate. Large predators are absent (the largest carnivore in the area is the red fox *Vulpes vulpes*). Access to people is regulated and most areas are close to the public (including lek areas).

Two leks were formed by fallow deer during the study period. The larger (lek 1) is named '*Stacca del Gatto*' and is located in the western part of the estate. The second lek (lek 2), called '*Fossacci*', lies north to the former at an approximate distance of 3.2 km.

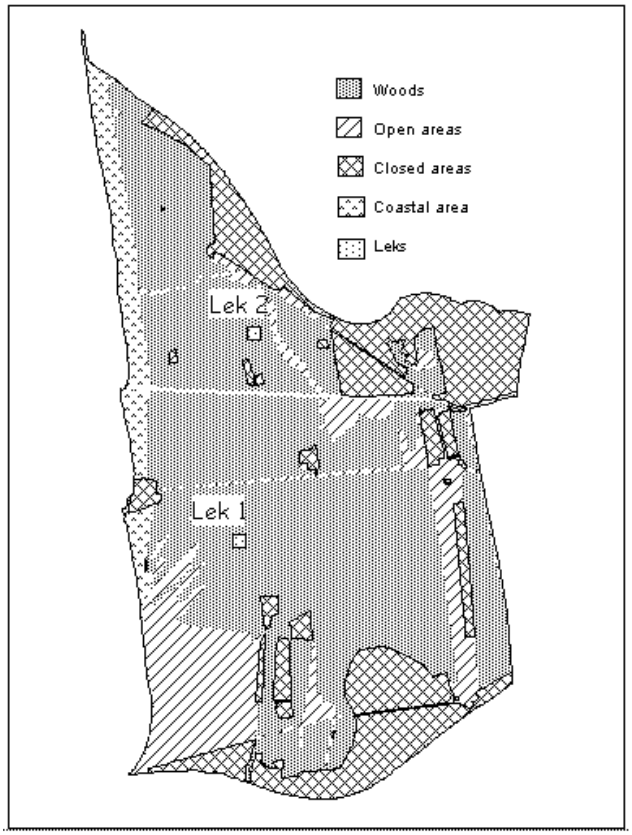

FIGURE 33 . The study area. Lek locations are indicated by squares (from Apollonio et al. 2003)

Study population

The fallow deer population inhabiting the San Rossore estate was founded in the sixteenth century or earlier (Simoni 1910). From then on, no successive introduction was done. Deer from Sardinia were probably used to found the San Rossore population (Simoni 1910). Culling and live trapping are used as demographic control and realized by the estate warders during winter.

The population size is estimated yearly by spring censuses, after correction based on the results of the previous year. The overall consistency of the population varied between 750 up to over 1500 deer in the period 1983-2001 (Fig. 34).

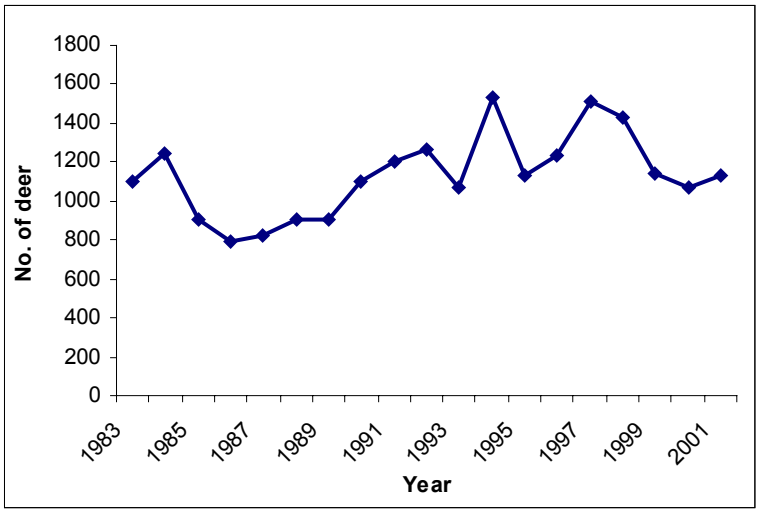

FIGURE 34. Estimated number of fallow deer in the study area (1983-2001). Corrected census data are from Apollonio et al. (2001).

Field activity

Lekking behaviour in the San Rossore fallow deer population is under study since 1985. In this period three different leks were detected in the area. Just two lasted until the start of the present study. During the rut (the time comprised between the first and the last observed copulation), observation were conducted daily, from dawn to dusk, from hides or elevated blinds, in order to monitor each lek (territory occupancy, competitive behaviour, copulations, etc.). Leks were watched continuously by skilled observers and volunteers from the beginning of October until the end of the rut. Some animals were ear-marked and/or radio-collared, and thus recognizable to the observer (Fig. 35). Individual identity for non-marked bucks was even defined on the basis of coat colour and antler shape. Every half hour, observers recorded lek composition and the behaviour of territorial bucks (i.e. fighting and mating), together with the number of females in lek territories.

Adult does frequented lek areas only for mating and spent up to 36-48 hours there, after which they went back to feeding or resting areas. Every year lek territories were mapped and territory owners identified. Different behavioural activities were recorded during lek attendance. All the observed copulations were recorded. Since both in lek sites lay in woodland, many copulations by territorial bucks were missed by the observers, as well overnight activities. Thus, in considering the individual reproductive success, I assume that the observed copulations reflect the actual proportions among males in a lek.

FIGURE 35. Radio-collared fallow buck in the study area (photo: G. Caleo).

3.4 Methods

Sample collection

Sampling was designed as follows: 1) to sample most males in a lek, 2) to collect as much adult buck samples as possible, 3) to collect other class samples in accordance with their estimated proportion in the population. Calves were not sampled to do not generate bias due to contemporary sampling of several mother-offspring pairs.

Samples were obtained from live captured or naturally dead animals between 1999 and 2003. Most lekking males (n=15) were sampled in one occasion (October 1999), in which they were immobilized, fitted with radiocollars and marked with plastic ear-tags. A blood aliquot was collected via jugular veni-puncture, a small piece of ear was cut, and some hair tufts were plucked to each animal. Age was defined on the basis of teeth consumption and ranged between 4 and 10 years. Captures interested two leks. Other specimens (n=88) were collected during culling activities in winter. In this case only ear and plucked-hair samples were collected. The last source of samples was represented by dead animals (n=10). They were found usually at the beginning of winter, the death being often consequence of the enormous energy expenditure during the rut. A small piece of skeletal muscle was collected from dead individuals. Jaws were taken from each animal and teeth examined for accurate age estimation.

All samples were kept at –20°C until used for genetic analysis.

DNA isolation

Three types of samples were used for DNA extraction: blood, muscular and ear tissue, and hair follicles. Genomic DNA was isolated from blood and tissue using the QIAamp Blood kit (Qiagen), according to manufacturers' instructions. In extracting DNA from hairs a minimum of 10 follicles were used recurring to the Chelex-100 resin protocol (Walsh et al. 1991). Final DNA concentration in each extract was measured through a GeneQuant spectrophotometer (Pharmacia Biotech). Aliquots were diluted in order to obtain an approximate concentration of 10 ng/µl.

Microsatellite selection and amplification

As already mentioned, fallow deer populations have a low genetic diversity. In order to obtain a set of microsatellites useful to this study, a total of 26 microsatellite loci was tested (Tab. 12). They had been isolated in cattle, sheep, goat and reindeer, for which they resulted among the most polymorphic. Primer sequences were either published or available in the ARKdb public database (www.thearkdb.org).

Microsatellite	Source species	Chromosome	Amplification in fallow deer	No. of alleles	Allele size range	Reference	Primer sequence (5' - 3')
BM6444	*Bos taurus*	2	polymorphic	3	116-120	Bishop et al. 1994	CTCTGGGTACAACACTGAGTCC TAGAGAGTTTCCCGTCCATCC
CSSM014	*Bos taurus*	4	polymorphic	4	136-152	Moore et al. 1994	AAATGACCTCTCAATGGAAGCTTG AATTCTGGCACTTAATAGGATTCA
CSSM016	*Bos taurus*	16	monomorphic	1	148	Moore et al. 1994	GATGCAGTCTCCACTTGATTCAAA AGAGCCACTTGTTACACCCCAAAG
ETH2	*Bos taurus*	5	polymorphic	4	184-198	Solinas Toldo et al. 1995	CCCACAGGTGCTGGCATGGCC CCATGGGATTTGCCCTGCTAGCT
HEL1	*Bos taurus*	15	aspecific	-	-	Kaukinen & Varvio 1993	CAACAGCTATTTAACAAGGA AGGCTACAGTCCATGGGATT
ILSTS006	*Bos taurus*	7	aspecific	-	-	Brezinsky et al. 1993	TGTCTGTATTTCTGCTGTGG ACACGGAAGCGATCTAAACG
ILSTS028	*Bos taurus*	3	polymorphic	2	156-158	Kemp et al. 1995	TCCAGATTTTGTACCAGACC GTCATGTCATACCTTTGAGC
INRA005	*Bos taurus*	12	monomorphic	1	135	Vaiman et al. 1992	CAATCTGCATGAAGTATAAATAT CTTCAGGCATACCCTACACC
MAF033	*Ovis aries*	9	polymorphic	1	111	Buchanan & Crawford 1992a	GATCTTTGTTTCAATCTATTCCAATTTC GATCATCTGAGTGTGAGTATATACAG
MAF214	*Ovis aries*	16	aspecific	-	-	Buchanan & Crawford 1992b	AATGCAGGAGATCTGCAGGGACG GGGTGATCTTAGGGAGGTTTTGGAGG
NVHRT16	*Rangifer tarandus*	-	monomorphic	1	157	Roed & Midthjell 1998	ATTCTAAGCCCAAATAATCTT TCTAAGGGGTCTGTGTCTT
NVHRT21	*Rangifer tarandus*	-	polymorphic	4	173-179	Roed & Midthjell 1998	GCAGCGGAGAGGAACAAAAG GGGGAGGAGCAGGGAAATC
NVHRT30	*Rangifer tarandus*	-	polymorphic	2	196-200	Wilson et al. 1997	CACTTGGCTTTTGGACTTA CTGGTGTATGTATGCACACT
OarAE16	*Ovis aries*	13	aspecific	-	-	Penty et al. 1993	CTTTTTAATGGCTCGGTAATATTCCTC CATCAGAGGAATGGGTGAAGACGTGG
OarCP20	*Ovis aries*	21	no amplification	-	-	Ede et al. 1995	GATCCCCTGGAGGAGGAAACGG GGCATTTCATGGCTTTAGCAGG
OarCP26	*Ovis aries*	4	polymorphic	3	138-146	Ede et al. 1995	GGCCTAACAGAATTCAGATGATGTTGC GTCACCATACTGACGGCTGGTTCC
OarFCB48	*Ovis aries*	17	polymorphic	3	152-167	Buchanan et al. 1994	GACTCTAGAGGATCGCAAAGAACCAG GAGTTAGTACAAGGATGACAAAGAGG
OarFCB266	*Ovis aries*	7	monomorphic	1	203	Buchanan & Crawford 1993	GGCTTTTTTTCCACTACGAAATGTATCCTCAC CACCACATACCAAACACACAGCCTGC
oMHC1	*Ovis aries*	20	polymorphic	5	187-212	Groth & Weatherall 1994	ATCTGGTGGGCTACAGTCCATG GCAATGCTTTCTAAATTCTGAGGAA
RT1	*Rangifer tarandus*	-	monomorphic	1	216	Wilson et al. 1997	TGCCTTCTTTCATCCAACAA CATCTTCCCATCCTCTTTAC
SPS113	*Bos taurus*	14	polymorphic	3	140-144	Slate et al. 1998	CCTCCACACAGGCTTCTCTGACTT CCTAACTTGCTTGAGTTATTGCCC
SR-CRSP-1	*Capra hircus*	-	polymorphic	3	136-140	Arevalo et al. 1994	TGCAAGAAGTTTTTCCAGAGC ACCCTGGTTTCACAAAAGG
TGLA110	*Bos taurus*	2	polymorphic	2	153-155	Georges & Massey 1992	GTATTTTCAGACAGCCCGCTGGTGTC CAGCATTTACTTATACACACTCACCTGC
TGLA122	*Bos taurus*	21	monomorphic	1	123	Barendse et al. 1994	CCCTCCTCCAGGTAAATCAGC AATCACATGGCAAATAAGTACATAC
TGLA126	*Bos taurus*	20	monomorphic	1	106	Georges & Massey 1992	TTGGTCTCTATTCTCTGAATATT CTAATTTAGAATGAGAGAGGCTTCT
TGLA387	*Bos taurus*	20	monomorphic	1	119	Georges & Massey 1992	CAAAGTCTTAGAATAAACTGGATGG GTCCCTTTGTTTACTTTGATAAAAC

TABLE 12. Microsatellite loci screened for polymorphism in the San Rossore fallow deer population.

The amplification of each locus was attempted over a minimum of 10 different samples, according to the described PCR conditions. Whenever reaction conditions were not previously reported for that locus, a general protocol was used. A final volume of 10 µl contained 0.5 units of RedTaq polymerase (Sigma), 1 x RedTaq reaction buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer (one of the pair was labelled with a fluorophor) and 3 µl of DNA dilution (10-100 ng). The amplification run in a Biometra Thermal Cycler, starting with 3 min at 95°C denaturation step, followed by 35 cycles of 45 sec at 92°C, 45 sec at 55°C and 30 sec at 72°C, and a final extension step of 10 min at 72°C. Such starting conditions (basically annealing temperature) were sometimes changed to optimize the amplification of specific loci. All

recovered PCR products were then automatically analyzed using a 310 or a 3100-Avant ABI PRISM automatic sequencer (Applied Biosystems). The softwares GENESCAN, GENOTYPER and GENEMAPPER were used to determine allele size.

Of the 26 microsatellite primer pairs tested, 21 amplified a specific PCR product showing the typical microsatellite peaks pattern (Tab. 12). The remaining loci failed to amplify or showed a complex amplification pattern, including several aspecific products. Among the successful loci, 9 were monomorphic and 12 polymorphic. Three of these latter were discarded because having few alleles, close in size and producing confused patterns.
Finally, a set of 9 microsatellite loci was selected for the analysis. After the choice, PCR conditions for each of the selected microsatellites were optimized (number of cycles, annealing temperature, $MgCl_2$ and primer concentration). A fully description of such conditions is reported in Table 13.

Microsatellite	Dye	T_a (°C)	Primer (μM)	$MgCl_2$ (mM)	No. of cycles
BM6444	HEX	62-57[a]	0.16	2.8	40
CSSM014	TET	55	0.40	2.6	35
ETH2	TET	62	0.12	2.6	35
NVHRT21	HEX	62-58[a]	0.20	2.3	40
NVHRT30	HEX	60-54a	0.20	5.0	35
OarCP26	HEX	60-55[a]	0.16	2.1	35
OarFCB48	FAM	60	0.20	2.3	35
OMHC1	FAM	66-60[a]	0.10	3.0	35
SR-CRSP-1	HEX	58-50a	0.20	4.0	35

[a] touchdown PCR protocol

TABLE 13. Selected microsatellite loci and amplification conditions.

Animal classification

Before processing microsatellite data, all sampled individuals were divided into different categories. The first classification was referred to sex and age. Animals were classified as adult bucks (MA), subadult and yearling bucks (MS), and adult females (FA)(see the section *Sex and Age Classes* at page 80). A second subdivision took into account the geographic location where each specimen was captured. The area was divided into north (N) and south (S), respect to the position of the Fiume Morto river. Consequently samples were assigned to each of the two sub-areas. The last classification involved only adult bucks and refers to their behaviour respect to leks. '*Territorial*' (T) were considered males defending a territory in a lek for at least 3 consecutive hours during at

least one breeding season between 1996 and 2001. Three hours is the minimum time after which bucks in the lek were seen to copulate (Apollonio et al. 1990). Every marked buck which was never observed defending a lek territory was classified as '*nonterritorial*' (NT). In so doing, I assume that all territorial males in a lek were observable and recognizable.

Statistical analysis

Microsatellite data were analyzed using the software CERVUS 1.0 (Marshall et al. 1998), to obtain an estimate of the basic population parameters, specifically average number of alleles per locus (A), expected (H_e) and observed hererozygosity (H_o) and polymorphism information content (PIC, Botstein et al. 1980). As the presence of null alleles at some loci may potentially distort several statistics based on allele frequencies, I also calculated the locus-by-locus expected frequency of null alleles according to Summer and Amos (1997). Allele frequencies and all the above mentioned values were estimated for the overall population and for each category separately.

Deviations from Hardy-Weinberg equilibrium were assessed using GENEPOP 3.2a (Raymond & Rousset 1995). Levels of significance were calculated by an exact-probability-test based on the Markov chain method, according to Guo and Thompson (1992). One thousand randomizations were used in the calculation. A global test across loci was performed by the Fisher's method. Alternatively, probabilities of accordance to HWE were obtained for each locus by χ^2 test using the program BIOSYS 2 (Swofford & Selander 1997), and across loci by summing single-locus χ^2 values and degree of freedom and obtaining the global P-value, as suggested by Ryman and Jorde (2001).

Excess or defect of heterozygotes were instead tested for significance by a score test (U-test) implemented in GENEPOP. Significance levels were corrected for multiple comparisons using the sequential Bonferroni adjustment (Rice 1989). Deviations from linkage equilibrium were evaluated by the program F-STAT 2.9.3 (Goudet 2001), using a G-statistic to test the null hypothesis of independence of genotypes among loci. Even in this case, the software applies a sequential Bonferroni correction to assess the significance of each pairwise P-value. This software was also employed to test differences among classes for different statistics (heterozygosity, F_{IS}, relatedness). To assess the significance of the obtained differences, a permutation scheme is used by the program. Samples are allocated at random to the different groups (keeping constant the number of samples in each category) and the given statistic is calculated for each group from the randomized data set. The P-value of the test is referred to the proportion of randomized data sets, over 1000 permutations, giving a larger difference among the compared groups than the observed one.

Wright's F_{IS} index, modified according to Weir and Cockerham (1984) was estimated using GENETIX 4.04 (Belkhir et al. 2001) and their significance was assessed as percentage of values

minor than the value obtained across 1000 permutations. Confidence intervals are estimated by bootstrapping procedure, using 1000 iterations.

In order to evaluate genetic similarity among samples, two approaches were used. An allele sharing index was calculated for all pairs of samples. Intra-class indexes were obtained by averaging all pairwise values referred to a given category. The second approach was based on the calculation of genetic relatedness. Three different relatedness estimators were used: Queller and Goodnight's R (R_{QG}), Lynch and Ritland's R (R_{LR}), and Wang's R (R_W). The software SPAGEDI 1.1 (Hardy & Vekemans 2002) was used for their calculation. Allele frequencies for the entire population were used as reference in the computation.

Since sampling is male-biased, a reference sample was created with 47 genotypes of randomly chosen individuals, represented by unknown adult bucks, subadult bucks and adult does, in the same proportion as they are found in the population (3 MA: 14 MS: 30 FA). All parameters estimated for each category in the population were also computed for this reference sample.

Genetic variability was also evaluated individually for territorial bucks and associated to their age (at the time they held lek territories) and reproductive success (overall and yearly number of observed copulations).

3.5 Results

Genetic diversity

One hundred and eleven samples were analyzed over 9 polymorphic loci. The overall level of polymorphism was very low for microsatellite markers (Tab. 14). The polymorphic loci employed for the analysis showed between 2 and 5 alleles, and an expected heterozygosity comprised between 0.061 (NVHRT30) and 0.667 (OarCP26). The PIC (polymorphism information content) was in the range 0.059-0.590 (mean 0.358). The overall expected heterozygosity was 0.415 ± 0.060, whereas the mean number of alleles per locus was 3.56. The expected frequency of null alleles, calculated according to Summer and Amos (1997), ranged between negative values and 0.213.(locus CSSM014). On the whole, 32 alleles were detected over nine loci. Most loci showed a single prevailing allele, present in the sample at high frequency ($p > 0.50$). On the other hand, 10 alleles recurred at a negligible frequency ($p < 0.05$). Due to this skewed distribution of allele frequencies, many individuals in the population were homozygous for the most common allele at several loci.

							Exact test			χ^2-test		
Microsatellite locus	No. of genotypes	A	He	Ho	PIC	Null exp freq	HWE	Hdef	Hexc	HWE	χ2	d.f.
BM6444	109	3	0.316	0.220	0.289	0.182	0.000	0.000	1.000	0.000	68.6	3
CSSM14	108	5	0.585	0.380	0.502	0.213	0.000	0.001	1.000	0.000	78.6	10
ETH2	106	5	0.379	0.368	0.341	0.026	0.616	0.504	0.520	0.974	3.3	10
NVHRT21	107	4	0.335	0.308	0.306	0.068	0.149	0.460	0.545	0.219	8.3	6
NVHRT30	95	2	0.061	0.063	0.059	-0.008	1.000	1.000	0.922	0.772	0.1	1
OarCP26	102	3	0.667	0.676	0.590	-0.008	0.292	0.620	0.380	0.267	4.0	3
OarFCB48	106	3	0.501	0.434	0.379	0.070	0.142	0.094	0.932	0.320	3.5	3
*OMHC1^*	*96*	*4*	*0.520*	*0.542*	*0.428*	*-0.025*	*0.976*	*0.725*	*0.310*	0.983	*1.1*	6
SR-CRSP-1	90	3	0.366	0.378	0.327	-0.035	0.205	0.233	0.779	0.231	4.3	3
mean	***104.9***	***3.56***	***0.415***	***0.374***	***0.358***	***-***	***0.000***	***0.000***	***1.000***	***0.000***	***171.7***	***45***

TABLE 14. Variability at the nine microsatellite loci. Accordance to the Hardy-Weinberg equilibrium is reported, referring to exact test and χ^2 test per locus and across loci (HWE, probability of accordance to the Hardy-Weinberg equilibrium; Hdef, probability of heterozygote deficit; Hexc, probability of heterozygote excess; d.f., degree of freedom).

The level of microsatellite diversity for the considered categories is shown in Tab. 15. Observed and expected heterozygosity for the reference sample were 0.360 and 0.392 respectively . Referring

to H_e, a considerable, but not significant (P = 0.14), difference was found between sexes (0.422 in males, 0.387 in females), and in males values decreased from MA (0.431) to MS (0.396). On the contrary, values of H_o were similar between MA and FA (0.380 and 0.384, respectively), but both were higher than MS (0.339). Among adult males, the observed variability was similar between territorial and nonterritorial bucks (H_o = 0.395 and 0.405, respectively; P = 0.84). In Figure 36 the observed heterozygosity of territorial bucks is compared to those of adult females, subadult males and a random sample of adult males (not marked nor identified). No difference in heterozygosity values resulted significant after 1000 permutations between the considered categories, even in relation to the area (north/south) in which animals were sampled.

	Class	N	A	SD	He	SD	Ho	SD	HWE	Hdef	Hexc
SEX	M	81	3.56	1.01	0.422	0.063	0.369	0.019	0.000	0.000	1.000
	F	30	2.78	0.67	0.387	0.058	0.384	0.031	0.471	0.475	0.509
AGE CLASS	MA	61	3.56	1.01	0.431	0.063	0.380	0.022	0.000	0.002	0.999
	MS	20	2.56	0.73	0.396	0.066	0.339	0.036	0.053	0.003	0.998
	FA	30	2.78	0.67	0.387	0.058	0.384	0.031	0.471	0.478	0.519
LEK BEHAV.	T	23	3.11	1.05	0.424	0.060	0.395	0.036	0.017	0.105	0.890
	NT	13	3.11	1.05	0.476	0.069	0.405	0.049	0.181	0.021	0.971
AREA	S	81	3.44	1.13	0.417	0.061	0.384	0.019	0.000	0.004	0.996
	N	25	3.00	0.50	0.412	0.054	0.362	0.033	0.007	0.006	0.995
REF. SAMPLE		47	2.89	0.60	0.392	0.058	0.360	0.024	0.018	0.056	0.947

TABLE 15. Variability and accordance to Hardy-Weinberg expectations (Fisher's method) by different classes of fallow deer. Sex, age class, behaviour on the lek and sampling area were considered.

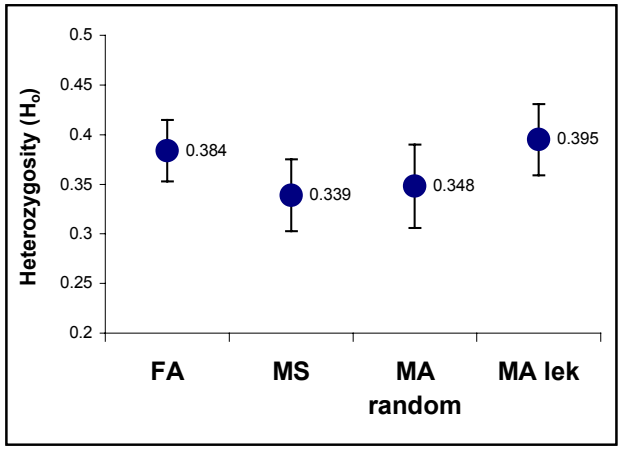

FIGURE 36. Comparison of observed heterozygosity estimated for adult females (FA; n = 30), subadult bucks (MS; n = 20), a random sample of adult bucks (MA random; N = 15) and territorial males in the two leks (MA lek; n = 23). Means ± standard deviations are reported.

Hardy-Weinberg and linkage equilibrium

The overall observed heterozygosity in the population resulted significantly lower than expected according to Hardy-Weinberg equilibrium (Tab. 14), both by Fisher's method (P = 0.000) and combined χ^2-test (χ^2 = 171.7; df = 45; P < 0.001). Even the reference sample deviated significantly from HWE (Fisher's method; P = 0.018). The distribution of genetic diversity among the considered classes revealed an interesting difference in relation to the sex (Tab. 15). Bucks showed a pronounced difference between H_o and H_e (Fisher's method; P = 0.000), whereas females did not show any difference (Fisher's method; P = 0.471). Considering separately adult and subadult bucks, only the formers violated significantly the equilibrium (Fisher's method; P = 0.000) and among them only those territorial in a lek (Fisher's method; P = 0.017). No discrepancy was instead observed between animals captured in different areas (N vs. S); both deviated significantly from the equilibrium (Fisher's method; P = 0.000 and P = 0.007, respectively).

The population was not in Hardy-Weinberg equilibrium due to a heterozygote deficit (exact-test + Fisher's method, P = 0.000). In particular, the male component showed a significant lack of heterozygotes respect to HW expectations (exact-test, P = 0.000) and a significant coefficient of inbreeding (average F_{IS} = 0.126; percentage of values < obs. value across 1000 permutations = 100%). This was not true for females (exact-test, P = 0.475, and F_{IS} = 0.008, P = 0.530). The deficit of heterozygotes was observed both in adult (MA) and in subadult (MS) males (Tab. 16).

	Class	N	F_{IS}	1000 bootstraps (95% C.I.)	1000 permutations % val >	1000 permutations %val <	AS	R_{QG}
SEX	M	81	0.126	(0.052 - 0.193)	0.0	100.0	0.619	-0.016
	F	30	0.008	(-0.101 - 0.064)	46.9	53.1	0.689	0.028
AGE CLASS	MA	61	0.119	(0.047 - 0.173)	0.1	99.9	0.605	-0.010
	MS	20	0.149	(-0.065 - 0.294)	1.9	98.1	0.658	0.039
	FA	30	0.008	(-0.094 - 0.071)	42.5	57.5	0.689	0.028
LEK BEHAV.	T	23	0.069	(-0.088 - 0.165)	15.9	84.1	0.609	0.048
	NT	13	0.154	(-0.015 - 0.235)	3.3	96.7	0.572	-0.014
AREA	S	81	0.080	(0.010 - 0.141)	1.6	98.4	0.642	-0.002
	N	25	0.124	(-0.010 - 0.212)	1.6	98.4	0.629	0.001
REF. SAMPLE		47	0.083	(-0.020 - 0.170)	4.6	95.4	0.678	-0.031
TOTAL		111	0.099	(0.040 - 0.153)	0.1	99.9	0.639	0.000

TABLE 16. Coefficient of inbreeding (F_{IS}) of different fallow deer classes. Confidence intervals (95% C.I.) are established by 1000 bootstraps. Significance is tested over 1000 permutations and expressed as the percentage of values in permutations higher or lower than the observed one. Allele sharing (AS) and Queller and Goodnight's relatedness coefficient (R_{QG}) are reported as measures of genetic similarity within categories.

Respect to the reproductive behaviour, territorial bucks slightly deviated from HWE and had a low value of F_{IS} (F_{IS} = 0.069, P = 0.159), whereas non-territorial adult males showed a significant heterozygote deficit (exact-test, P = 0.021) and were significantly inbred (F_{IS} = 0.154, P = 0.033).
Two loci seemed to be responsible of the global lack in heterozygosity: BM6444 (P=0.000) and CSSM14 (P=0.000). For them, the expected frequency of null alleles, estimated according to Summer and Amos (1997), was not negligible (Tab. 13).
No significant association was detected among loci in the population, after Bonferroni correction for multiple tests. Thus, even loci attributed to the same chromosome (e.g. CSSM014 and OarCP26, Tab 12) were assumed unlinked.

Genetic relatedness

Allele sharing was maximum among females (0.689). In males, it was higher among young males (0.658), and lower among territorial (0.609) and nonterritorial (0.572) adult males (Tab. 16).
The three coefficients of relatedness, estimated according to Queller & Goodnight (1989), Lynch & Ritland (1999) and Wang (2002), had similar values. They were close to zero, for all considered categories. Referring to the Queller and Goodnight's coefficient (R_{QG}), the overall value obtained in the sample was 0.000. Average relatedness among territorial males was on the whole 0.048, and particularly -0.002 in lek 1, ranging each year between –0.207 (in 1997, n = 3) and 0.068 (in 1999, n = 9). In lek 2, the same coefficient amounted on average to –0.136. No difference was observed between territorial and non-territorial bucks (Fig. 37).

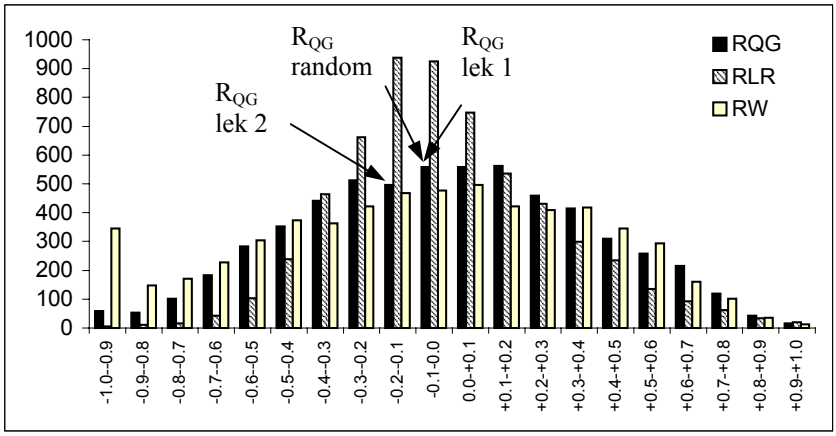

FIGURE 37. Distribution of three coefficient of relatedness in the San Rossore fallow deer population. R_{QG} (Queller & Goodnight's relatedness), R_{LR} (Lynch & Ritland's relatedness) and R_W (Wang's relatedness) are considered. Values of R_{QG} obtained for territorial males in lek 1, for territorial males in lek 2 and for a random sample of the population are shown by arrows.

Individual heterozygosity and possible correlates

Between one and ten sampled bucks were territorial in lek 1 in the years 1996-2001. In lek 2 between 1 and 4 sampled bucks were observed holding territories during the same breeding season. Yearly data referred to the sample of territorial bucks are summarized below.

LEK1

Year	1996	1997	1998	1999	2000	2001	Total
No. of sampled territorial bucks (T)	1	3	3	9	8	10	***18***
Av. age of territorial bucks	6	7.7	8.7	7.9	8.6	7.7	***7.8***
No. of bucks achieving copulation (%)	0 (0%)	2 (67%)	3 (100%)	6 (67%)	5 (63%)	2 (20%)	***7 (39%)***
No. of observed copulations by T	0	17	9	66	19	4	***115***
Av. heterozyosity (9 loci)	-	0.352	0.352	0.399	0.362	0.381	***0.415***
Av. relatedness (R_{QG})	-	-0.207	-0.207	0.068	0.030	0.026	***-0.002***

LEK 2

Year	1996	1997	1998	1999	2000	2001	Total
No. of sampled territorial bucks (T)	1	1	1	1	4	2	***5***
Av. age of territorial bucks	7.0	8.0	8.0	9.0	7.0	9.5	***8.0***
No. of bucks achieving copulation (%)	0	0	1 (100%)	1 (100%)	2 (50%)	1 (50%)	***3 (60%)***
No. of observed copulations by T	0	0	22	2	18	5	***47***
Av. heterozyosity (9 loci)	-	-	0.222	0.222	0.401	0.333	***0.421***
Av. relatedness (R_{QG})	-	-	-	-	-0.136	-0.195	***-0.136***

Within the sample of territorial bucks, individual heterozygosity ranged between 0.111 and 0.714 over 9 loci. The average heterozygosity for lek 1 males was 0.415, whereas it was 0.421 for territory-holders in lek 2. The six most successful males over the study period (performing more than 10 observed copulations) had values matching the overall distribution (Fig 38).

Adult bucks attended a lek territory, as they were 5 to 11 years old (Fig. 39), with 76% of cases in the range 6-9 years. The age of territorial bucks averaged 8 years, both for lek 1 and lek 2. Buck age did not seem to be influenced by heterozygosity (Fig. 40), since the mean age of territorial bucks, as they held a lek territory, was not correlated with their heterozygosity (Spearman's correlation coefficient, $r_S = -0.317$, df = 20, ns).

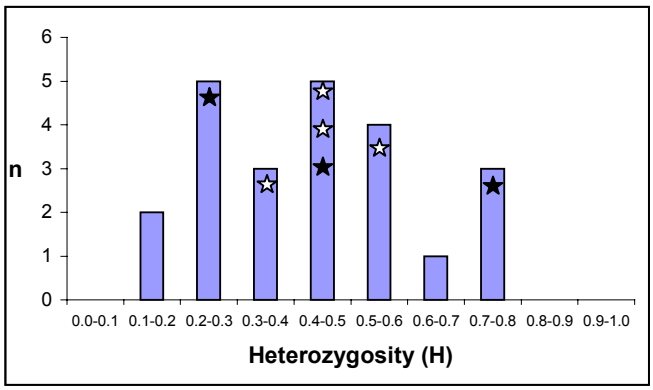

FIGURE 38. Distribution of individual heterozygosity of territorial males in the two leks. Stars indicate values corresponding to the most successful males in the sample (empty stars are used for lek 1, solid stars for lek 2).

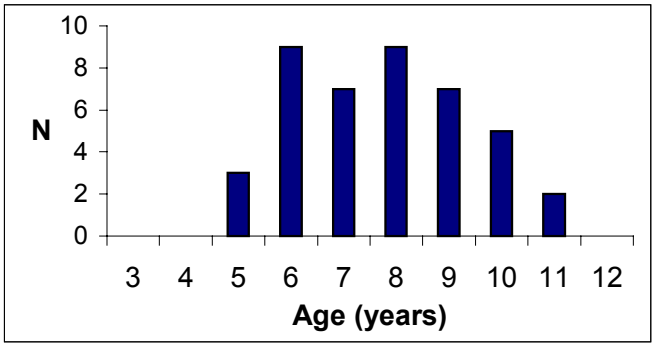

FIGURE 39. Age distribution of sampled males holding territories on the two leks.

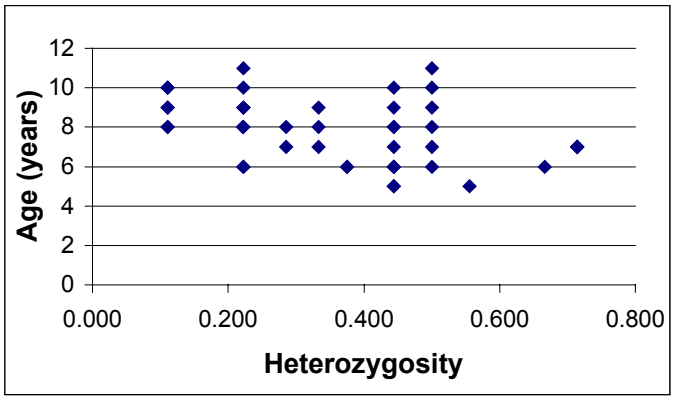

FIGURE 40. Comparison between age of territorial males and heterozygosity.

Only 10 males (out of 26 sampled) were observed to copulate on lek (Fig. 41). Seven bucks (39% of sampled individuals) achieved copulations in lek 1 territories, over a six-year period. Three out of 5 (60%) sampled bucks copulated on lek 2. On the whole, 162 copulations (115 in lek 1 and 47 in lek

2) were observed during six ruts (1996-2001), referring only to territorial bucks included in my sample. Just six dominant bucks accounted for 85% of all observed copulations.

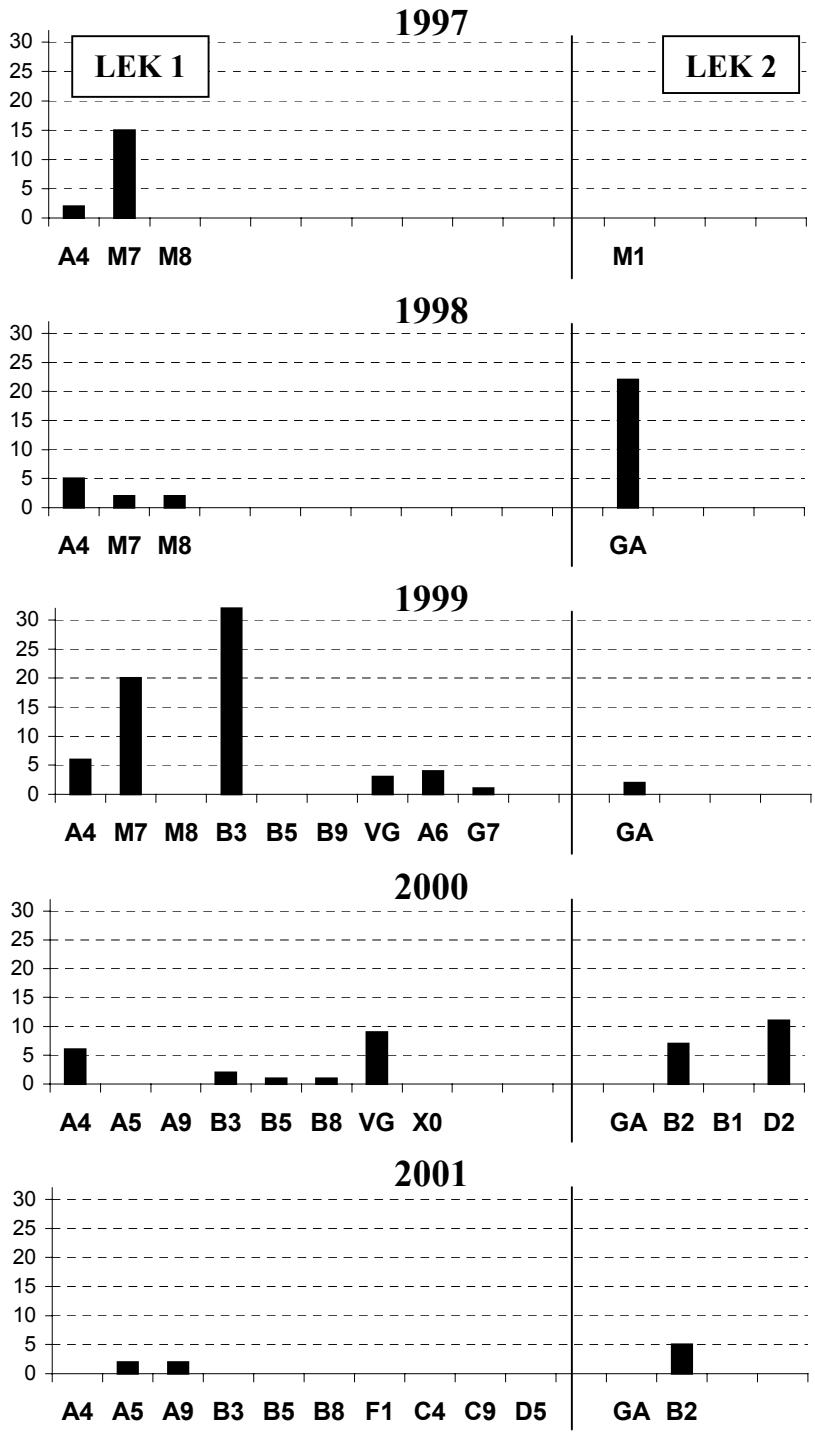

FIGURE 41. Distribution of observed copulations achieved by territorial bucks in the sample (1997-2001). Only males holding territories in a lek are taken into account for every breeding season. In 1996 no copulation by sampled bucks in the lek was recorded.

A maximum of 32 copulations were observed to be achieved by the same male during a rut. No territorial bucks achieved a high number of copulations (>10) for two consecutive years (Fig. 41), despite of the constancy of presence in a lek (in 81% of cases a male holding a lek territory in one year, held a territory in the same lek the following year).

No correlation was found between the number of copulations per breeding season and individual heterozygosity (Spearman's correlation coefficient, r_S = -0.208, df = 21, ns), whereas the former was positively correlated to the age of territorial bucks (Spearman's correlation coefficient, r_S = 0.491, df = 20, $p < 0.05$, Fig. 42).

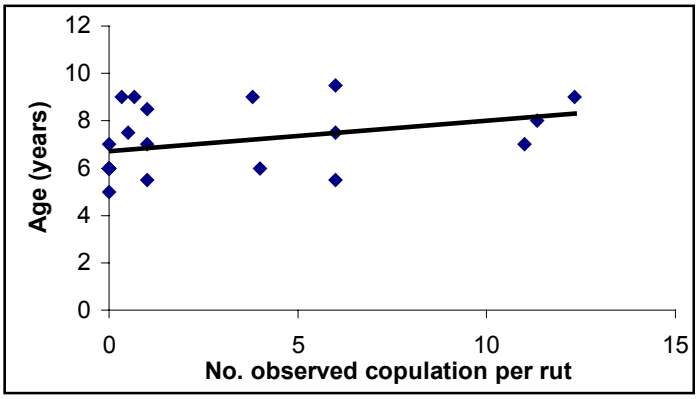

FIGURE 42. Average age of territorial bucks and number of achieved copulations per year.

3.6 Discussion

Despite extensive effort, the evolution of lekking as mating system in mammals, like in other taxa, remains a controversial issue. This study represent, as far as I know, the first attempt to explore the genetic bases of lek formation in a mammal species.

Fallow deer represent an ideal species for the study of lekking behaviour, as it shows an enormous plasticity in the adoption of mating strategies, lek representing just one of several alternatives bucks have to mate.

Fallow deer in San Rossore constitute now an isolated population of more than 2000 heads. The presence of leks in the population is known since 1970s (local warders, pers. comm.). During the rut, leks become centres of attraction for reproductively mature bucks and does. Females in oestrus leave their traditional home ranges and move to one lek for mating purpose (Ciuti et al. 2003). A small proportion of bucks holds a territory on a lek and, during this time, suspends feeding (Apollonio et al. 1989a). They come to the lek from different areas (M. Apollonio, pers. comm.) and, once occupied the territory, they defend actively their position from concurrent males for as long as 20 days (Apollonio et al. 1989a). Most males are not able to gain a territory and remain as satellites, occasionally challenging territorial bucks and attempting to intercept does approaching the lek. Other bucks adopt a low-risk strategy, defending single territories (Apollonio et al. 1992). Lekking behaviour produces a high skew in male reproductive success, with a few dominant territorial bucks monopolizing copulations. A dominance hierarchy is established among territorial bucks, from which a high variation in copulatory success arises (Apollonio et al. 1989a,b; Festa-Bianchet et al. 1990). Lek attendance was found to be the main correlate of reproductive success (Apollonio et al. 1989a). During the rut, each buck visits only a single lek (no radio-collared individuals frequented more than one lek per mating season) and, if successful, he tends to maintain his territory from one year to the next (Apollonio et al. 2003). In other words, successful territories are more likely to be held by the same buck in different years. Competitions are frequent in San Rossore leks, but non-contact aggressive interactions are the most important form of agonistic behaviour and males seem to be able to reciprocally assess fighting ability (Festa-Bianchet et al. 1990).

Although a number of studies ascertained ecological and evolutionary aspects of lekking in fallow deer, no study so far used a molecular approach to investigate its biological significance. One major problem in understanding the evolution of lek-breeding is how benefits from lekking can exceed the enormous cost of intra-sexual competition. If a so costly mating system is conserved within the San Rossore population, it should produce benefits either at the individual or at the population level.

The first beneficial effect coming in mind concerns the reproductive success of bucks joining the lek. Nevertheless, not every lekking male succeed in mating, but only a minority of them gets attention by females and does copulate (Apollonio et al. 1989a). Thus, it is not clear which benefit unsuccessful territorial bucks obtain by defending a lek territory. One solution to this apparent enigma may be to look at the reproductive success over a life span. The defence of an unsuccessful territory through one or more years could in fact assure access to a more successful territory in future years (Apollonio et al. 1992). An other possibility is to explain in terms of kin selection. If close relatives lek together, the reproduction of any of them would produce a gain in the inclusive fitness of all his kin. Indeed, the Hamilton's rule states that any behaviour of an individual, increasing the genetic fitness of another at the expense of its own, could be favoured by selection if the product of the fitness gain associated to this behaviour and the relatedness coefficient between the recipient and the actor outweighs the cost of the behaviour itself (Hamilton 1964). Kokko and Lindström (1996) considered for the first time this as a possible mechanism promoting lek formation and enabling its maintenance. Since then, only few studies were addressed to ascertain levels of relatedness in lekking species. In three bird species, leks were proved to be formed by related males. In peacock (*Pavo cristatus*, Petrie et al. 1999), black grouse (*Tetrao tetrix*, Höglund et al. 1999) and white-bearded manakins (*Manacus manacus*, Shorey et al. 2000, Höglund and Shorey 2003), birds in a lek are more related than expected. The aggregation of related males may be a consequence of their natal phylopatry, despite of female dispersal, as demonstrated in black grouse (Höglund et al. 1999).

In other bird species, co-operative behaviour in leks was proved not being the result of kin selection. Male long-tailed manakins (*Chiroxiphia linearis*) have an extraordinary courtship display which takes place in traditional arenas (leks): two males (defined alpha and beta) co-operate in a complex active display, which attract females, but only the alpha does normally copulate (McDonald and Potts 1994). This form of altruism was not explained by genetic data, as alphas and betas were not relatives.

An alternative hypothesis of lek formation would take into account the heterozygote advantage.

Brown (1997) proposed a theory according to which females prefer more heterozygous males, because the progeny sired by them would be genetically more diverse and would have a higher fitness. In other terms, heterozygosity would reflect high genetic quality, that may be referred to strength, parasite resistance, parental effort, ability in collecting food, etc.. Regard to this theory, lek would represent an aggregation of males displaying honestly their good genes. By virtue of their genetic quality, more heterozygous bucks would be stronger and more resistant to starvation,

parasites and injuries. During the rut they would join in a cluster, become territorial and attract females. Other males, with a worse body condition, might try to parasitize their attractiveness as satellites or to establish single territories in proximity of the lek. Nevertheless, copulations would be monopolized by territory-holders in the lek.

In this figure, female choice is the selective force, acting on the genetic quality of males. Brown's theory imply that females are able to recognize heterozygous males. One possibility could be represented by the effect of heterozygous genes on developmental stability, determining the symmetry of bilateral traits (Mitton 1993). In the case of fallow deer, does would thus prefer more symmetrical males, which would be those with higher levels of average heterozygosity. However, some studies failed to detect any relationship between heterozygosity and developmental stability (reduced fluctuating asymmetry) in polygynic species (Weatherhead et al. 1999). A second possibility is that heterozygosity acts on the expression of sexual traits, producing larger or more striking ornaments. The hypothesis of a mate choice based on heterozygosity-dependent sexual traits was tested without success in some bird species (e.g. Aparicio et al. 2001). Another variable which can correlate with heterozygosity is individual viability. In 17 out of 21 animal species, superior survival of individuals was associated with heterozygosity (Allendorf & Leary 1986). Since age and body weight often correlate with reproductive success (Clutton-Brock 1988, Apollonio et al. 1989a, Balmford et al. 1992), one can predict that viability, heterozygosity and fitness are strictly correlated. A further mechanism could be in act, by virtue of which the selectivity of the accession to lek territories by fallow deer bucks address female choice. In lek competitions among males, more heterozygous bucks would achieve an advantage, asserting their dominance over less heterozygous competitors. Dominant males would thus hold better territories on the lek and be preferred by females.

The two working hypotheses (Tab. 17) considered in the study can thus be summarized as follows:

1. lek is an aggregation of highly related adult bucks
2. lek is formed by fit, highly heterozygous adult bucks

The first empirical problem to face in attempting to test the mentioned hypotheses was the extremely low genetic variability of the species. As resulted in the past for other genetic markers (Pemberton & Smith 1985, Hartl et al. 1986, Randi & Apollonio 1988, Scandura et al. 1998), even microsatellites succeed in detecting just low levels of variability in fallow deer (Poetsch et al. 2001, Say et al. 2003). During the screening, out of 26 microsatellite loci, highly polymorphic in most bovids and other cervids, only 12 showed 2-5 alleles in fallow deer. The nine of them selected for

the investigation had a combined PIC of 0.358 and a mean H_e of 0.415. As consequence, the average similarity among genotypes was high.

Referring to the tested hypotheses, my data do not support the hypothesis of kin selection in fallow deer leks (Tab. 17). Levels of relatedness were on average very low within the sample of territorial males and not significantly different from those estimated for a random sample of the San Rossore population, nor from nonterritorial males. A second estimator of genetic similarity, allele sharing coefficient, was lower among territorial males than among other classes like adult females or subadult males, although higher than the average value obtained for nonterritorial males. According to this, intra-group F_{IS} was not significantly different from zero (F_{IS} = 0.069, ns) for territorial bucks, unlike instead observed for overall adult and subadult males and for nonterritorial bucks. Moreover, the high turnover among lekking males during the same rut, the variance in lek composition from one year to the next and the weak probability one male has to hold a lek territory for successive years seem to exclude an important role of kin selection.

As regards heterozygosity, the deficit of heterozygotes in the male component of the population was intriguing, as compared to a female component in Hardy-Weinberg equilibrium. Nevertheless territorial bucks are the only class of males, for which a similar deficit was responsible of the departure from HWE. Moreover, they do not seem to contribute to the significantly high value of male F_{IS}. This suggest a lower effect of inbreeding over this class respect to the others taken into account. Looking at figure 36, a progressive increase in H_o is obtained considering random subadult males, random adult males, and territorial adult males, although the differences did not resulted significant over a set of 1000 permutations. Probably the limited overall variability affects the power of the test, constraining heterozygosity values within a very short range. But a further interpretation should be considered. A highly polygynic mating system, like lek, implies that a small non-random fraction of the gene pool of adult males is sampled in each generation and transmitted to the next one. The monopolization of reproductive success by few males results in high levels of relatedness within the cohort of offspring. This process, creates ever more related cohorts and progressively erases genetic variability to the population. Consequently, a subdivision of the population in age classes is expected to generate low variable young classes respect to adult ones. Only sexual selection for heterozygotes can modify this figure, balancing the effect of non-random mating on gene frequencies.

If a high average heterozygosity is responsible of a major overall viability, territorial males should be represented by individuals under a strong selective pressure. The high costs associated to holding a lek territory could be only sustained by genetically advantaged bucks. Random males and subadults would undergo decreasing levels of this selective force, thus resulting less variable. If this

is true, a relation between age and heterozygosity is expected in territorial males. Older bucks would have a high proportion of heterozygous loci, representing the key factor enabling them to survive, despite of the costs of mating. My data do not directly confirm such prevision, as a rather opposite trend is observed, considering individual heterozygosity and the age of each territory-holder (Fig. 40). However, every year, territorial bucks were on average 8 years old, thus confirming an important role of individual viability.

Finally, no direct relationship was detected between heterozygosity and reproductive success, expressed in terms of number of observed copulations. In fallow deer, copulations performed by males were demonstrated to be a good estimator of their reproductive success and rather they can underestimate the actual variance in male reproductive success (Say et al. 2003). Consequently, the high reproductive skew observed in fallow deer cannot find a genetic justification, at least in the San Rossore population.

On the basis of the results obtained in this study, some new elements can be added to the knowledge of lek formation in fallow deer. Previous studies in this enclosed population have reported the existence of traditional leks, with a stable location from year to year. Territory occupancy is ruled by male-male competitions and a dominance hierarchy is established among males. Body size and body condition influence the capacity of a male to hold a lek territory (Apollonio et al. 1989a). Females seem to prefer only particular territories and have a memory of past matings (Apollonio et al. 1992, 2003). Copulatory success by lekking bucks is not correlated to dominance and fighting rate, but is slightly dependent on their age (Fig. 42). The fact that males come from every area of the preserve to the leks suggests a sort of 'tradition', which can arise from a common origin (kin). As territory-holders are not close relatives, a different mechanism should promote lek formation. Moreover, kin selection cannot explain the behaviour of several males, which join a cluster of territorial bucks, with a slight or no chance at all to achieve copulations.

The hypothesis of lek evolution by heterozygosity advantage implies the role of active female choice. In fact, females should be interested (and base their preference) to one or more condition-dependent male traits, which are in turn expression of the male's genetic quality (Kotiaho et al. 2001). Under this hypothesis, through their choice, practically, females select the (good) genes to transfer to their offspring, thus maximizing their own fitness. This process will promote in some cases the evolution of exaggerated male traits (like antlers in fallow deer or tail in peacock) which, although nonadaptive *per se*, are maintained by selection because indicating the high overall quality of the male's genotype (Houle & Kondrashov 2002).

In some lek-breeding ungulate populations, females were observed to select territories, rather than mates, while males compete for territories preferred by females (Deutsch & Weeks 1992, Clutton-

Brock et al. 1993, Bro-Jørgensen 2002). This competition may lead 'best' males to gain access to the 'best' territories, where they will be joined for mating by a number of receptive females. A similar mechanism was proposed also for San Rossore fallow deer leks (Apollonio et al. 1989a, 1992).

However, in other cases, evidences were reported that females in fallow deer are not selective in their mating preferences (Clutton-Brock et al. 1989). The choice, indeed, may be addressed by the harem size (the number of females present in a male territory): oestrus females choose larger harems in the lek by 'copying' the movement or the mate choice of other females (Clutton-Brock & McComb 1993). It was suggested that sexual harassment by nonterritorial bucks is the major factor inducing females to move into the lek, and leads ultimately to the evolution of lek mating in this species (Clutton-Brock et al. 1992). But, as observed by Carbone and Taborsky (1996), this assessment was affected by the lack of an accurate measure of the harassment costs experienced by females when visiting the lek. In a case in which they were accurately measured, the topi antelope (*Damaliscus lunatus*), harassment avoidance was excluded as explanation of lek evolution in that species. This aspect has been not investigated so far in the San Rossore population.

Summarizing, the results obtained in this study, combined to evidences collected in previous years in the same study area, suggest the following figure for lekking in the San Rossore fallow deer population:

1. unrelated male fallow deer in October aggregate in clusters with the only purpose of reproduction and suspend feeding activities;
2. leks are formed in traditional areas, which maximize the access of females (where several female home ranges overlap or where many female routes intersect – M. Apollonio pers. comm.);
3. oestrus females leave their usual herd and move towards a lek for mating; they prefer large aggregations (maybe to avoid sexual harassment by subordinate males);
4. the first receptive females select either male phenotypes or lek territories with particular features
5. other females visiting the lek prefer to join large harems, thus generating a high variance in harem size and mating rate among territories;
6. bucks compete for the most successful territories; age and body size influence the outcome of such competitions;
7. winning males hold territories for a few days, during which they achieve a number of copulations;
8. high heterozygosity (at random nuclear loci) do not influence mating success.

Further studies are necessary to understand: 1) which is the females' benefit (in terms of direct or indirect fitness) in mating on lek rather than in single male territories close to their usual home ranges, and analogously 2) which is the benefit for males joining the lek, but holding unsuccessful territories.

HYPOTHESIS	EXPECTATION	EVIDENCES	RESULT
❶ related bucks during the rut join in a lek to attract females; all benefits by the reproduction of few, who monopolize matings (kin selection)	territorial bucks in a lek are on average more related than other individuals in the population	negative average values of R_{QG} for territorial males on the two leks	***not supported***
	territorial bucks have a high level of inbreeding	territorial males had F_{IS} positive but lower than nonterritorial males	
❷ bucks compete for getting a lek territory; only stronger and healthy individuals succeed and become attractive for females; body condition is related to individual heterozygosity	mean heterozygosity is higher among territorial bucks than among non-territorial ones and within the population	heterozygosity is not significantly higher in territorial bucks than in other categories	***not supported***
	heterozygosity correlates with viability, thus older territorial bucks are more heterozygous than young ones	no correlation between individual heterozygosity and age of territorial bucks	
	heterozygotes are selected by female choice, thus they have major copulatory success	no correlation between individual heterozygosity and male copulatory success	

TABLE 17. Hypotheses verification on the basis of evidences provided by the present study.

- Chapter 4 -

Conclusions

The present study was intended for empirically evaluate the application of microsatellite analysis to the study of social and population structure in large mammals. In order to appreciate the possible advantages offered by microsatellites respect to other molecular markers, I selected as study cases two species, representing two different orders (*Carnivora* and *Artiodactyla*), which implied some methodological complications:

1) the Italian wolf is subject to conservation efforts (legally protected since 1976), is present at low density, is shy and elusive to humans. All these features make difficult to sample wild free-ranging wolves as much as dead wolves. This limitation has prevented for a long time from studying possible peculiarities in the ecology and behaviour of wolves in Italy, respect to their conspecific inhabiting northern regions of North America and Eurasia.
2) fallow deer is one of the most genetically impoverished ungulate species so far known. This feature prevented the study of some interesting behavioural traits in fallow deer populations with the help of traditional molecular markers. This limitation is exacerbated in small isolated populations, where the level of homogeneity among individuals is expected to be higher.

In the first case, the use of microsatellites enable the recourse to a non-invasive sampling approach. Short tandem repeats, indeed, are small regions amplified by PCR and thus their analysis is applicable to every kind of DNA source. Few molecules of DNA are necessary to start the reaction and even highly degraded DNA can be used. Therefore, wolf scats, shed hairs and blood spots collected on the snow represented the most important DNA source in the study.

However, the employment of non-invasive samples generates further problems. First, one should check for the source of errors associated to the analysis of low-quality samples. Second, one should verify that the sampled individual belongs to the species under study. Third, one has to be sure that genetically indistinguishable samples belong to the same individual in the population. Both this latter steps are possible by the combination of genetic information and statistical procedures, validating results. The former is the more problematic, and I overcame it adopting a strictly selective quality-control process (ultimately determining the reject of almost half microsatellite profiles), in order to reduce, under a reasonable threshold, the occurrence of scoring errors (false alleles and allelic dropout).

In such a way, a data set may be obtained with a sample size, sufficient to test several hypothesis concerning the social and population structure of the species under study.
In the case of the wolf study, I analyzed more than 300 samples to obtain 52 high-confidence unique genotypes. This data set was then used to measure the level of population subdivision, to determine pack structure, and to reconstruct possible genealogies.

Concerning the study on fallow deer, the high levels of polymorphism at microsatellite loci are the key feature of this markers. After a large screening including many of the most polymorphic loci found in related species, a panel of loci was detected, which were moderately variable in fallow deer. They were used to analyze 111 individuals belonging to an isolated fallow deer population adopting lek as mating system. On this way, it was possible, for the first time, to test two hypothesis related to lek formation: kin selection and heterozygosity advantage.

The application of microsatellite markers in the two studies revealed their plasticity and usefulness under different methodological and theoretical constraints.

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