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Applications of landscape genetics for wildlife conservation and management

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RIASSUNTO

Nell'ultimo decennio, l'uso di marcatori molecolari in grado di rilevare polimorfismi a livello del DNA ha acquisito sempre maggiore importanza nella genetica e nello studio delle popolazioni animali. I microsatelliti sono i più diffusamente impiegati, per la loro facilità d'impiego e il loro elevato polimorfismo, che li rende altamente informativi. I marcatori sono strumenti interessanti ed utili per evidenziare la variabilità genetica di specie, razze e popolazioni, per indagare la struttura delle popolazioni, per determinare distanze genetiche fra razze e individui e anche per la definizione di metodi di tracciabilità genetica al fine di identificare l'origine di prodotti animali destinati all'uomo, questione di particolare importanza data l'esigenza oramai diffusa di sicurezza da parte del consumatore. Essi sono decisivi per la costruzione di mappe genetiche e fisiche e sono sempre più studiati e impiegati a sostegno dei piani di selezione e conservazione. Consentono inoltre l'applicazione di test di paternità e maternità, e possono quindi contribuire al controllo delle informazioni genealogiche.

L'obiettivo generale di questo lavoro è stato l'applicazione dell'analisi con microsatelliti ad una popolazione naturale di capriolo distribuita sul territorio delle province di Trento e Belluno, con l'individuazione di nuclei di sottopopolazioni da poter, eventualmente, utilizzare a fini gestionali. Infine, è stata condotta un'indagine sull'interazione fra le caratteristiche del paesaggio e la struttura genetica delle (sotto)popolazioni di capriolo identificate.

Il primo contributo sperimentale comprende la messa a punto di un panel di 25 marcatori molecolari microsatellite per il capriolo (*Capreolus capreolus*) e la sua applicazione per l'identificazione della struttura genetica della popolazione di capriolo nelle province di Trento e Belluno, nelle Alpi orientali. La popolazione di capriolo è stata caratterizzata geneticamente per stabilire il livello di diversità genetica e per ricercare evidenze di un'eventuale strutturazione interna. Sono stati analizzati 657 campioni provenienti da capi abbattuti nelle province di Trento e Belluno nel corso delle stagioni venatorie 2003-2004 (per i campioni di Belluno) 2007-2008 e 2008-2009 (per i campioni di Trento). La caratterizzazione genetica effettuata sul campione analizzato ha dimostrato un forte deficit di eterozigosi. Sono stati applicati diversi approcci statistici per l'identificazione di eventuali sottopopolazioni e per l'identificazione di ipotetiche barriere. L'applicazione di un approccio statistico di tipo Bayesiano, utilizzando i software STRUCTURE e GENELAND, ha consentito di rilevare la presenza di sette sottopopolazioni, spazialmente separate, nell'intera area di studio. L'identificazione di ipotetiche barriere è stata effettuata tramite l'analisi delle componenti principali (PCA), utilizzando il software SURFER.

Il secondo contributo sperimentale rappresenta un'applicazione della disciplina denominata "landscape genetics", che consiste nello studio dell'interazione fra le caratteristiche del paesaggio e

processi microevolutivi quali il flusso genico, la deriva genetica e la selezione. L'associazione fra struttura genetica e conformazione del territorio è stata quindi ulteriormente approfondita nel tentativo di identificare le variabili che hanno un ruolo maggiore nell'influenzare il flusso genico. Sono state calcolate tra ogni coppia di individui due tipi di distanze geografiche: la distanza euclidea (la lunghezza della linea retta che unisce un individuo ad un altro) e la distanza di minimo costo (la traiettoria che massimizza l'utilizzo dei corridoi di bosco per spostarsi da un luogo ad un altro). Sono state, successivamente, calcolate entro ciascuna popolazione le correlazioni fra le matrici di distanza genetica ottenute con GENEPOP e le corrispondenti matrici di distanze geografiche utilizzando due approcci statistici, il Mantel test e il Partial Mantel test. Queste correlazioni sono state verificate andando a considerare vari modelli del paesaggio, che hanno preso in considerazione diversi parametri quali la presenza di bosco, la presenza di insediamenti urbani, ecc. I risultati hanno dimostrato che tutte queste variabili incidono sulla connettività della popolazione. E' stato messo in rilievo, inoltre, un differente impatto della struttura del territorio sui due sessi. Purtroppo, l'esiguo numero totale di femmine disponibili per ogni sotto-popolazione ha impedito un'adeguata analisi di questi sotto-campioni e il suo confronto con gli altri..

In conclusione, i risultati di questo lavoro hanno messo in luce, entro un'area geograficamente abbastanza limitata, l'esistenza di 7 sottopopolazioni di capriolo spazialmente separate che possono essere la base per la definizione di unità di gestione su base ecologica e non amministrativa. Inoltre, hanno fornito indicazioni a scala di paesaggio sulle relazioni fra la specie e l'uso e la morfologia del suolo. Da un punto di vista generale, inoltre, possiamo concludere che questo approccio è sicuramente molto promettente sia per studiare la struttura genetica e spaziale, e quindi evolutiva, delle popolazioni di animali selvatici, sia per affrontare con un criterio innovativo le relazioni fauna-ambiente.

Il campionamento, se si tratta di specie cacciabili, è semplice e con costi modesti si possono ottenere numerosità consistenti. La possibilità di georeferenziare la localizzazione del singolo campione e di descrivere l'ambiente con strumenti di tipo GIS permette poi di collegare le informazioni genetiche a quelle ambientali e spaziali. Con l'ormai consolidata disponibilità di software GIS e basi cartografiche approfondite, e con la prevedibile diminuzione dei costi e l'affinamento delle indagini sui marcatori genetici molecolari, le applicazioni di landscape genetics potranno certamente estendersi e fornire indicazioni sulla storia recente, sugli scambi genetici e sulla dipendenza dai fattori ambientali delle popolazioni selvatiche.

SUMMARY

In the last decade, the use of molecular markers revealing polymorphism at DNA level has played an increasing role in animal genetic and population studies. Amongst others, microsatellites have become the most widely employed markers, due to their easy use and to their high polymorphism that provides a large degree of information. Molecular markers are interesting and useful tools to assess genetic variability of species, breeds and populations, to infer population structure, to estimate genetic distances between breeds and individuals and also to define traceability methods for the identification of the origin of animal products for human consumption, a particularly important issue considering the widespread consumer demand for food safety. They are essential for the construction of genetic and physical maps and are increasingly used to assist selection and conservation plans. Moreover, they allow paternity and maternity tests, which can be a valid support to check genealogic information. Recently, molecular genetic markers have found wide application in the study of the interaction between landscape features and gene flow in natural populations.

The first part of this thesis is aimed to apply an individual-based approach, with a panel of 25 microsatellites developed for roe deer (*Capreolus capreolus*), to examine the genetic structure of a natural roe deer population distributed over the the provinces of Trento and Belluno (north-esastern Italy. Georeferenced samples from a total of 657 roe deer, harvested in the hunting seasons 2003-2004 (for the samples of Belluno), and 2007-2008 and 2008-2009 (for the samples of Trento) were used.The results showed a significant heterozygosity deficit. The application of a Bayesian statistical approach, using the STRUCTURE and GENELAND programs, detected the presence of seven spatially separated subpopulations. The identification of hypothetical barriers was carried out by principal component analysis (PCA) using the software SURFER.

The second part of the thesis is an application of the discipline "landscape genetics" assess whether the main landscape features that can be assumed as being relevant for roe deer ecology are associated with gene flow boundaries between subunits and with gene flow within subunits. Pairwise inter individual genetic distances (a_r) were calculated with GENEPOP. To consider spatial and landscape distances between each pair of individuals, we calculated the Euclidean distance (the length of the straight line that connects one individual to another) and a "least cost distance" (the trajectory that maximizes the use of wooded corridors). To take into account other landscape features, we assigned a relative cost to each distance, which varied according to the proportion of potential obstacles to roe deer movement (high elevation areas, open areas, and urban areas). Finally, we analyzed, within each subpopulation, the correlation between pairwise genetic distances and the various geographic distances using Mantel test and Partial Mantel Test. The results showed

that linear and least cost distances were correlated with gene flow in almost all populations, with slightly better values for least cost distances. Correlations improved when distances were weighed for land use and morphology costs, confirming that the assumed landscape features have an incidence on landscape connectivity for roe deer. In addition, the results suggested a difference in gene flow between males and females, although this indication should be better explored with a larger females sample.

In conclusion, this approach is certainly very promising for studying the genetic and spatial structure of wild animal populations, and for identifying landscape features limiting gene flow. In the specific case of the studied roe deer population, the results obtained can help in devising ecologically meaningful management units and in understanding the species movement patterns-habitat features at a landscape scale. The increasing availability of specific GIS tools and and geographic databases, and the expected analytical improvements and cost reduction for molecular genetic markers, the applications of landscape genetics can certainly expand and provide information on microevolutionary processes and patterns of movement of wild animal populations

CHAPTER 1

Molecular genetic approaches: what differs between livestock and wildlife?

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1.1 Introduction

The first biological level of differentiation between all organisms is DNA. Available genomes of domesticated animals range from 1 (chicken) to around 3 Gb (dog, cattle, horse) (Hillier *et al.*,2004; Lindblad-Toh *et al.*, 2005; Elsik *et al.*, 2009; Wade *et al.*, 2009), and among DNA sequences there is an enormous proportion of genetic variation. Exchanges during crossing over or mutations cause new DNA rearrangements and variability, generating differences between individuals and groups. The resulting variation is shaped by a number of factors, including individual reproductive success, exchanges between population units, population size, and natural and artificial selective pressures. By examining variation of appropriate genetic markers in relation with demographic and geographic parameters, information can be obtained about population and evolutionary processes (Sunnucks, 2000). In addition, provided suitable information on genes and regulatory regions are identified, relating their variability to phenotypic parameters may help understanding the genetic basis of natural and artificial selection.

With the development of PCR (Polymerase Chain Reaction; Mullis *et al.*, 1986) methodology and subsequently with the rapid advances in analytical techniques and platforms for genotyping, revolutionary perspectives became reality for research. In animal science, geneticists integrated phenotypic records with information on variability of genetic markers to improve selection plans for commercial traits and against diseases and to inform actions for conservation of farmed biodiversity. In Conservation biology, the advances in population genetics led to the emergence of the discipline of Conservation Genetics (Frankham, 1995; Meffe and Carrol, 1997; Frankham, 2010), which integrates the traditional demographic approach with population genetics for the conservation of biodiversity. In Landscape ecology, Landscape Genetics (Manel *et al.*, 2003; Storfer, 2007; Holderegger and Wagner, 2008) developed as a discipline integrating landscape features with spatial patterns of genetic variability to understand the influence of landscape on gene flow and microevolutionary processes.

This paper intends to provide a general overview of the differences and similarities of the applications of molecular genetic markers in what we could broadly define as the conservation and management, including commercial exploitation, of farmed and wild animal resources. In fact, genetic approaches concerned with economic or recreational use of domestic species and those concerned with conservation and management of natural resources have already inter-changed knowledge. For instance, many molecular markers used for wild species were originally developed for domestic species; moreover, quantitative genetic applications and statistical approaches and software (fore example to infer population structure and kinship), which were developed for animal breeding, are now being used to decipher natural selection. With the rapid progresses in genome

sequencing and gene mapping technologies, these interchanges are likely to increase.

1.2 Molecular genetic markers

Genetic markers are specific chromosomal regions that can be easily identified using biochemical techniques; they are mainly variation in the DNA sequence from a single base (SNP) to longer sequence changes, such as micro and minisatellites, allowing to detect genetic differences among individuals in a population. The advent of genetic tools such as restriction enzymes, methodologies as the polymerase chain reaction (Mullis *et al.*, 1986) and the growing abundance of DNA sequence data, coupled with automated high-throughput assays, have originated a rapid evolution in several classes of molecular markers (Table 1), including restriction fragment length polymorphisms (RFLPs), variable number tandem repeats (VNTRs), microsatellite and single nucleotide polymorphisms (SNPs). On the basis of the type of information at a single locus, molecular markers can be classified in three main categories (Vignal, 2002): the bi-allelic dominant, in which the dominant allele masks the recessive when heterozygous (e.g. RAPDs, AFLPs); the bi-allelic co-dominant (RFLPs, SSCPs), in which both alleles are "observable"; and the multi-allelic co-dominant (STRs). Another classification can be designed on the basis of the three main types of variation at the DNA level: single nucleotide mutations (SNPs); insertions and deletions; variations in the number of tandem repeats of a basic pattern (VNTRs).

Mitochondrial DNA (mtDNA) is another important source of information especially in population studies as it is inherited as a haploid from the mother and event of heteroplasmy are very rare (Troy *et al.*, 2001). Analysis of mtDNA together with nuclear markers as well as analysis on the Y chromosome is now being exploited to better understand population structure and history of domestic and wildlife species.

In conclusion, during the last two decades, molecular techniques applied to animal genetics moved from the analysis of very few markers (RFLP, SSCPs etc.) to hundreds of microsatellites and, nowadays, to several thousands SNPs. At present, sequencing whole genomes is technically and economically affordable, also for the most important livestock species, but it is limited by the huge bioinformatics effort needed for analyzing data.

1.3 Molecular genetic markers in animal breeding and genetics

In the last 20 years of the past century two revolutions influenced animal breeding and genetics (Green, 2009). First, developments in computing technology allowed the application of the BLUP methodology to large pedigree and performance databases, and this opened the way to continual progress in prediction methods, improvement in accuracy of breeding value estimates, and inclusion

of new traits (Koots *et al.*, 1994a,b). Second, the new generations of molecular genetic markers allowed looking into genetic code: using microsatellite markers, it was possible to search for regions of the genome harboring genes containing polymorphisms, and associations of these polymorphisms with traits of interest where then identified using statistical analysis of phenotypic records. The inclusion of this genetic information in the new "Marker Assisted Selection" (Soller, 1994) was expected to produce a series of fundamental advantages over the traditional phenotypic approach (Dekkers, 1999): heritability potentially equal to 1 (assuming no genotyping errors and no influence of environmental effects on molecular genetic information), reduction of generation intervals, thanks to the availability of genetic information for selecting at an early age, possibility of obtaining genetic information on both sexes for sex-limited traits, and on a larger number of individuals for traits that are expensive or difficult to record or that require phenotypic measurement post-mortem, as carcass and meat quality traits. To identify genes affecting traits of interest, two main approaches are used: the candidate gene and the genome-wide scan approaches (Russo and Fontanesi, 2001; Russo *et al.*, 2007). In the candidate gene approach, knowledge from species that are rich in genome information (e.g., human, mouse) and/or knowledge of the physiological basis of traits of interest, is used to identify in the species of interest genes that presumably influence economic traits, and gene polymorphisms are detected. Associations of these polymorphisms with the trait of interest are then identified using statistical analysis of phenotypic records. In the genome scan approach, linkage and/or linkage disequilibrium mapping are used to identify QTL (quantitative trait loci) in the genome. The latter, then, requires fine mapping approaches to identify genes and/or mutations involved in the traits under study. These approaches can be complementary, with a genome scan identifying regions of the genome that harbour potential QTL, followed by further investigation of genes known to be located in that region using the candidate gene approach.

Although these approaches resulted in the implementation of genetic tests for a variety of traits, concerning congenital defects, disease, milk and meat production and quality (Dekkers, 2004), progress for many traits has been far lower than that initially expected (Dekkers, 2004; Spötter and Distl, 2006, Green *et al.*, 2007). Reasons for this can be various (Weller, 2001; Kirkpatrick, 2002; Spotter and Diestl, 2006; Sellner *et al.*, 2007), and include inabilities in fine-mapping QTL regions, inadequate approaches in mapping procedures, insufficient statistical power and sample size when relating polymorphism to phenotypic records or only a partial role of QTL in determining the expression of the character.

In 2001 Meuwissen *et al.* published a pivotal scientific paper. They extended the concept of using single or multiple selected genetic markers in a breeding program into the concept of Genome Wide Marker Assisted Selection (GWMAS). They hypothesized that if one had sufficient genetic markers

to cover the entire genome of the breeding animal, it should be possible to explain all genetic variation for a trait (and indeed for all measured traits) by the variability of the genetic markers.

At present genomic selection is already used especially in dairy cattle; by using dense SNP panels, genomic breeding values (GEBV) are calculated as the sum of effects of each marker, across the entire genome, contributing to the variation of the trait under study. This approach potentially captures all the QTLs involved in the trait and they are initially estimates in a reference population with phenotypic information. Subsequently, in the following generations, only marker information is required to calculate GEBV. The reliability of GEBV seems to be significantly greater than the reliability of parental average breeding values which induced, already, some dairy breeding companies to market bull teams for commercial use based on their GEBV at 2 years of age (Hayes *et al.*, 2008).

Molecular markers are used not only for animal breeding and selection but their use is essential in the conservation of biodiversity in livestock species. In fact, in the last few decades there was a spread of specialized breeds (chickens, pigs and cows), which led, first to an increase in livestock production, but on the other to a continuous loss of biodiversity (FAO, 2007). The loss of biodiversity results in the loss or disappearance of unique adaptive attributes. Maintain the diversity of races is not only a public good but it is also important from the evolutionary point of view, because maximizing a wide range of allelic combinations that arise from long processes of adaptation and interactions with the environment. To minimize further loss of biodiversity that the international community has become aware of the need to promote specific actions for the conservation of genetic resources, or to set up and applied, at least for the species most at risk, patterns of conservation. Their main purpose is to maintain a pure genetic resources available, through the establishment of a group of animals starting (founders) and the maintenance of this population through planned matings.

In 2007, FAO published that on 19 per cent of the 7600 documented mammalian and avian domestic breeds are in danger of extinction and 9 per cent are already extinct. In particular, conservation is essential for 4 main reasons: exploit economic value of bio resources, to maintain ecosystem services, aesthetic purposes, right of all plants and animals to life. However, genetic erosion not only threatens the local breeds with decreasing population sizes, but also the highly selected breeds that are becoming genetically uniform by reproducing only through a few top breeding animals. In fact, biodiversity is genetic diversity and the basis of life on earth (Ajmone-Marsan and GLOBALDIV Consortium, 2010). Several of the seven major criteria for conservation of livestock proposed by Ruane (1999) are also relevant for wildlife: the species, the degree of endangerment, adaptation to a specific environment, traits of economic importance, unique traits,

cultural, historical and social value, and genetic uniqueness of the breed. For wildlife and livestock is important to consent a flexible selection of advantageous traits, adaptation to a changing the environment and avoiding inbreeding depression. In all this cases genetic variation is essential. Several wild species or populations are in danger of extinction, because human demographic and cultural development involves habitat loss, fragmentation of ecosystems or hunting. Although selection of livestock is partially artificial, there are several interesting parallels with wildlife genetics. Surveys of genetic diversity on the molecular level of wildlife and livestock are based on the same categories of genetic markers (Vial *et al.*, 2003). In fact, several molecular studies on the diversity of wild species were on the basis of markers identified originally in livestock species, like the bovine markers used for deer.

1.4 Genetic authentication of species, breed, and individual: traceability and forensic

Authentication of species, breed, individual and geographical origin of animals or of their products is important for a variety of reasons. Marketing of animal products with known and assured provenance has become a necessity in recent years following various food safety scares (e.g. Bovine spongiform encephalopathy, avian flu, food toxinfections). In addition, marketing strategies for many brands of local or high quality animal products rely on assurance of geographical provenance, breed and even farm and individual origin for increasing added value (Gandini and Villa, 2003). On the other end, fraudulent and/or illegal use of low value products instead than high value products is a risk, especially in preparation of processed foods of animal origin. These issues have led to an increasing implementation of traceability systems, which can be defined as systems used to trace an animal product by identifying the various steps in the food chain, “from farm to fork”. Since DNA is stable, intrinsic to animal cells, and highly variable among species, breeds and individuals, DNA-based approaches have the ability to overcome the uncertainties and limits of the conventional traceability procedures (for reviews see Dalvit et al., 2007, and Fontanesi, 2009). It is interesting how, in addition to endogenous DNA for tracing the intrinsic origin of products, exogenous DNA may be used for labeling these products for external characteristics, as year, batch, producer, etc. (Fontanesi , 2009). With the future trend for increasing consumption of wild ungulate meat, species traceability will likely become an important issue also for marketing of game meats (Fajardo et al., 2010; Ramanzin et al., 2010). But game meat authenticity not only relates to the industrial economic profit resulting from illegal trading, but also to public health risks (e.g. zoonoses or allergies). For example Chomel et al. (2007) evidenced that scientist believed that HIV derived by consumption of ape meat infected with Simian Immunodeficiency Virus.

Genetic authentication of animal specimens or products is important also for forensic applications. Wildlife DNA forensic (Ogden et al., 2009; Alacs et al., 2010) is an applied discipline that has emerged from a combination of conservation genetic and forensic genetic to help in contrasting the illicit procurement, transport, and distribution of live animals and their food products. Wyler and Sheikh (2008) estimated that profit of illegal trade of animals and products exceed 20$ billion annually. Forensic applications (Budowle et al., 2005) are most relevant for endangered and strictly protected species, but obviously may also be used against illegal harvesting of species subject to exploitative management (for example fish) or recreational hunting. Wildlife legislation usually operates within political boundaries (national and regional borders), but species distributions is determined by environmental features and other factors that rarely match with such boundaries (Ogden et al., 2009). This leads to the necessity of identifying different geographic origins within species. For example, to manage marine protected areas methods are required that permit to identify illegally harvested stocks from those taken legally from a different place (Ogden, 2008)

A variant of individual identification is the testing of relationships between two or more individuals, such as parent-child, full sibs, half-sibs, or unrelated individuals, have to be tested. This can contribute to the reconstruction of pedigrees (see previous section), but has important commercial implications for horses and cattle (Vignal et al. 2002), and also for companion animals as dog and cat (Lipinski et al., 2007). Paternity tests are also useful in extensive farming conditions where multiple sire mating strategies are utilized and cattle producers would find otherwise very difficult to assign sires to progeny and to establish the reproductive performance of each bull (Van Eenemann, 2010).

Methods for authentication of species, breed, and individual, and for identification of geographic provenance, use different approaches and markers but are similar across disciplines. For species authentication, PCR-based analysis of DNA fragments which differ in their sequences between species are mostly used, and mitochondrial DNA is preferred because it is easier to amplify from highly processed and/or degraded tissue (Mafra et al., 2008; Fontanesi et al., 2009; Fajardo et al., 2010). In particular, the most commonly used universal markers are the mitochondrial cytochrome b and the cytochrome oxidase I genes (Pfunder et al., 2004). These procedures are each able to identify a limited number of species, and therefore are difficult or costly and time consuming to apply when the range of potential species is unknown and possibly wide. However, recent developments in microarray analytical technologies are encouraging development of methods that can screen a wide range of species (Peter et al., 2004; Chisholm *et al.*, 2008, Kochzius et al., 2010). For example, the development of DNA barcoding should be applied for species identification of animal products of different origin (Consortium for the Barcode of Life,

http://www.barcoding.si.edu/). Using a "deterministic approach" (Milanesi & Negrini, 2003; Fontanesi, 2009), breeds or sub-specific groups may be recognized with simple diagnostic tests if specific molecular markers, or genes that have certain alleles fixed in that breed, may be identified. Examples are the analysis of mutations in the MC1R gene proposed by Russo et al. (2007) for authentication of mono-breed Parmigiano Reggiano produced from Reggiana milk, or the use of fixed alleles for coat colour (Fontanesi, 2009), or the nuclear melanocortin receptor 1 (MC1R) gene for discriminating between wild boar and domestic pig meats (Fajardo et al., 2010). A second approach follows a "probabilistic method", where breeds are genotyped with a set of microsatellites or SNPs, and individuals are assigned on the basis of genetic distances between the breeds or of allele frequencies of each breed (Dalvit et al., 2007; Negrini et al, 2008). The assignment probability depends on the number of markers used and on the genetic distances between breeds, and especially with microsatellites might be unsatisfactory. However, the use of high throughput genotyping technologies based on the analysis of commercial chips including thousands of SNPs will greatly improve the allocation probability. Also the inability of this method to discriminate breeds for products composed by mixtures of several/many animals might be overcome by the use of a deterministic approach (Fontanesi, 2009).

1.5 Understanding the genetic bases of evolution in natural populations

In Conservation Biology, knowledge of the genetic basis of phenotypic traits and of the selection pressures acting on them would dramatically increase understanding of the evolution, and hence conservation of wild populations (Garant and Kruuk, 2005; Allendorf, 2010; Frankham, 2010). This knowledge is also necessary for informing exploitative management or recreational hunting strategies, that otherwise might create potentially negative artificial selection pressures (Coltman et al., 2003b; Allendorf et al., 2008). To this purpose, applications of quantitative (phenotypic) genetic in wild populations have been for a long time impeded by the lack of pedigree records to obtain heritability estimates. Pedigree reconstructions became possible with the availability of microsatellite panels, but, given the accuracy allowed by the limited number of loci considered, remain mostly limited to few populations subjected to intensive monitoring, where at least mothers are known from field observations of mother-son dyads (Kruuk et al, 2000; Coltman et al., 2001; Coltman et al., 2003a; Réale et al., 2003; Poissant et al., 2008, but for a review see Kruuk, 2004). Molecular markers may be used to obtain "pedigree-free" measures of genetic relatedness (for reviews see Jones and Harden, 2003), but Thomas et al. (2002) and Garant and Kruuk (2005) concluded that these approaches (using microsatellite markers) are less useful to estimate quantitative genetic parameters than explicit pedigree reconstruction. The availability of molecular

markers has allowed QTL and candidate gene approaches also in wild populations (Slate et al., 2002; Vasemagi and Primmer, 2005; Da Silva et al., 2009; Luikart et al., 2008; Johnston et al., 2010), but constraints limiting these approaches are understandably much more important than for livestock.

One fundamental topic for conservation of small, isolated population is inbreeding depression (Reed, 2003; Hedrick, 2000; Blomqvist et al., 2010; Frankham, 2010). Availability of microsatellite markers allowed to indirectly estimate inbreeding coefficients through heterozygosis measures (Coltman and Slate, 2003) and to relate them with fitness and phenotypic traits, but the genetic origin of inbreeding depression is still unclear and the meaning of such heterozigosity-fitness correlations for explaining inbreeding depression has been questioned (Balloux et al, 2004, Grueber et al, 2008; Ruiz-Lopez et al., 2009). On the opposite, outbreeding depression is a concern when of gene flow is established between previously isolated populations, for instance with animal translocations or habitat modifications, because it might result in chromosomal or genic incompatibilities between hybridizing taxa or reduced adaptation to local environmental conditions (Edmands et al, 2007). Presently, molecular markers may be used to identify hybrid individuals and relate hybridization with changes in fitness or other phenotypic traits (Senn and Pemberton, 2009; Randi, 2005), but offer a limited capability of explaining the genetic mechanisms underlying these effects.

In conclusion, molecular genetic markers allowed introducing quantitative genetic approaches in natural populations, but progress has been hampered by difficulties in pedigree estimates and uncertain reliability of pedigree-free approaches, and there are presently few estimates of quantitative genetic variation in threatened species (Frankham, 2010). However, the "genomic Revolution" is expected to make available wide genome maps for numerous species, which on one side will improve marker-based pedigree reconstructions, and on the other will allow improved estimates of genetic relatedness (Allendorf et al., 2010; Frankham, 2010). Therefore, we may expect that the prediction by Moore and Kukuk (2002) of an imminent revitalization of quantitative genetics will be confirmed. On the other hand, additive genetic variance may explain only part of the variability in adaptation and evolution of natural populations. To this purpose, the limited number of markers available has so far hindered molecular approaches to identify QTLs. List of candidate genes will increase with the expected much wider genome mapping, but a very interesting perspective is that of identifying genomic regions responsible for local adaptation: when comparing different populations these regions will appear as outliers from the patterns of neutral regions, which are determined primarily by genetic drift and gene flow (Joost et al., 2007; Allendorf et al., 2010). Two crucial questions in conservation biology that have been so far inadequately understood

are inbreeding and outbreeding depression, and this is an area where genomic might provide decisive progress by unveiling the loci involved and their genetic mechanisms (Allendorf et al., 2010).

1.6 Monitoring genetic structure in wild populations

Conservation genetics plays a fundamental role in defining the appropriate taxonomic and population units that need separate conservation and management. A fundamental concept to this purpose is the "evolutionary significant unit" (ESU; Moritz, 1994; Bowen, 1999; Crandall et al., 2000; Moritz, 2002). Early definitions of ESU, as for instance that of Ryder (1986) as " one or a set of conspecific populations with a distinct long-term evolutionary history mostly separate from that of other such units of the same species", were mainly based on patterns of reproductive isolation and aimed at defining "intraspecific phylogroups" (Avise and Walker, 1999). In this regards, ESUs might be considered as validated "subspecies" (see for instance the italic roe deer, see Randi et al., 2004). More recently, the concept has been expanded to include divergence in adaptation, as in the definition of Allendorf et al., (2010): "A classification of populations that have substantial reproductive isolation which has led to adaptive differences so that the population represents a significant evolutionary component of the species". Neutral markers are normally used to measure genetic distance and infer reproductive isolation. Mitochondrial haplotypes are especially powerful for identifying potential ESUs (Avise, 1995; Moritz, 1994) because their typically fourfold smaller effective population size (compared with haplotypes at autosomal loci), and because of the special relevance of matrilines to population demography. Criteria suggested for recognizing ESUs may be very general, as in the suggestion that ESUs should contribute substantially to the overall genetic diversity within a species, or more explicit in that ESUs should be identified as groups of populations "reciprocally monophyletic for mtDNA alleles and also differing significantly for the frequency of alleles at nuclear loci" (Moritz, 1994). Furthermore, recognition of the role of adaptive divergence is presently more a theoretical than a feasible issue, given the difficulties of identifying differences in adaptation. Frankham (2010) proposed the agreeing on definitions of ESUs as one of the priorities for conservation genetics. In any case, the concept that intraspecific groups showing sufficient genetic divergence should be regarded as conservation units, irrespective of their recognition as subspecies, is an important achievement e remains valid.

In management actions for conservation or exploitation (for instance hunting) of natural populations a critical need is that of defining appropriate management units (MUs). Management units may be considered as units of a population that are demographically independent (Moritz, 1994), and therefore may be subject of specific monitoring and management actions. They differ from ESUs in

that ‘the focus of the management unit is on contemporary population structuring and short-term monitoring rather than historical factors’ (Fraser and Bernatchez 2001). In practice, MUs are in most cases devised on the basis of administrative boundaries, or of obvious barriers (rivers, mountain ridges, urban areas, etc.), but this is rarely an ecologically meaningful criterion. Normally, the number of migrants that must be exchanged between two populations to avoid genetic divergence is lower than the number that is needed for ensuring demographic synchrony (Avise, 2004). Hence, a genetic approach may resolve situations where geographical and administrative approaches are inadequate or ambiguous: if significant differences in allele frequencies at neutral marker loci are observed between two spatially separated groups of individuals, these groups may qualify as distinct MUs (Zannése et al., 2006).

For many species, human induced landscape changes have produced fragmentation of suitable habitats, and human pressure (for instance hunting) has caused local extinctions. From an hypothetic original population that was more or less homogeneously distributed over a large area, this has produced either few smaller, isolated populations or a “metapopulation”. A metapopulation is “a collection of populations of a species found in differing geographic locations and with restricted gene flow (exchange of individuals and genes) between the populations” (Allendorf et al., 2010). In these small population units, drift may produce a reduction in heterozygosis and allelic richness, which is considered negative for the evolutionary potential and viability. Therefore, for an informed management of small isolated populations and metapopulations, information on gene flow and genetic variability within and between populations are needed. This is usually achieved by using molecular markers, most frequently microsatellites but also mitochondrial DNA and SNPs, to calculate allelic richness, observed and expected heterozygosis, population effective size (Schwartz et al., 2006). In metapopulations, exchange rate of individuals between populations units might also be assessed (Storfer et al., 2007). A strategy for the conservation of species with a fragmented distribution is in fact that of increasing range with artificial reintroductions in suitable areas were the species had gone extinct, or to reinforce local populations to increase genetic variability (Storfer, 1999). Source populations for translocated individuals should be chosen on the basis of their genetic variability and genetic distance from the receiving ones.

In synthesis, in conservation and management of wild animal biodiversity, molecular genetic markers have a fundamental role for identifying Evolutionary Significant Units, Management Units, and Metapopulations, and allow temporal monitoring of the genetic variability between and within these units. This information can be used to inform conservation and management plans by defining priorities (for instance when limited resources obligate to choose few among many units for conservation actions), addressing the appropriate units for coordinated population demographic

monitoring and active management actions (for instance for setting differentiated harvest quotas), and planning translocations aimed at increasing gene flow. Theoretical approaches and methods are well developed, but for some issues are still debated, in particular that of how to define units within species that are sufficiently differentiated to require separate management (Frankham et al, 2010). This task will be probably easier in the future, when the genotyping of many more neutral loci will greatly improve understanding of the patterns of reproductive isolation and demographic history of populations (Schwartz et al., 2006), while genomic approaches for studying functional genes will allow quantifying the extent of adaptive divergence among them (Allendorf et al., 2010).

1.7 Using genetic approaches for the monitoring of demographic and spatial parameters in natural populations

Use of molecular genetic markers for individual identification may provide information on population size and on demographic parameters as mortality and recruitment (Schwartz et al., 2006). Traditionally, these parameters are estimated by capturing, marking and releasing a sample of individuals of the population, which are then monitored (usually with field observations). However, this approach is costly and time consuming, and cannot be applied to species that are elusive of difficult to capture. With the genetic approach (Prugh et al, 2005; Schwartz et al., 2006; Bhagavatula and Singh, 2007; Marucco et al., 2009), DNA analysis of samples of faeces, hair and feathers collected in field surveys is used to identify the different individuals and to produce estimates of population size at each survey, and of mortality and recruitment between successive surveys. Measuring individual reproductive success is a critical issue in monitoring demographic parameters: for females, it may be estimated from field observations of marked individuals, but this is rarely possible for males. Genotyping males and mother-sons dyads within a population may permit estimates of male reproductive success in wild populations.

Using molecular markers and procedures for species (and other groups) assignment, the same non-invasive extensive sampling approach followed by genetic analysis may be used to monitor spatial distribution (Balestrieri et al., 2008; Fernandez et al., 2006), which for rare and elusive species is normally very difficult to estimate due to the low probability of detection and the poor reliability of visual observations and other signs of presence (tracks, etc.).

In conclusion, applications of non-invasive sampling and genotyping are in many cases (rare and elusive species) the only opportunity for monitoring demographic and spatial population parameters, or can be integrated with other traditional approaches to improve efficacy of experimental protocols. With the reduction in costs and availability of simpler tests, these approaches could find useful applications also in the monitoring of species that are easier to study

with conventional approaches. However, genetic applications in these fields are relatively recent, and a critical issue is consequently the improvement of sampling strategies, experimental design, and statistical methodologies (Schwartz et al., 2006). In addition, to increase the detective power of non-invasive sampling, it will be necessary to improve quantity and quality of DNA obtained from faeces, hair and feathers, which is often unsatisfactory (Waits & Paetkau, 2005).

1.8 Understanding the effect of landscape on gene flow

A recent development in conservation genetics is the emergence of the new field of "Landscape genetics". Manel et al (2003), in their inaugural article, defined landscape genetics as an "amalgamation of landscape ecology and population genetics". Later, Storfer et al (2007) defined landscape genetics as "research that explicitly quantifies the effects of landscape composition, configuration and matrix quality on gene flow and spatial genetic variation". More recently, Holderegger and Wagner (2008) expanded the definition of landscape genetics to "an amalgamation of population genetic data, adaptive or neutral, with data of landscape structure". The distinction between neutral and adaptive genetic data is important: in general only neutral markers (e.g. microsatellites, amplified fragment length polymorphisms) are used (Manel et al., 2003; Storfer et al., 2007), because their variability reflects gene flow but is uninfluenced by selective pressures that change allele frequencies in genes responsible for adaptive traits. However, a genomic approach could distinguish between neutral and adaptive loci, making in theory possible the understanding both of factors affecting gene flow and of those influencing local adaptation (Allendorf et al., 2010). The incorporation of landscape matrix into landscape genetics is an important difference to population genetic. In landscape genetics this matrix of habitat, morphological, climatic (etc.) features is considered as a major cause of biological and ecological processes occurring at the landscape level (Holderegger and Wagner, 2008). Therefore, while population genetics studies the genetic variability between and within (sub)population units, landscape genetics will identify (sub)population units but also describe spatial patterns of genetic variability (isolation by distance, clines, genetic boundaries, random patterns), and investigate on the causes that determine such units and patterns. Examples of questions that could be addressed are, for instance, whether particular landscape features act as effective barriers to movement (Valvo et al., 2009; Coulon et al., 2006; Luo et al., 2010) or which are the ecological features facilitating or conversely those limiting gene flow between the different units of a metapopulation (Murphy et al., 2010), etc. This is important to define geographic units for animal conservation and management, but also to understand the effect of antrophic infrastructure such as motorways, bridge, etc. on ecological corridors (Kuehn et al., 2007). In prospective landscape genetics should be used also to identify environmental and

landscape drivers of disease spread. In particular, landscape features influencing aetiological agents spread by tracking host and/or their movement should be investigated across multiple spatial scales (Archie et al., 2009). This can lead to important application also for health, for example studying epidemiology of zoonoses.

The two key steps of landscape genetics are the detection of spatial genetic patterns, with the analysis of genetic data from many individuals (or spatially separated populations) whose exact geographical location is known, and then the correlation of genetic patterns with landscape features analysed with GIS approaches. Landscape genetics is a rapidly evolving, interdisciplinary field where approaches and statistical methods of different disciplines (landscape ecology, population genetics, spatial analysis) can be used. Manel et al, (2003) first reviewed the statistical tools to identify spatial genetic patterns and correlate them with landscape features, and Guillot et al. (2009) updated this issue; Excoffier and Heckel (2006) described the variety of computer software available; Storfer et al. (2007) reviewed the questions commonly addressed in the landscape genetics literature, and provided guidelines for sampling design; Holdereger and Wagner (2008) reviewed the future role of landscape genetics, with specific reference to the understanding of adaptive genetic variation. For the purpose of this study, a synthetic overview of the main statistical approaches (table 2) is given in what follows.

1.8.1 Bayesian clustering approaches and Assignment tests

A basic step in landscape genetic is that of defining (sub)population units and locating them spatially without any a *priori* (sub)population definition (Storfer et al, 2007). Usually, a number of individuals of the species of interest are sampled over the study area and the geographical location of each individual sampled is recorded. Bayesian clustering approaches are derived from traditional assignment tests and use individual genotypes to assign individuals to populations (Paetkau et al., 1995). These methods (e.g. STRUCTURE software [Pritchard et al., 2000; Falush et al., 2003]) attempt to minimize Hardy-Weinberg and gametic disequilibrium to group individuals into populations, and assign to each individual a probability of belonging to each population sampled. Individuals are then mapped using their geographic coordinates to evaluate the spatial distribution of each population (Coulon et al., 2006). In continuously distributed populations, spatial-assignment tests are used to identify genetic boundaries between sub-population units. A widely used approach is that of GENELAND (Guillot et al., 2005; Guillot et al, 2008), that integrates spatial contiguity of individuals with a Bayesian genetic assignment. As a result, individuals are assigned to the likely sub-populations not merely on the basis of their genotype, but also of their geographic locations,

and boundaries between sub-populations are identified more clearly than with STRUCTURE (Coulon et al., 2006).

1.8.2 Mantel's test and regression analysis

Mantel's (1967) test is a regression in which the variables are themselves distance or dissimilarity matrices summarizing pairwise similarities among sample locations. In particular in landscape genetics, it is a regression between genetic differentiation (between pairs of individuals) and their geographical distance (Manel *et al.*, 2003, Guillot et al, 2009). This is a method for detecting isolation by distance patterns (isolation by distance indicates the increase of genetic distance between individuals or populations with increasing spatial distance), which is generally used as the first step in exploring spatial patterns of gene flow and inferring the presence of drift or barrier effects (Hutchison and Templeton, 1999; Manel *et al.*, 2003; Guillot *et al.*, 2009). Partial Mantel tests are multiple regressions that are often used when the analyses aim at identifying among a set of landscape variables those that explain significant levels of the genetic distance among individuals (Manel et al, 2003; Epps *et al.*, 2005; Stevens *et al.*, 2006; Guillot *et al.*, 2009).

1.8.3 Ordination (Canonical Correspondence Analysis)

A limitation of the Mantel and Partial Mantel tests is that they cannot quantify the amount of variation of genetic distances explained by the different environmental factors (Manel et al, 2003). To this purpose, canonical correspondence analysis (CCA; Ter Braak, 1986), a method in which a regression model is inserted in the ordination model with the result that the ordination axes appear in order of variance explained by linear combinations of independent variables, has been used to identify the influence of continuous variables and gradients on genetic variations (Angers et al., 1999; Manel et al, 2003; Storfer et al, 2007).

1.8.4 Spatial autocorrelation

Spatial autocorrelation happens when the value of a variable is dependent on the values of the same variable in neighbouring locations (Getis, 2010). This approach is used to test as the genotype of an individual is influenced by the genotype of other individuals in relation to their spatial distances (Manel et al., 2003; Miller et al., 2010). Spatial autocorrelation has been used also to identify clines (Sokal and Thomson, 1998). This method is very useful to detect the scale at which spatial patterns occur, but it is unable to locate genetic discontinuities (Manel et al., 2003)

1.8.5 Multivariate analyses and synthesis maps

Another approach to identification of spatial patterns is the use of principal component analysis (PCA). In statistics, PCA is a mathematical procedure that transforms a number of possibly correlated variables into a number of uncorrelated variables, the principal components, related to the original variables by an orthogonal transformation. It is used to reveal simple patterns within a complex set of variables. In population genetics PCA was first used by Menozzi et al. (1978) to detect axes of human variation, and is frequently used in human genetics. In landscape genetics PCA aimed at synthesizing all the variation for many loci in the study area. Finally, the interpolation of the major principal components leads to a synthesis maps (Piertney et al., 1998; Hanotte et al., 2002). Principal component analysis may also be coupled with clustering to identify populations. To this regard, it is considered to be more powerful than classical methods when large numbers of markers are available to detect fine population structuring (Lee et al., 2009).

1.9 Landscape description

The approaches synthesized above require maps or a set of landscape variables to be related with patterns of genetic variability (Manel et al, 2003), and this is accomplished using GIS software. For example Coulon et al. (2004) used GIS to calculate least cost distance paths, which maximized interception of woodland patches and minimized that of open fields, to mimic movement routes of roe deer across a heterogeneous landscape, and found using partial mantel tests found that least cost distances were more related to gene flow than linear distances. Least cost distances including different landscape features are usually used in partial mantel tests (Manel et al, 2003; Epps et al., 2005; Stevens et al., 2006; Guillot et al., 2009). Modelling of animal movement is in continuous development. McRae and Beier (2007) borrowed the electrical circuit theory approach to incorporate effects of multiple pathways connecting individuals in the analysis of gene flow. Spear et al. (2010) used Resistance Surface models, which produce maps in which each spatial units is assigned a value indicating the cost for an individual of crossing it. In metapopulations of Columbia spotted frog (*Rana luteiventris*), Murphy et al. (2010) applied gravity models, originally developed in transportation and economic geography, to integrate spatial distance, source-sink dynamics and resistance surfaces in modelling gene flow.

In summary, landscape genetics is an interdisciplinary field in rapid movement. We have rapidly delineated above the main methods used, but conceptual approaches and statistical procedures are continuously evolving. In the future, these developments will allow accommodating larger number of markers and finer landscape descriptions and analyses in the understanding of factors influencing gene flow and ecological and evolutionary processes. To this regard, the possible inclusion of

adaptive variation could be revolutionary, permitting the understanding of the spatial variability of selective pressures and adaptation (Allendorf et al., 2010).

1.10 What differs between livestock and wildlife?

There is no doubt that the advent of molecular genetic markers has profoundly innovated research in livestock science and conservation biology. Applications in livestock science have been favored by the economic interests of productive traits and the ability of controlling environment, recording genealogies, and measuring phenotypes over, in certain cases, most of the breeds. A major use of molecular markers is in genome mapping, comprising the identification of loci or regions (QTL) that are responsible for traits of interest within marker assisted selection schemes (or, in the future, genome wide assisted selection). This field is in rapid evolution, but has resulted in the availability of a variety of tests concerning congenital defects, disease, and productive traits. In addition, molecular markers are used for describing population genetic structure between and within breeds, which is essential for designing conservation plans of livestock biodiversity. To this purpose, methodological guidelines have been recently produced (CGRFA/WB, 2010). Finally, molecular markers allow fingerprinting, which is used in traceability of animals and their products through the commercial and food chain, and in forensic applications such as parentage assignment and individual traceability. Microsatellites are widely used, but SNPs and genome-wide approaches will replace them in many applications in the future, enabling researchers to address questions that are now intractable (Dekkers, 2004; Sellner et al., 2007). Despite of the great potential and the promises of the "genomic revolution", this progress will depend on the availability of sufficiently powerful bioinformatic and statistic support, of resource populations for measuring traits, especially those that are difficult to define and measure (for instance longevity and adaptability), and of adequate funding.

In conservation biology, understanding of the genetic bases of natural and human induced selection and of adaptation would be a revolutionary advancement. However, since in natural populations it s impossible to record pedigrees and difficult to measure phenotypes over sufficiently large samples, these aspects cannot be fully deciphered. Molecular genetic markers allowed to partially overcome these limitations and, in the recent years quantitative and molecular assisted approaches have been proposed, but advancements are limited to model species or few isolated and intensively monitored wild populations. Genomic approaches might circumvent the need for pedigrees and heritability estimates, and are particularly useful for traits, such as fitness, that have a complex genetic architecture. According to Allendorf et al. (2010) "…This coming explosion of information will transform our understanding of the amount, distribution and functional significance of genetic

variation in natural populations…". However, the present interest in genome projects is only marginally towards wild animal populations (genome projects stored in http://www.genomesonline.org are 66% in biomedical, 18% in biotechnology, 10% in environmental, 5% in agricultural and 1% in evolutionary). In addition, funding opportunities but mainly availability of resource populations for phenotype measures will be important limitations. Therefore, we expect that progress will be slow. Despite of this, the molecular genetic markers and the future genomic advances will bridge the gap in methodological approaches that has so far limited progress in knowledge in wild as respect to domestic species.

Where conversely molecular markers have found wide and innovative application in conservation biology is in the description of biodiversity, providing researchers and managers with effective tools for the identification of intraspecific units worth of conservation and in the management and monitoring of threatened species and populations. In addition, landscape genetics is a rapidly expanding, area that is unveiling how features of the landscape in which populations live shape patterns of gene flow. This has fundamental implications for the understanding of functional connectivity and, ultimately, of evolutionary processes. The perspective of incorporating adaptive variation in these approaches is one of the most exciting among those expected from the genomic revolution (Allendorf et al., 2010).

In any case, molecular genetic markers have opened new research fields in conservation biology. These fields require an interdisciplinary approach and are rapidly moving forward, with exchanges of conceptual and methodological approaches. This ebullience differs from the more standardized approaches used for livestock, where aims are less diversified and evolution of species is mostly under human control. For instance, panel of microsatellites, the most widely used markers presently used which have been standardized for domestic species (CGRFA/WB, 2010), largely differ between studies with the same wild species, making comparisons and meta-analyses almost impossible. Furthermore, there is still debate and lack of agreement over the different approaches that can be used in landscape genetics (Storfer et al., 2007; Guillot et al, 2009). Therefore, we think that progress in the next future will be mostly dependent on the identification, sharing and standardization of appropriate experimental designs, genetic markers panels, and statistical approaches.

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TABLES

Table 1. Description and application of molecular markers in animal science

Marker	Description	Application	Use: H/C/F[1]
RFLP	**Restriction Fragment Length Polymorphism**. When at the nucleotide level a change occurs, a restriction endonuclease recognition site is created or deleted. If the DNA sequence acquires or loses the ability to be cleaved by a particular restriction endonuclease and consequently the digestion with the relevant restriction enzyme will generate two shorter fragments or a longer fragment..	Construction of genetic maps, revealed the chromosomal locations of genetic elements	H
RAPD	**Randomly Amplified Polymorphic DNA**. It is based on the amplification of multiple and random segments of the genome by short (c.a. 10 bp) PCR primers of arbitrary sequence. The amplicons can be separated on agarose gels stained with ethidium bromide. Allelic variation consists on the presence or absence of particular amplification products.	Population typing, Pedigree analyses, Phylogenetic studies, Genetic mapping	H
AFLP	**Amplified Fragment Length Polymorphism**. A more sensitive method for detecting DNA polymorphism, as opposed to the relatively coarse-grained resolution of RFLP. Following restriction enzyme digestion of DNA, a subset of the DNA fragments are selected for PCR amplification and visualization. It one can screen many loci simultaneously. Because it is a dominant marker, analysis requires some assumptions about heterozygote frequencies.	Detecting phylogenetic signal in poorly differentiated taxa	H
SSCP	**Single strand conformational polymorphism**. Single-stranded (denaturated) DNA molecules a few hundred bp in length often assume different conformations even when differing by as little as one base pair. These distinctive conformations can be detected by electrophoresing PCR-amplified molecules through neutral polyacrylamide gels. Under appropriate conditions (notably low temperature and non-denaturing conditions), DNA strands fold into structures that migrate according to their shape.	genotyping to detect homozygous individuals of different allelic states, detect variations in different strains of a organism	C
STRs	**Micorsatellites (Short tandem repeats)**. Repeats of nucleotide sequences. The tandem units can be dinucleotides, trinucleotides or tetranucleotides. The apparent mutation process is by slippage replication errors, where the repeats allow matching via excision or addition of repeats. Because this sort of slippage replication is more likely than point mutations, microsatellite loci tend to be hypervariable. The usual procedure is to use an oligo (e.g., AC10) as a probe, screen a genomic library and then sequence positive clones to develop primer pairs that can be used to amplify the target DNA with PCR.	Study of population genetic, map construction, QTL, forensic application, Landscape genetics	C / F
SNPs	**Single nucleotide polymorphism**. This marker is a DNA sequence variation occurring when a single nucleotide (A, T, C, or G) in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual. For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide. Almost all common SNPs have only two alleles.	QTL, individual traceability, Landscape genetics	C / F

[1] H= Historical use; C = Current use; F =Future use

Table 2: Statistical methods and software for landscape genetics

Spatial pattern identified	Method types	Software	References
Isolation by distance	Linear regression	Genepop	Raymond and Rousset, 2007
Populations assignment	Bayesian clustering	Structure Partition	Pritchard *et al.*, 2000 Dawson and Belkhir, 2001
Cline, Isolation by distance, Random	Spatial autocorrelation and correlogram	AIDA SGS R SPAGeDI GeneAlEX	Bertorelle and Barbujani, 1995 Degen *et al.*, 2001 Hardy and Vekemans, 2002 Peakall and Smouse, 2005
Identify barriers	Interpolation	No specific software	Murphy *et al.*, 2008
Identify barriers	Assignment tests and algorithm models	No specific software	Manel *et al.*, 2007
Visual insight on pattern concerned	Synthesis map	No specific software	Hannotte *et al.*, 2002
Correlation between genetic and geographic matrixes	Mantel and Partial Mantel test	Genepop R GeneAlEX FSTAT	Raymond and Rousset, 2007 Peakall and Smouse, 2005 Goudet, 2001
Relationships between landscape features and gene flow	Resistance surface	GIS	Spear *et al.*, 2010
Landscape connectivity	Circuit theory	GIS	McRae and Bier, 2007
Functional connectivity	Gravity models	GIS?	Murphy *et al.*, 2010

CHAPTER 2

Roe deer (*Capreolus capreolus*)

2.1. Roe deer

The roe deer is an ungulate belonging to order Artiodactyla and to the family of Cervidae. It is only European representative of the subfamily of Odocoileinae and the genus *Capreolus*. In this genus belong two species *Capreolus capreolus* and *Capreolus pygargus*. The roe deer is a small telemetarcarpalian deer with a mean body length and mass which vary among populations from 100 to 145 cm and from 18 to 49 Kg, respectively (Ramanzin, 2001). Roe deer moult twice a year, once in spring and once in autumn. Coloration in winter is light grey to dark brown, with a large white caudal patch. In summer, the coat becomes reddish to red-brown and the white caudal patch is less conspicuous or is absent. Males are slightly larger than females and have three-tined antlers, which are tuberculate. Basal rosettes or burrs are well defined. Antlers are shed in autumn or early winter and begin to re-grow immediately afterwards. The skull is small (population average value ranging from 180 to 245 mm) but relatively elongated, with a maximum width (75-106 mm) less than half its length. The karyotype (2n = 70-84) comprises 70 main chromosomes plus, in Siberian roe deer only, 1-14 accessory B-chromosomes (Danilkin, 1996).

2.1.1 European roe deer (Capreolus capreolus)

Capreolus capreolus (Linneaus, 1758) is distinguished from *C. pygargus* (Pallas, 1771) by its shorter body length (population average between 100 and 126 cm), smaller cranium (condylobasal length averages 180-200 mm) and shorter antlers (length 17-26 cm, span 7-14 cm). The average body weight is 18-32 kg. The length of the tooth row of the lower mandible is between 58 and 66 mm. Auditory bullae of European roe deer skulls are small and do not protrude from the bullar fosse. When in summer coat, the hair of the head and the metatarsal gland is grey or grey-brown. Unlike the Siberian roe deer, B-chromosomes are absent, but two additional blood serum antigens are present. This species consists of a single taxonomic group which is widely distributed in Europe (not farther than the Volga) and is also found in Asia Minor. However, a variant form, *Capreolus capreolus garganta* (Meunier, 1983) from the south of Spain differ from typical European roe deer in coloration as well as some body proportions. The taxonomic status of this form requires further detailed investigation.

2.1.2 Siberian roe deer (Capreolus pygargus)

Capreolus pygragus (Pallas, 1771) is larger, with a total average population body length of between 127 and 145 cm, an average body mass of 32 – 49 Kg, a bigger skull (condylobasal length 201-231 mm), a lower tooth row of average length 71-76 mm, and antlers generally longer than 27 cm with a span averaging in excess of 17-20 cm. Auditory bullae of Siberian roe deer are larger and protrude

noticeably from the bullar fossa. In summer coat, the hair of the head and metatarsal gland, like that of the rest of the body, is generally reddish.. The chromosome set is distributed through Eastern Europe and Asia but comprises two separate subspecies: *Capreolus pygargus pygargus* (Pallas, 1771) and *Capreolus pygargus bedfordi* (Thomas, 1908).

2.2 Distribution

The roe deer populations of western Europe had experienced a decline due to deforestation, cattle breeding and intensive hunting, prior to the nineteenth century. By the end of nineteenth century, roe disappeared from numerous parts of the European territories of the former URSS. The twentieth century saw great changes in the distribution of *Capreolus*, particularly in URSS. This reduction in range reached its peak in the 1920s and may have been largely due to the wars of 1914-1920. However, as early as the 1930s an increase in range of roe deer again became evident, due to more moderate levels of hunting, a warmer climate with only limited snow depths, and intensive exploitation of the closed-canopy coniferous forests which were subsequently replaced by deciduous tree.

During the twentieth century we have witnessed the most severe restriction in the geographical distribution of the genus *Capreolus*, its fragmentation into several sections, and its subsequent recovery to practically its former limits, with an additional expansion into northern Europe. After the second world war, due to certain protective measures, the absence of large predators and the availability of suitable habitat, the number of roe deer greatly increased in western and central Europe. Populations colonized all suitable biotopes, including open fields, and expanded substantially towards the north, in particular, on the Scandinavian peninsula

2.2.1 Distribution of European roe deer in Italy

In Italy the distribution range extends for about 110,000 km^2 (Carnevali *et al.*, 2009, Figure 1). Two large sub-areas can be identified: the first extends continuously along the Alps (where the species has already colonized all the potentially suitable area) and the Ligurian and Lombard Apennines, as far as the provinces of Genoa, Pavia and Piacenza; the second extends along the Apennine chain, between the provinces of Parma and Massa Carrara and the provinces of L'Aquila and Pescara (the southernmost limit), including the provinces of Pisa, Siena, Livorno, Grosseto, Viterbo and Rieti on the western side, and those of Pesaro-Urbino, Ancona, Ascoli Piceno, Macerata and Teramo on the eastern side. In the last years the range has expanded in Piedmont (where the species has also occupied low hill and plain areas) and in the central Apennines (where the Roe Deer is now well distributed in northern Lazio, in the provinces of Viterbo and Rieti, in Umbria and in all the

mountain and hill areas of Abruzzo). In southern Italy, its presence is limited to small isolated populations that originated from ancient larger ones, or it is the result of recent reintroductions. In the Alps and in the central-northern Apennines the Roe Deer populations originated through natural immigration from central Europe, as well as through expansion of residual autochthonous populations or through reintroductions of individuals from northern Europe (*C. c. capreolus*). The reintroduced populations include that of Abruzzo, Lazio and Molise National Park and neighboring areas, and that of Sila, both derived from releases started in 1970. The first release led to the colonization of all the provinces of Abruzzo. Other reintroductions were carried out in the last few decades in the Majella e Monti Sibillini National Park, in the Gran Sasso e Monti della Laga National Park and in the provinces of Ascoli Piceno, Pescara, Verona and Imperia. Southern Italy and southern Tuscany are occupied by Roe Deer belonging to the Italian subspecies (*C. c. italicus*), a relict form that occurred in central-southern Italy in the past (Festa, 1925). Also the populations that occur in the Castelporziano Presidential Estate (Rome), in the Umbra Forest (Gargano, Puglia) and in the Orsomarso area (Pollino, Cosenza), as well as the reintroduced populations of Cilento e Vallo di Diano National Park and of the Tolfa Mountains (Rome), belong to the Italian subspecies. On the contrast, the small population occurring in the Sila National Park consists of European individuals (*C. c. capreolus*) originated from releases started in the early 1970s. The range of the Italian Roe Deer in Tuscany has not been precisely determined yet, but it approximately covers the southern part of the province of Siena and all the province of Grosseto (apart from the Amiata area) (Randi *et al.*, 1998; Lorenzini *et al.*, 2002; Vernesi *et al.*, 2002; Randi *et al.*, 2004). The Roe Deer is currently present in 67 provinces (out of 107 provinces, 63%), it is widespread in 54 of them and it is represented by smaller and isolated populations in the other 14 (Meriggi et al., 2008)

2.2.2 Distribution in Belluno and Trento provinces

Between the 17th and the beginning of the 20th century, deforestation and direct persecution led to the partial or total extinction of some mammalian species in Italy. Mainly since the 1950s there has been a partial and gradual recovery due to both spontaneous re-colonization and re-introductions. A remarkable increase in Roe deer (*Capreolus capreolus*) density and diffusion has been recorded in most of the Alps in recent years. In the Belluno province, roe deer is almost present everywhere. His adaptability and the small size make it possible to colonize all suitable habitats, so that it can meet the cultivated valley floor to the edge of the alpine meadows (Ramanzin, 2001). The abundance of roe deer in this province, as it appears from the census carried out in alpine hunting reserves, is stable, the stability had been achieved at the end of the 1980s in endalpic district, including areas where had maintained the historical presence of species, and later in the other two,

where the area was re-colonized in after war (Ramanzin, 2001). Overall, the available data indicate that the species has therefore not only completed the colonization but, also, apparently, the phase of numerical expansion in the Belluno province. The density on the three districts are on quite similar, with values between 4 and 6 heads/100 ha. The endalpic district, where the environmental conditions are less favorable to the species, present a little disadvantage compared to the other two. In fact, these averages hide large variations in reserves, with estimated density values ranging from a minimum of just over more than 1 and a maximum of 15 heads/100 ha (Ramanzin *et al.*, 1998). It should therefore be very low average values for the known ability of this species. The factors that contribute to this wide diversity are numerous and include natural causes, but certainly even the intervention of man.

The abundance of roe in the Autonomus Province of Trento has more uniform distribution. In particular, Roe deer had an impressive resurgence with an increase from 5,350 counted in 1965 to 26,016 in 2008. Roe deer is distributed present in all municipalities of the Autonomus Province of Trento, although the density varies across districts from 4.92 heads/100 ha to a maximum of 9.8 heads/100 ha.

2.3 Social organization and migration

The social organization of a roe deer population varies appreciably through the year. Generally, the majority of deer are observed alone or in small family groups during summer but in winter larger groups or herds are more common. Perhaps 60-90 % of all summer observations are of solitary individuals, and groups of four or more animals are extremely rare (Danilkin, 1996). At this time of year, pairs commonly comprise a doe and her kid, two kids, or a rutting adult buck with a doe, but less frequently a pair of yearlings, or occasionally an adult buck with a younger buck, may be observed. There deer together most often constitute a family group of a mother with her two or three individuals may account for about a half of all observations of roe deer. During winter the situation is somewhat different, when mixed groups consisting of four or more animals are most commonly observed. The social organization of roe deer population may depend to a large extent on habitat-related factors such as the availability and distribution of food resources and cover, but intrinsic social factors may also be important. Average group size varies in response to a variety of factors, increasing with habitat openness, population density, falling temperature and deepening snow cover, as well as with decreasing area of feeding grounds. Winter groups begin to form during autumn, generally through the aggregation of two family groups which may be related, or at least occupy adjoining ranges, and possibly some other yearlings. Subsequently, subadult and adult bucks may join these groups, although older bucks on some cases remain solitary throughout the

winter. In this season, territoriality and aggressive behavior between bucks are relaxed; groups containing large numbers of males, and even exclusively male groups of up to seven individuals may be observed. Large herds of several hundred roe probably occur only during winter, in areas of locally high density, after the mass migrations which follow heavy snowfall. Large groups are most frequently formed at feeding sites in response to perceived danger and, when disturbed, tend to follow one another. Groups formed in such situations may remain together for several hours, days or, in extreme cases, weeks but more generally, the composition of large groups changes almost continuously, despite an apparent stability. Small groups are the most stable unit and radiotracking of individual deer has shown that members of a family group generally remain together throughout the winter. Sudden disturbances may lead to the fragmentation of these families but within a few days they reform, generally at feeding sites. Occasionally, kids become detached from their mother and may join some other family or group, however, in most cases, they are met with aggression and so may remain alone throughout the winter, joining other groups only when in danger. Subadults may also be solitary, or perhaps group in pairs, over winter, while individual adult bucks fluctuate in their grouping tendencies, joining and leaving different groups regularly. The family unit forms the core of roe deer group structure and serves as the basis for the social organization of the entire population. To better understand the social organization of deer is an important distinction is made between the terms: individual range, home range, kidding range and territory. The individual range is a set of all home ranges of an individual, including its migration routes, over its lifetime (lifetime range). seasonal migrations may be a common strategy exhibited by roe deer living in mountain areas to avoid deep snow, and that variability in climatic conditions might be responsible for the development of variable spatial strategies (Ramanzin *et al.*, 2007). The home range is the total area where an individual (or group) remains for a certain length of time (Mustoni, 2002). The kidding range is an area protected from other roe deer by a pregnant female, before giving birth until some time after delivery. A territory is that part of a male's home range with defined boundaries which are defended.

The spatial structure of a roe deer population, as well as the overall social organization, changes considerably over the annual cycle but is relatively stable within two periods of considerable duration. During summer, the period of territoriality and reproductive activity, the deer (which are primarily solitary) are dispersed over the population range. In contrast, during winter when the bucks are no longer territorial, the deer are mainly aggregated into groups which are concentrated on feeding ranges. This structure is complicated by the fact that in early autumn and spring, the change between territorial and non territorial behavior is asynchronous, so that these two periods

overlap. Moreover, in summer, the territorial behavior of individual deer differs according to sex and age.

2.4 Roe deer and landscape genetics: why?

Most studies of landscape genetics use roe deer (*Capreolus capreolus*) as animal model. Roe deer is useful model for landscape genetic approaches to ungulate conservation and management for a variety of reasons. The availability of harvested specimens offers a very good opportunity of collecting large numbers of samples, spatially distributed and georeferenced over wide areas (Coulon *et al.*, 2004; Zannése *et al.*, 2006, Valvo *et al.*, 2009). In addition, this species distribute over an ample variety of habitats, which enables to test statistical models and procedures designed for identifying fine scale and cryptic discontinuity patterns (Coltman *et al.*, 2003). Roe deer have also a significant ecologic, economic and social impact. This species experienced a spectacular expansion across Europe during the last fifty years (Andersen *et al.*, 1998) and are still increasing in Italy (Toso, 2000a, 2000b). Public authorities and wildlife managers are thus facing new management problems. High ungulate densities are related to an increase in road accidents, which are rapidly increasing in various European countries (Groot Bruinderink and Hazebroek, 1996; Dal Compare *et al.*, 2007). Finally, the expanding distribution and growing densities of roe deer lead also to an increasing demand for sustainable harvest strategies. A better understanding of animal mobility patterns, landscape connectivity, and genetic population structuring may greatly help in identifying management units and options on a sound ecologic basis.

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FIGURES

Figure 1 : Distribution of ore deer in Italy

CHAPTER 3

Genetic Structure of Roe deer Population

3.1 Introduction

The improvements in molecular genetic tools and the molecular population genetics, combined with existing or new statistical tools (e.g. Bayesian approaches) has led to the expansion of molecular genetics to new fields, such as a conservation and management of biodiversity and wildlife (Hedrick, 2004; Pearse and Crandall, 2004; Garant *et al.*, 2005; Diniz-Filho *et al.*, 2008). These properties change on larger spatial and temporal scales are effective markers of gene flow and population history, even in species with limited genetic variation (Galan *et al.*, 2002).

Microsatellites (Chambers and McAvoy, 2000; Li *et al.*, 2002) are neutral and indifferent to selective pressure from environmental factors which could alter the spatial genetic patterns, and their high mutation frequency produces genetic discontinuities even with partial and/or short term isolation. They, for their properties (multi-allelic, codominant), are generally considered to be the most powerful molecular marker currently available for population genetic studies (Galan *et al.*, 2002). In recent years, the importance of microsatellites for population genetics, linkage mapping, and parentage studies has increased significantly. This interest in wildlife population biology questions is caused by the possibility of quantifying connectivity and isolation among populations, or species determination and population structure of unknown biological tissue and by the possibility to differ between individual animals (Cattaneo *et al.*, 1999; Gauglich *et al.*, 1994). For example, various microsatellites useful for Cervids have been published (Galan *et al.*, 2003; Vial *et al.*, 2003), but panels size and analytical procedures might be further improved.

The European Roe deer (*Capreolus capreolus*) is the most widely distributed and numerous cervid in Europe, due to a demographic explosion over recent decades, and it is one of the most representative species of the ungulate fauna from the Mediterranean area (Galan *et al.*, 2002).

Roe deer is a useful model for landscape genetic approaches to ungulate conservation and management for a variety of reasons. The availability of harvested specimens offers a very good opportunity of collecting large numbers of samples, spatially distributed and georeferenced over wide areas (Coulon *et al.*, 2004; Zannése *et al.*, 2006). In addition, the ungulate distributes over an ample variety of habitats, which enables to test statistical models and procedures designed for identifying fine scale and criptic discontinuity patterns (Coltman *et al.*, 2003).

Roe deer has also a significant ecologic, economic and social impact. In fact, it experienced a spectacular expansion across Europe during the last fifty years (Andersen *et al.*, 1998) and are still increasing in Italy (Toso, 2000a, 2000b). Public authorities and wildlife managers are thus facing new management problems. High ungulate densities are related to an increase in road accidents, which are rapidly increasing in various European countries (Groot Bruinderink and Hazebroek, 1996; Dal Compare *et al.*, 2007). Finally, the expanding distribution and growing densities of roe

deer lead also to an increasing demand for sustainable harvest strategies. A better understanding of genetic population structuring may greatly help in identifying management units and options on a sound ecologic basis. For this reason, recently, many study investigated the genetic structuring of a roe deer population (Wang and Schreiber, 2001; Vernesi *et al.*, 2002; Coulon *et al.*, 2006). The aims of the present study are: 1) to produce microsatellite panels for the roe deer; 2) to suggest optimised protocols (multiplex PCR), for roe deer; 3) to compare different statistical approaches to defining the population (sub)structure and studying gradients and discontinuities in gene flow in the roe deer population in the bordering provinces of Trento and Belluno (north-eastern Italian Alps).

3.2 Materials and methods

3.2.1 Study Area

Study area comprises the bordering provinces of Belluno and Trento, in the north-eastern of Italy. Belluno and Trento cover 3667 Km^2 and 6300 Km^2, respectively (Figure 1). In general, the two provinces are characterized by an high variability in terms of landscape features, land use and habitat suitability for roe deer.

Belluno presents the altitude ranging from 167 to 3327 m a.s.l. from south to north. This province comprises agricultural crops and deciduous forests with abundant precipitation in the south area, and coniferous forests with mountainous climate in the north one (Zannèse *et al.*, 2006). Roe deer are present over the entire province. At the beginning of the 1950s there where only small scattered populations in the north, but by the end of 1970s the occupied all suitable habitats. Cull records are available from 1990. The number of animals shot increased threefold from 1990 to 1992 because of new rules that allowed females to be shot (only males were previously hunted) then stabilized at about 0.8 roe deer shoot 100 ha-1 suitable habitat, until 2005 (Ramanzin 2007).

Trento province contains a predominantly mountainous with plains limited to small areas in the valley, trails from the main rivers of the province (e.g. Adige). The forests are mainly made up of conifers. The strong antrophic presence influences the distribution of deer. Roe deer has increased its density more than one order of magnitude in the last fifty years (by c. 2000% Hudson *et al.*, 2001). The species is present across the entire province, with a density ranging from 4,92 heads/100ha to 9,8 heads/100ha (Forest Service and Wildlife PAT, 2010).

3.2.2 Animals

Samples collection was realized with the help of the wildlife management authority of the Belluno (CPP) and Trento (ACT) provinces and of the local hunting districts (HD). This ensured an intensive and spatially representative sampling. With the co-ordination of CPP and ACT, members of HD was provided with test-tubes filled with ethanol 95% and instructed on how to collect a small

fragment of tongue tissue and record location of shooting, sex and age class (fawn, yearling, "adult") of the individuals they harvest. A total of 657 roe deer (*Capreolus capreolus*) were included in this study, shot during the hunting season 2004 – 2009 in the provinces of Trento (306) and Belluno (351). Management of roe deer in based on hunting districts coordinated between provinces (Valvo *et al.*, 2009). Each deer was georeferenced with the geographic coordinates of the shooting location (Figure 2).

3.2.3 DNA extraction

Genomic DNA was extracted from a small piece of ear punch or tongue tissue using a modified PURGENE DNA tissue kit (Gentra System INC) protocol and Maxwell 16® DNA Purification Kits (Promega Corporation U.S.A.), respectively. The Maxwell® 16 Instrument purifies samples using MagneSil® Paramagnetic Particles (PMPs), which provide a mobile solid phase that optimize capture, washing and elution of the target material. DNA concentration are determined by using Quant-it DNA High Sensitivity Assay Kit (Invitrogen). This assay is high selective for double strain DNA and it is indicated for genomic DNA quantification, for PCR products and for genotyping. The method is based on fluorimetry and DNA quantification is done by using QubitTM fluorimeter.

3.2.4 Microsatellite polymorphism, PCR conditions, sequencing analysis

Many studies have developed microsatellite markers for wildlife (Poetsch *et al.*,2000; Balloux *et al.*, 2002; Bonnet *et al.*, 2002; Galan *et al.*, 2003; Vial *et al.*, 2003; Thullin, 2006). The research unit has already tested a panel of 11 microsatellite markers on roe deer (Zannese *et al.*, 2006; Valvo *et al.*, 2009). This panel has been enriched using 25 microsatellite markers. The loci were amplified in singleplex "touch-down" PCR" from ear samples and in multiplex PCR from tongue samples. Singleplex amplification were performed according to Vial *et al.* (2003). Multiplex PCR amplification were conducted in 20 µl volume containing 10 µl of dried DNA, 4 µl of Buffer, 0.2 µl $MgCl_2$, 0.8 µl of dNTPs, 0.5 µl of each primer (with a fluorenscent label on the forward primer from Proligo, in accordance with the suggestions made by Vial *et al.*, 2003), 0.2 µl Phusion DNA polymerase.

Singleplex PCR amplification were conducted in a P x 2 Thermal Cycler (Thermoelectron Corporation). Multiplex amplification were performed in GeneAmp PCR System 9700 (Applied Biosystem), with the following cycling conditions: 30 sec at 98 °C, 45 cycles composed of 7 sec denaturing at 98 °C, 15 sec annealing at Tm, 30 sec extension at 72 °C, and 7 min at 72 °C to ensure complete extension. Fragments were analyzed with Beckman Coulter CEQ 8000 automated sequencer and scored with the Fragment Analysis software (Genetic Analysis System v.9, Beckman Coulter).

3.2.5 Statistics and Genetic data

Summary statistics were calculated using MSA (Microsatellite analyzer) v.4.05 (Dieringer *et al.*, 2003) and Genetix v.4.01 (Belkir *et al.*, 1998). F-statistics were calculated using F-stat v.2.9.3 (Goudet *et al.*, 2001) defining groups of individuals and then using their genotypes to compute variance in allele frequencies.

Then, we used Genepop v.4.0 (Raymond and Rousset, 1995) to test for linkage disequilibrium between pairs of loci (Markov chain parameters 10.000 dememorizations, 600 batches, 5.000 iterations per batch) with Hardy-Weinberg exact test for all loci combined and for each locus. We evaluated whether there was significant genetic structure within the study area that might concord with spatial structure in environmental heterogeneity.

Population structure and the most likely number of clusters (K) in the data set were analyzed using the software STRUCTURE v2.3.3 (Pritchard *et al.*, 2000). Analysis was performed using an admixture model with correlated allele frequencies (Falush *et al.*, 2003); to obtain a representative value of K for modelling the data, we ran 50 independent runs of the Gibbs sampler for each K between 1 and 10 with a burn-in period of 500.000 iterations followed by 1.000.000 iterations for data collection. We performed these parameters to assess convergence of ln Pr(X|K). The large burn-in and iteration number were chosen to avoid problems of convergence.

The most likely number of K clusters fitting the data was established by plotting the ln Pr(X|K) over the 50 independent runs for each K, as suggested by (Pritchard *et al.*, 2000). The resulting individual membership coefficients for runs with the largest ln Pr(X|K) were displayed using DISTRUCT (Rosenberg, 2004).

GENELAND software (Guillot *et al.*, 2005) was then used to analyze the genetic structure of sub-populations, taking into account also the spatial information associated to each individual (i.e. spatial coordinates implemented in a bi-dimensional map of the study area). We ran 100 Monte Carlo chains with 200.000 interations each with parameters values maximum rate of Poisson process, 300; coordinates noise, 250 m; minimum number of populations, 1; maximum number of populations, 30; maximum number of nuclei in the Poisson-Voronoi tessellation, 500; thinning, 100; Dirichlet model of frequency (Guillot *et al.*, 2005). Finally, we selected the 10 top chains on the base of average of log posterior density.

In order to visualize the partition in genetic differentiation among all samples of roe deer, a NJTree dendrogram was built with the software MEGA 4.1 (Kumar *et al.* 2008) from a chord distance matrix (Felsenstein, 1984), as recommended by Takezaki and Nei (1996), and computed by Genetix v.4.01 (Belkir *et al.*, 1998).

The genetic variation gradient across the study area was analysed by a principal component analysis (PCA), implemented on R software (Jombart, 2006). PCA is a spatially constrained multivariate analysis based on Moran's statistic that maximizes the global variance (Thioulouse *et al.*, 1995). It uses the individual genetic data as the variables on the first – axis principal component scores (PCA1). This were also used to delineate any barriers to gene flow among the populations. The Kriging interpolation procedure was employed to interpolate hypothetical PCA1 scores with a regular grid that encompasses the whole sampling area. For this process we used a grid-based graphics program, Surfer (Golden Software, Inc. 2007).

3.3 Results

3.3.1 Summary statistics

Summary statistics for each marker used (chromosome localization, size range, expected and observed heterozygosity, number of alleles per marker and Fis) are given in table 1.

The total number of alleles detected was 150 and the mean number of alleles per locus was 6 ranging from 3 (NVHRT48, IDVGA8, BL4) to 10 (MAF70). Allelic richness, an estimate of the number of alleles per locus corrected for sample size was 5.999. Mean gene diversity was 0,705 ranging from 0.,340 (CSSM41) to 0,838 (MAF70).

A significant ($P < 0,001$) departure from Hardy-Weinberg equilibrium (HWE) was observed at each *loci* and in populations (Het obs = 0,623, Het exp = 0,704). Observed heterozygosity among loci ranged from 0,239 to 0,808 and expected heterozygosity from 0,339 to 0,837.

The F_{IS} estimates over all loci was 0,12 for roe deer populations. Then we used a range of different analytical approaches for identifying discrete genetic clusters across the entire study area. Population genetic structure was detected by different methods: assignment of individuals to populations identified with the Bayesian software STRUCTURE; estimating the number of panmictic groups and locating their spatial boundaries with GENELAND; identification of genetic variation gradient and hypothetical barriers with PCA .

3.3.2 Population genetic structure: STRUCTURE

The STRUCTURE software was used to detect the population structure and the degree of admixture of individuals as it implements an algorithm which uncover the "hidden structure" of a population without using a priori knowledge about the number of clusters (populations) in a dataset. In figure 3 values of ln Pr(X|K) for each K and for each runs are presented. The most likely K is that where ln Pr(X|K) is maximize, the maximum values of ln Pr(X|K) reached a plateau starting from K = 9 while the mean values of ln Pr(X|K) among the 50 independents runs reached the maximum value

at K = 5 (Figure 3). Nevertheless, the most consistent values across runs were obtained with K = 5. We used another method to infer the appropriate number of clusters by calculating the ΔK statistic (Evanno *et al.*, 2005). Calculation of ΔK from the structure output produced a modal value of the statistic at K = 5 (Figure 4). Figure 4 shows the largest value of ΔK was at K = 5, a second mode was present at K = 3 and K = 8. The height of the modal values of ΔK indicates the strength of the population subdivision signal (Evanno *et al.*, 2005), here suggesting deep subdivision at K= 5, and less pronounced differentiation at K = 3 and K = 8. Results of STRUCTURE analysis are shown in figure 3 for K = 5. In figure 5 the probability of individual assignment to the five subpopulations identified with STRUCTURE is showed. Some individuals were not assigned to any subpopulations with more than 0,70 posterior probability of the population membership, indicating no clear population structures among them.

The STRUCTURE analysis indicated some possible obstacle to gene flow (Figure 6), such as Adige River, Fassa Fiemme valleys in north eastern part of Trento province (blue circles), and differentiation between North and South of Belluno Province. However, the existence of explicit geographical boundaries were unclear.

3.3.3 Population genetic structure: GENELAND (Guillot et al., 2005)

The distribution does not fully correspond to a geographic distinction, which explains why it was decided to further analysis using software GENELAND; it is a computer package that allows to make use of georeferenced individual multilocus genotypes for the inference of the number of populations and of the spatial location of genetic discontinuities between those populations (Guillot *et al.*, 2005). The GENELAND analysis indicated that the most likely value of K was 7 as the number of subpopulations in the total data set. Eight of the best 10 runs inferred seven populations (Table 2). Therefore, we named the 7 subpopulations as follows: right side (with respect the Adige river) of Trento province in the North (n = 69), right side of Trento province in the South (n = 63), left side of Trento province (n = 139), two small units located one in the hunting districts of Tesino – Primiero (n = 12) and the other one in the hunting districts of Fassa – Fiemme (n = 17), north of the Belluno province (n = 176) and south of the Belluno province (n = 181) (Figure 7).

To verify the genetic variation between the identified nucleus, deviations from Hardy–Weingberg equilibrium were detected in all subpopulations. All nucleus presented an Ho value lower than He, in particular the unit identified to left part of Trento province (Table 3). We detected the allelic number using allelic richness value because the subpopulations present different number of individual and because the two units identified (Fassa – Fiemme and Tesino – Primiero present a lower number of individual than other). Allelich richness value did not present differentiation between subpopulation, only Fassa – Fiemme and Tesino – Primiero presented low value (3,92 and

4,72, respectively). We found six allelic variant and they belonged to six different loci (CSSM41, BM848, OARFCB304 from Belluno samples and CSSM43, BM757, RT1 from Trento samples). Their frequencies ranging from 0,005 to 0,056.

Table 4 shows the genetic differentiation expressed in F_{ST} values between the different sub populations. Pairwise Fst values between the Sub-6 and other subpopulations (0,044–0,079) were higher than other Fst values in all pairs of subpopulations with value ranging from 0,012 (between subpopulations 2 and 4) to 0,035 (between subpopulations 5 and 7) (Table 4).

Figure 8 shows phylogenetic tree of seven sub-populations of roe deer identified by GENELAND. The tree obtained with the neighbour-joining shows as in our study area there are hypothetical barriers; the first one is along the west – east axis of the study area. This reveals two principal subdivision: sub-5 and sub-3 on the right of Adige river and sub-1 (Trento) with subpopulations of Belluno province (sub-2 and sub-4) on the left side. This result support the resistance to gene flow due to the presence of Adige river, as reported by other authors in different ungulates species (Crestanello *et al.*, 2009). From the figure it's evident that sub-6 (Fassa – Fiemme) and sub-7 (Tesino – Primiero) are differentiated with respect the entire sample. Sub-7, located to the left side of the Adige river, is more similar to sub-3 and sub-5, whose are instead located to the opposite side. We must considered that this subpopulation is particularly small, and more reliable results should be obtained with a larger sample. Genetic origin of Fassa – Fiemme subpopulation should be connected with the Bolzano province. Another barrier should be identified between sub-1 (Trento) and sub-2 and 4 (Belluno); the Dolomites peaks, with elevation higher than 3000 m asl, represent a clear barrier to gene flow.

3.3.4 Identification of genetic variation gradient and hypothetical barriers with PCA

A contour plot of the first – axis principal component show different level with a first level of the right side of Trento province border by the Adige. In general Figure 9 show a level of gradient that moves from west to east. Comparing the results obtained with GENELAND clustering with the synthesis map in figure 9 it's evident that Adige river (with the valley at high antrophic concentration and the motorway) contribute to genetic variation of roe deer populations. Also the barrier represented by Dolomites peaks between Trento and Belluno Province, and the differences between the north and the south of Belluno Province are supported. Finally, the small group of Tesino-Primiero different with respect the other individuals in the neighboring seems to be confirmed.

3.4 Discussion and conclusion

The panel of microsatellites markers and the sample size considered in this study is quite large compared to other studies on the same species (Table 5).

A comparison of results among authors is partially biased because the different locus used. Nevertheless, our sample of roe deer showed a significant heterozygosity deficit. Fis values calculated are higher than those obtained by Coulon *et al.* (2004) in France and by Targhetta (2006) in the Province of Belluno. It was lower than the Fis calculated by Zachos *et al.* (2006), but this last research was conducted at population level taking into account sites with large environmental variability and not connected. Explanations of the departure from HWE could be: the presence of null alleles, a significant level of inbreeding and the "Wahlund effect" (Coulon *et al.*, 2004 and 2006). The possibility that there are null alleles can not be excluded, as it can not be completely ruled out the hypothesis that there is a significant rate of inbreeding, given the relative stability of the home range of this species over time and the low dispersal ability of the same (Hewison *et al.*, 1998). Wahlund effect refers to reduction of heterozygosity in a population caused by existence of 'demi' semi-isolated or entity in the population (subpopulation structure). Also reintroduction of animals from other areas should contribute to the departure from to HWE. In past, probably some translocation of animals were done, but these kind of activities usually are not documented. Also for this reason, genetic analysis should be important to understand the origin of roe deer populations. The individual approach and the uniform sampling across the study area permit to obtain useful information for roe deer management at local level. Through the use of genetic data, we detected population structure of roe deer in the North-Eastern Italian Alps. Our analysis suggests that up to seven subpopulations are present in our study area. The investigation of genetic variability inside the seven identified subpopulation showed contrasting results. In the province of Belluno (2 sub-pop) Fis values was lower with respect the entire study area, showing a lower departure from the HW equilibrium. In Trento province is the opposite, with three sub-populations showing an higher Fis value. Roe deer of Trento province show a larger genetic variation and this should be due to the presence of landscape or antrophic resistance to gene flow. Also the evaluation of different genetic origin must be taken into account; in fact, in the second half of the 19th century the roe deer population of Trento province experienced a dramatic decline. During the last century the expansion of roe deer moved from the north to the south and some re-stocking from other areas is hypothesized.

The genetic difference among the identified subpopulations are characterised by Fst values ranging from 0,012 to 0,079. In general, Fst values below 0,05 are expected with current gene flow, values between 0,05 and 0.1 indicate that populations are semi isolated, and values above 0,1 suggest that

populations are isolated from each other (Wilson *et al.* 2003). In this paper, population 6 (Fassa Fiemme) is significantly differentiated from the others with pairwise Fst between 0,044 and 0,079 (Table 4). The other pairwise Fst are lower, indicating that the population structure identified do not lead to a differentiation among nucleus but should be useful to obtain management units. This result indicate also the ability of our microsatellites panel to identify mild genetic discontinuity with a wide sample.

In conclusion, the genetic structure of roe deer in Eastern Italian Alps is associated with different geographic areas, showing the relationships between gene flow and landscape features in wildlife. To understand the real effect of landscape to generate barriers or corridors for gene flow must be valuated with approaches of landscape genetics at subpopulation level. Also the differences between male and female must be considered (Coulon *et al.*, 2006), to verify if dispersion is sex biased. For these analysis see chapter 4. The results showed that different units should be defined inside the study area to improve the management of roe deer. In particular, up to seven subpopulations are identified, with different environmental and genetic characteristics. As a consequence, population dynamics should vary independently inside the different nucleus. The hunting and management policies of roe deer should be improved using distinct and more effective measures.

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FIGURES AND TABLES

Figure 1: Location of the study area. Belluno and Trento provinces, in the north-eastern of Italian Alps.

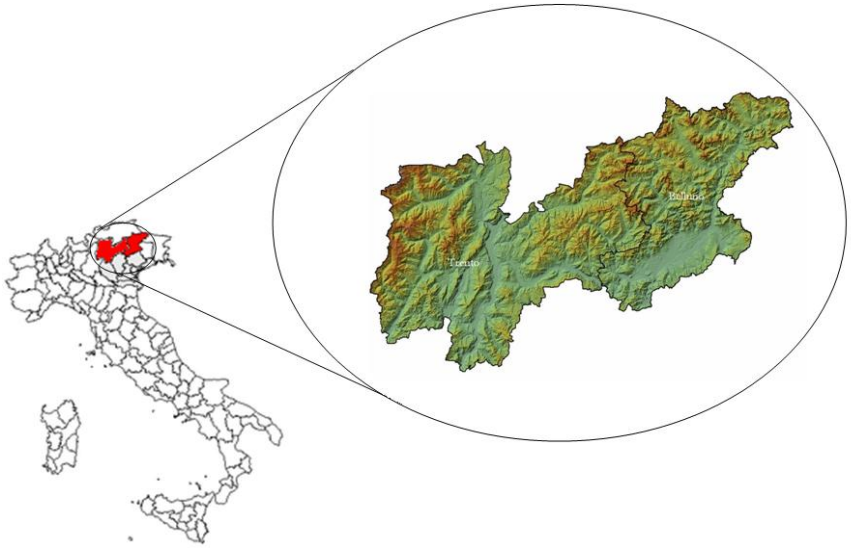

Figure 2: Maps of sites where roe deer were sampled for this study

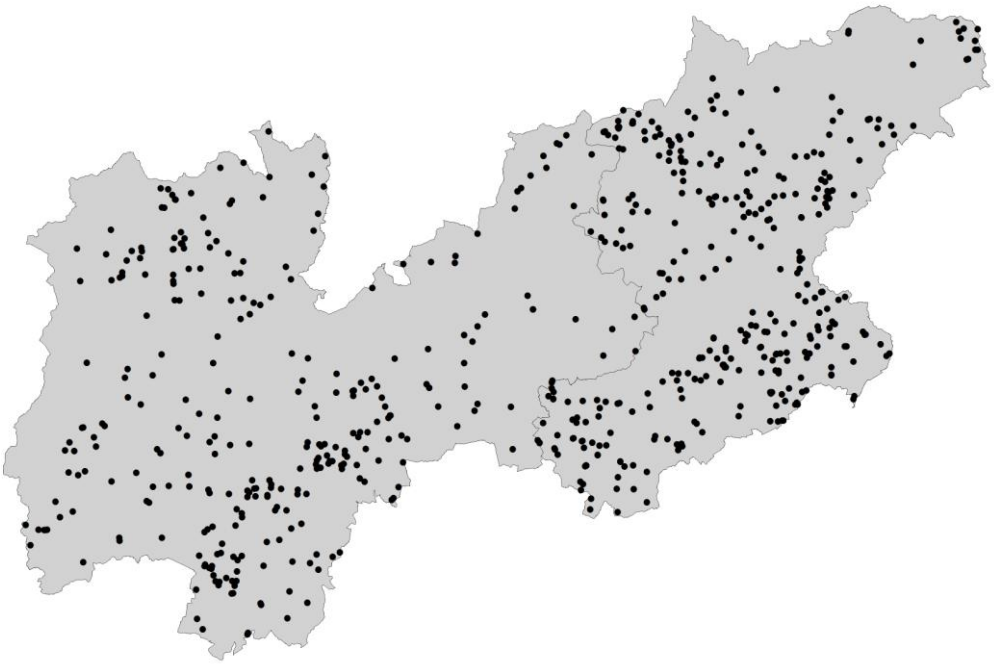

Figure 3: Ln Pr(G|K) values presented as a function of the number of clusters (Pritchard *et al.*, 2000). Values of ln Pr(G|K) within each K for all 50 runs are presented with blue crosses, the largest and mean ln Pr(G|K) within each K (among 50 runs) are presented with yellow and red circles respectively

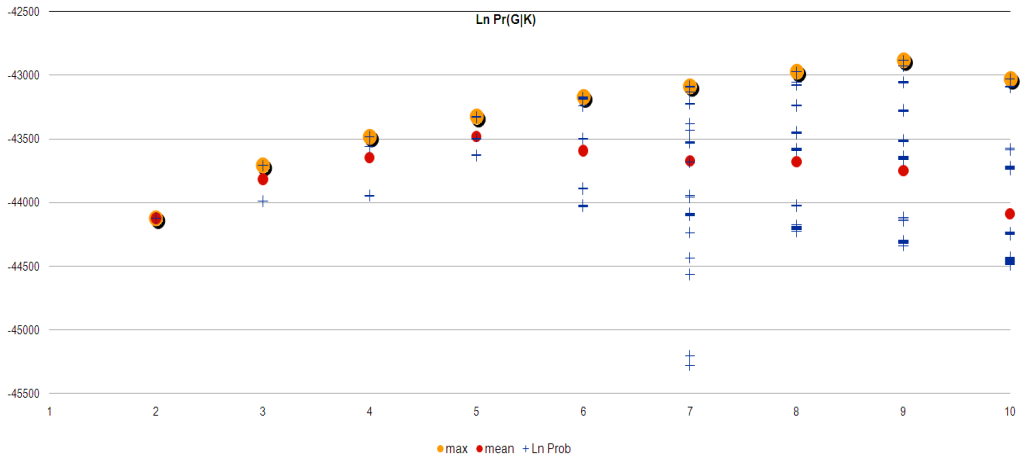

Figure 4: ΔK (a measure of the rate of change in the structure likelihood function) values as a function of K, the number of putative populations

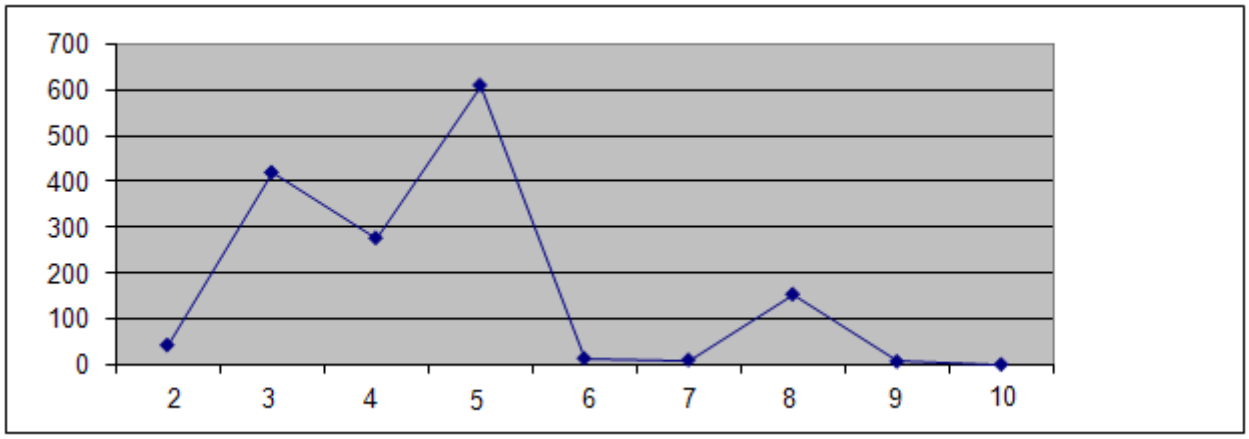

Figure 5: Graphical presentations of the population structure analyses for a sample of 657 roe deer. Each roe deer is represented by a single vertical line broken into K colour segments, with lengths proportional to the estimated membership of the inferred cluster. Among 50 runs, only graphical presentations for runs with the largest ln Pr(G|K) values are shown

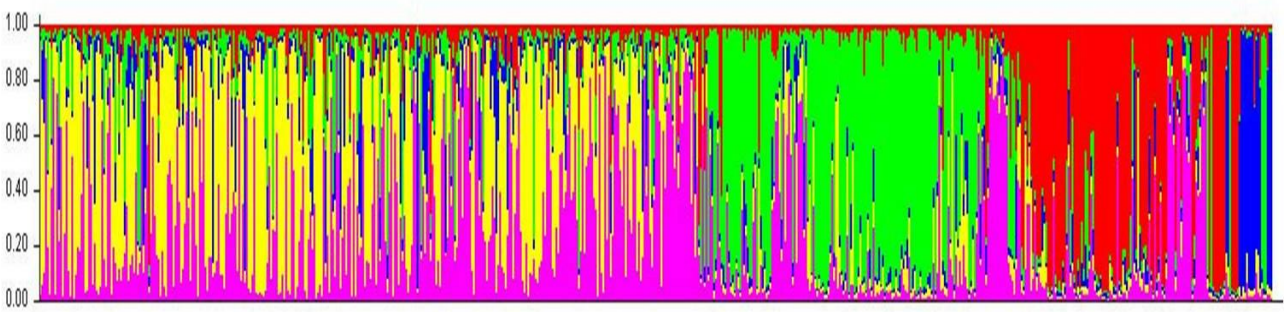

Figure 6: Graphical presentations of distribution of individuals belonging to the five subpopulations identified by STRUCTURE on the Trento and Belluno provinces. Each subpopulations of roe deer is represented by a different colour: sub-1 red, sub-2 green, sub-3 blue, sub-4 brown, sub-5 yellow.

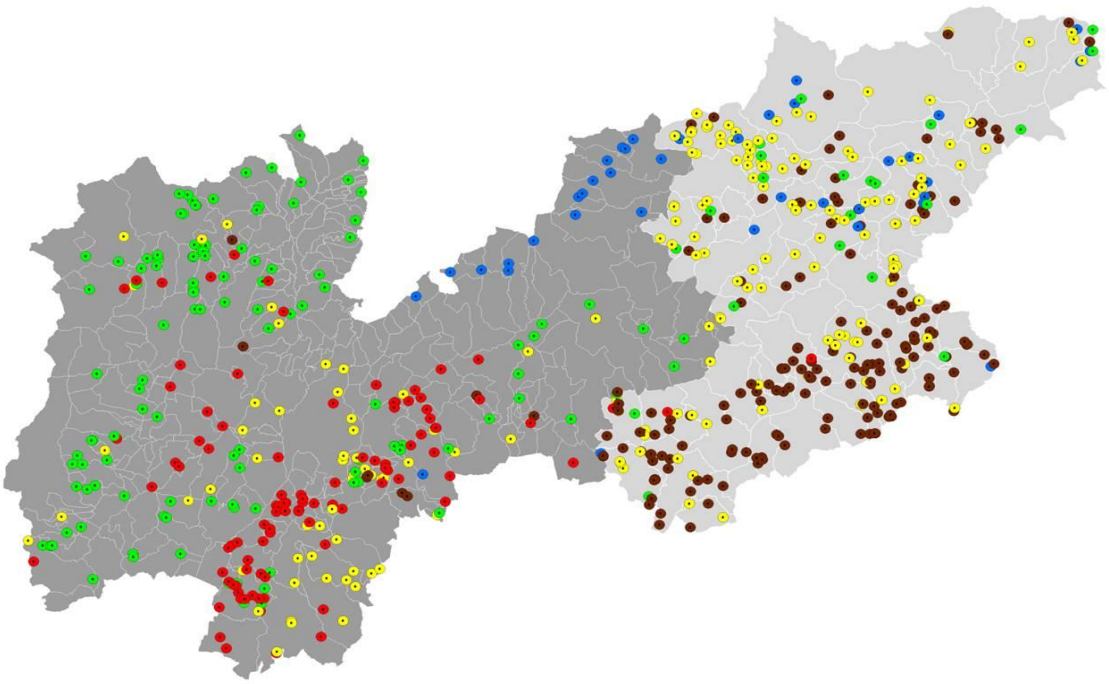

Figure 7: Graphical presentations of distribution of individuals belonging to the seven subpopulations identified by GENELAND on the Trento and Belluno provinces. Each subpopulations of roe deer is represented by a different colour.

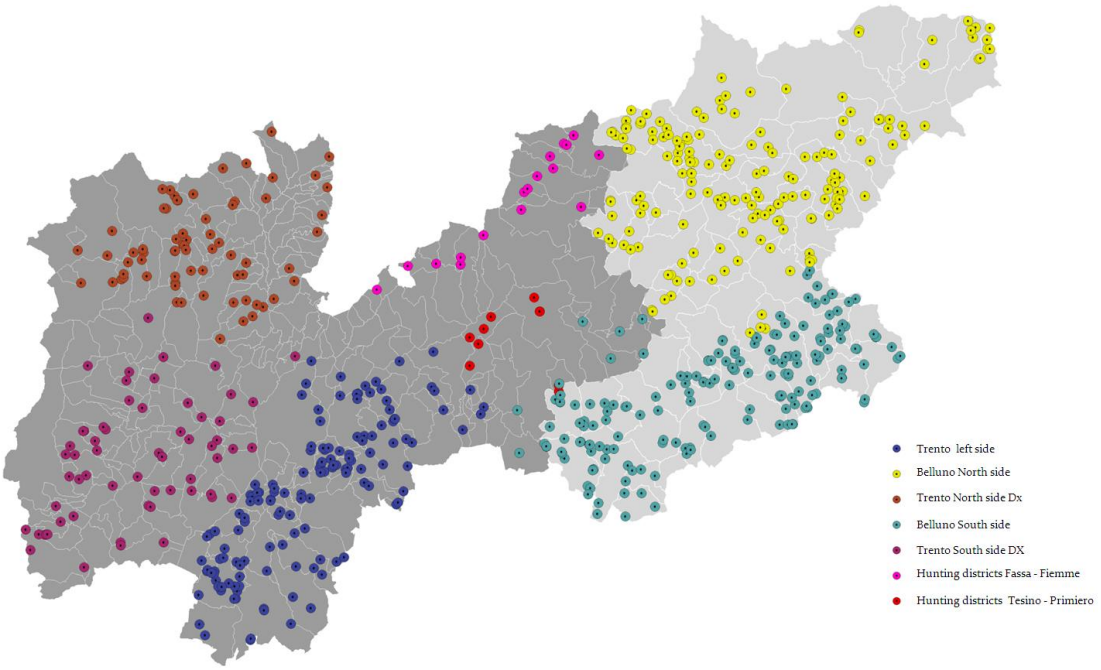

Figure 8: Reconstruction of relationships between seven subpopulations of roe deer identified by GENELAND

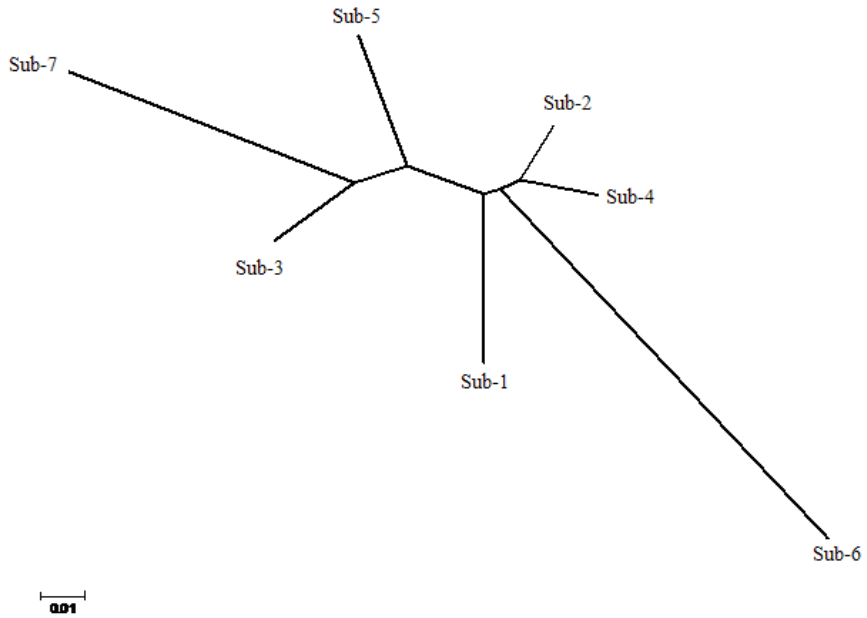

Figure 9: Synthesis map showing the PCA1 scores derived from the kriging procedure in 2 dimensions for the Roe deer populations

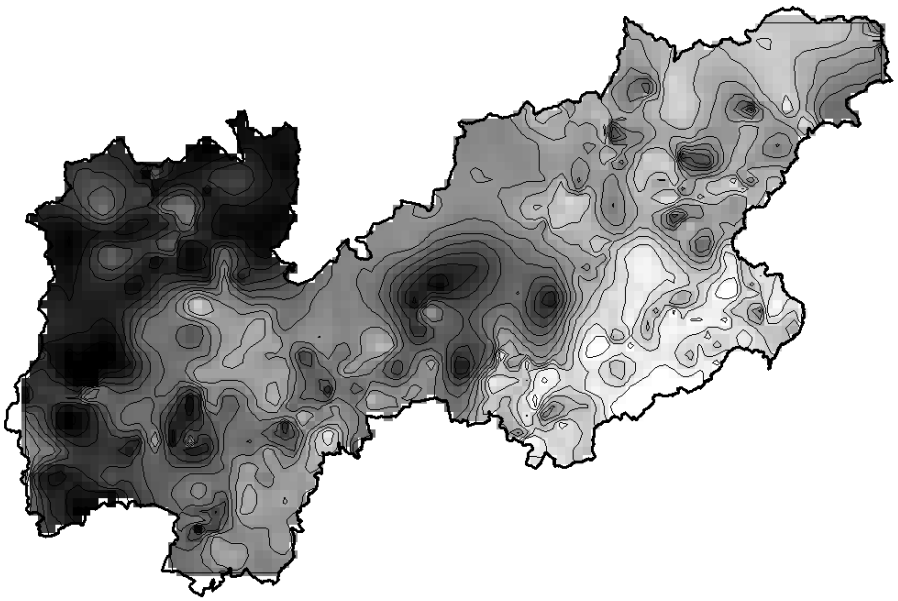

Table 2: Summary statistics of the 25 microsatellite loci analyzed in roe deer populations (657 animals); chromosomal location, expected (He) and observed (Ho) heterozygosity calculated for each locus, number of detected alleles (NA), and Fis

Locus	Fragment size	Ho	He	NA	Fis
CSSM43	238-244	0,536	0,536	4	-0,046
BM757	172-204	0,406	0,484	6	0,162
Roe 5	137-147	0,465	0,744	4	0,375
CSSM41	120-124	0,239	0,339	4	0,297
NVHRT48	86-90	0,502	0,590	3	0,151
BM848	356-368	0,652	0,774	8	0,158
BMC1009	276-286	0,665	0,730	6	0,090
HUJ1177	199-219	0,622	0,726	8	0,145
OarFCB304	158-177	0,797	0,817	9	0,025
BM1706	238-250	0,692	0,746	5	0,074
IDVGA8	209-225	0,379	0,413	3	0,081
CSSM39	177-183	0,679	0,718	5	0,056
BL4	135-155	0,565	0,631	3	0,105
SRCRSP1	127-151	0,755	0,751	7	-0,004
BM1818	249-267	0,729	0,798	6	0,088
INRABERN192	211-225	0,756	0,774	7	0,024
NVHRT16	175-181	0,662	0,756	9	0,124
MAF70	139-161	0,702	0,837	10	0,163
RT7	226-240	0,654	0,765	6	0,146
NVHRT21	159-173	0,706	0,829	9	0,149
ETH225	139-145	0,560	0,762	5	0,266
Roe6	91-109	0,688	0,819	6	0,161
ILST011	267-277	0,808	0,743	5	-0,087
RT1	236-242	0,708	0,744	7	0,050
Roe8	62-82	0,622	0,778	5	0,201
Mean		0,623 ± 0,137	0,704 ± 0,132	6,00	0,118

Table 2: Ten of the best runs obtained by GENELAND and the average of log posterior density

Run	Average log posterior density	Number of populations
5	-43115	7
3	-44193	5
1	-45119	7
4	-45223	7
7	-45335	5
2	-45360	7
10	-45437	7
8	-45476	7
6	-45468	7
9	-45507	7

Table 3: Summary statistics of the 25 microsatellite loci analyzed in seven subpopulations of roe deer identified by GENELAND; expected (He) and observed (Ho) heterozygosity, allelic richness (AR) and Fis

Population	Ho	He	AR	Fis
Trento left part (Sub-1)	0.550	0.715	6.32	0.231
Belluno North side (Sub-2)	0.634	0.704	6.56	0.099
Trento North side Dx (Sub-3)	0.614	0.716	6.24	0.149
Belluno South side (Sub-4)	0.636	0.690	6.52	0.073
Trento South side Dx (Sub-5)	0.603	0.718	6.12	0.167
Hunting districts Fassa – Fiemme (Sub-6)	0.569	0.636	3.92	0.104
Hunting districts Tesino – Primiero (Sub-7)	0.603	0.677	4.72	0.122

Table 4: Estimates of F_{ST} (Weir and Cockerham 1984) calculated from 25 microsatellite loci.

Population	Sub-1	Sub-2	Sub-3	Sub-4	Sub-5	Sub-6
Sub-2	0,020					
Sub-3	0,026	0,023				
Sub-4	0,025	0,012	0,030			
Sub-5	0,029	0,027	0,013	0,031		
Sub-6	0,047	0,044	0,068	0,044	0,046	
Sub-7	0,034	0,027	0,019	0,032	0,035	0,079

Table 5: Comparison of our results among authors considering: study area, N° STR (Short tandem repeat), N° of individuals, type of approach (I = individual or P = population), expected (He) and observed (Ho) heterozygosity, number of alleles (NA) and Fis.

Authors	Study area	N° STR	N° individuals	Approach (I / P)	Ho	He	NA	Fis
Coulon *et al.*, 2004	France	12	648	I	0.65	0.66	6.5	0.020
Zachos* *et al.*, 2006	Germany	8	105	P (5 populations)	0.55	0.76	9-18	0.271
					0.64	0.78		0.180
Targhetta, 2006	Belluno	11	369	I	0.56	0.58	6.7	0.044
Randi* *et al.*, 2004	Italian Alps	11	116	P (7 pop on Italian Alps)	0.67	0.74	4.7-6.4	na
					0.65	0.58		

*different populations considered; minimum and maximum values of the genetic variables are reported

CHAPTER 4

Relationships between genetic structure and landscape features in Roe deer

4.1 Introduction

The recent improvements in molecular genetic tools, combined with existing or new statistical tools, have led to the expansion of molecular genetics to new field, such as conservation and management of biodiversity and wildlife.

Landscape genetics aims to provide information about the interaction between landscape features and microevolutionary processes (e.g. gene flow, genetic drift and selection) (Manel et al., 2003; Holderegger and Wagner, 2006). The botanist De Candolle was the first to talk about of Landscape genetics and was the first author to distinguish between ecological and historical biogeography. De Candolle explained that the former depends upon "physical causes operating at the present time", and for the latter, upon "causes that no exist longer today" (Crisci, 2001). In 1860 Wallace during a travel in Malaysia archipelago, described in details the faunal discontinuities, so called "Wallace's line" (Wallace, 1860). This discipline seeks to detect two key steps: genetic discontinuities and the correlation of these discontinuities with landscape environmental features, such as barriers (e.g. mountains, gradient of humidity). Gene flow is the transfer of alleles of genes from one population to another. Migration into or out of a population may be responsible for a marked change in allele frequencies (the proportion of members carrying a particular variant of a gene). Immigration may also result in the addition of new genetic variants to the established gene pool of a particular species or population.

There are a number of factors that affect the rate of gene flow between different populations. One of the most significant factors is mobility, as greater mobility of an individual tends to give it greater migratory potential. Animals tend to be more mobile than plants, although pollen and seeds may be carried great distances by animals or wind.

Maintained gene flow between two populations can also lead to a combination of the two gene pools, reducing the genetic variation between the two groups. It is for this reason that gene flow strongly acts against speciation, by recombining the gene pools of the groups, and thus, repairing the developing differences in genetic variation that would have led to full speciation and creation of daughter species. Physical barriers to gene flow are usually, but not always, natural. They may include impassable mountain ranges, oceans, or vast deserts. In some cases, they can be artificial, man-made barriers which has hindered the gene flow of native plant populations. Barriers to gene flow need not always be physical. Species can live in the same environment, yet show very limited gene flow due to limited hybridization or hybridization yielding unfit hybrids.

Identifying potential gene flow barriers is a major focus of landscape genetics research. While all landscape features affect gene flow, particular structures such as roads (Riley et al., 2006), waterways (Antolin et al., 2006) or mountain ridges (Funk et al., 2005) are potentially impenetrable

barriers. Genetic data have been used to identify abrupt breaks in gene flow (Dupanloup et al., 2002; Manni et al., 2004) as well as more gradual transitions (Geffen et al., 2004). Barriers may also consist of microhabitats that prevent gene flow because they exceed a threshold for moisture, temperature or chemical tolerance for particular species (Palo et al., 2004). Therefore, barrier identification has important implications for ecological (Walker et al., 2003; Kreyer et al., 2004; Funk et al., 2005), conservation (Bhattacharya et al., 2003; Miller and Waits, 2003; Dodd et al., 2004) and evolutionary (Castella et al., 2000; Broderick et al., 2003; Cicero, 2004; Gee, 2004) investigations.

This is the main different with traditional phylogenetics and biogeography approaches that focus mainly on species diversity patterns at broad temporal and spatial scales while landscape genetics can resolve population substructure at finer scales and taxonomic levels. It will therefore help our understanding of the microevolutionary processes that generate genetic structure across space. This understanding provides essential information to investigate fundamental ecological issues, such as dispersal (Coltman, 2005; Coulon et al., 2006) and landscape connectivity (Coulon et al., 2004), and to address conservation of endangered species and metapopulations (Maudet et al., 2002; Hoglund et al., 2007; Segelbacher et al., 2008).

Landscape genetics has emerged as the result of researchers explaining observed spatial genetic patterns by using landscape variables. The most common spatial patterns described in the literature are: Clines, Isolation by distance, Genetic boundaries (discontinuities) to gene flow, Metapopulations and Random patterns. The identification of these spatial genetic patterns requires the collection of genetic data from many individuals (or populations) whose exact geographical location is known. Ideally, in a landscape genetics approach, the individual is the operational unit of study. However, this can be extended to populations (using allele frequencies) if enough populations can be sampled. The advantages of using individuals as the operational unit are to avoid potential bias in identifying populations in advance and to conduct studies at a finer scale. After sampling, genetic and statistical tools are used to determine the spatial genetic pattern and to correlate it with landscape or environmental features.

This work aims to analyze the interaction between landscape features and gene flow within the populations, to identify the factors that influence the genetic structure of the population; and to verify the presence of natural or antrophic barriers for gene flow in roe deer populations. This is important to understand how the landscape features can limit or facilitate the movements of individuals. This is useful to give an evolutionary significance to any internal subdivisions of the population, as determined by the landscape modelling the effects of landscape features on genetic structure of roe deer populations. We examined the relationship between genetic distances

(Euclidean and Least Cost Paths) calculated between pairs of individuals and different patterns of geographical distance, designed to incorporate the main features of the study area.

4.2 Material and methods

The relationships between genetic variability and landscape features were analyzed by the following steps: genetic distances between individuals were calculated by using the same sample of chapter 3. Then landscape features were calculated in GIS: in particular the resistance surface to gene flow was estimated considering a forest numeric model and an urban numeric model. Hypothetical barriers were tested by spatial overlapping of cartographic data and genetic structure identified with GENELAND. Pairwise geographic distances between individuals were calculated as Euclidean and least cost paths. Finally, the relationships between genetic distances and landscape features (length and average cost of paths) were tested with Mantel and Partial Mantel test.

4.2.1 Genetic data

Samples of 657 roe deer tissue were taken and used as a source of DNA for the genetic analyses, as described in chapter 3. A set of 25 microsatellite markers were used to examine the spatial distribution of genetic variability. We used GENELAND program (Guillot et al., 2005) that on the basis of a Bayesian clustering model, assigned individuals to subpopulations from its genetic information (Guillot et al., 2005).

4.2.2 Estimating individual pairwise genetic distances

Genetic distances between pairs of individuals, a_r, defined by Rousset (2000), were computed using GENEPOP (Raymond and Rousset, 1995). This a_r is a generalization of Fst / (1 - Fst), distance normally used to evaluate differences between populations. Hence, we obtained one matrix of pairwise inter individual genetic distances. The inter-individual matrices were calculated for all pairs of individuals of the seven main subsets identified by the software GENELAND. The analysis were also performed for the sub-samples of male and female of each subpopulation (with the exception of subpopulations 6 and 7 because the number of individuals is lower), with the aim to verify if dispersal is sex-biased in roe deer (Coulon et al., 2006).

4.2.3 Identification of barriers

To identify the barriers to gene flow, we used two land-use maps of study area. The land use map (2007) of the Belluno province (Veneto Region) and the land use map of Autonomous Province of Trento (Ufficio cartografico Trentino). We reclassified each map in order to obtain 11 land-use

categories grouped under the table 2. Then, we overlapped the management units obtained with GENELAND (seven sub populations) with digital terrain model (DTM) and land use maps, using ArcGIS® 10 (ESRI).

4.2.4 Landscape features and costs

We obtained information on vegetation cover, topography, slope and elevation for each district. Vegetation data were derived from aerial photographs and a forestry and land-use map (Ramanzin & Sommavilla 2004). First, we excluded non-productive land-cover types (e.g. rocks, lakes and glaciers, which are a priori unsuitable habitat for roe deer). Secondly, we then measured the percentage cover of different habitat types as beech, deciduous woods other than beech, thick coniferous woods, larch, Pinus spp., mixed woods, mountain pine and alder (scrub stands at high altitude), low altitude scrub, low altitude fields (including meadows and agricultural crops), alpine pastures and rocks with sparse vegetation. Slope and elevation data were derived from Digital Elevation Model (DEM) that is the representation of the distribution of elevation in a certain area, in digital format. In most practical applications involving modeling the surface is the surface of the ground.

Landscape structure of the area was described by a FNM (Forest Numeric Model), quantifying the extent of wooded habitat (distance to the nearest wooded patch and size of those patches) around a given pixel of the area (Hewison *et al*., 2001). The FNM assigns to each pixel a value between 0 and 250. A value of 250 indicates that there is no wooded habitat within 500 m around the pixel, whereas a value of 0 indicates that the pixel is completely surrounded by forest within a radius of 500 m. This radius of the neighbourhood considered was chosen with reference to the average size of a roe deer home range and according to previous analyses of landscape–roe deer relationships in this area (Ramanzin *et al*., 2007). This model, which is hence not simply binary, describes the degree of openness of the local landscape and can be interpreted as a measure of woodland connectivity.

The second type of model considered was the UNM or "urban numeric model. Also give each pixel values between 0 and 250, but in this case a value of 250 indicates the complete presence of urban settlements in a neighborhood of pixels of 500 meters radius. The methodology for determining the values of each pixel is the same of FNM.

4.2.5 Pairwise geographic distances and landscape features

To calculate the geographical distances between pairs of individuals, compared with genetic ones, we used the software ArCGIS® 10 (ESRI) and the layer of the locations of each individual sampled.

4.2.5.1 Euclidean distance.

This distance simply measures the length (in m) of the straight line separating one roe deer from another (Figure 1). It does not take landscape structure into account. We used a Digital Elevation Model (DEM) with a pitch of 25 meters.

4.2.5.2 Least cost distance

The 'least cost distance' measures the distance between two roe deer supposing that they maximize the use of wooded corridors to move from one location to another, as suggested by direct observations of marked animals (Vincent *et al*., 1998; Zanon et al., 2005), Figure 2. We computed this distance using ArcGis® 10, based on the FNM of the area. The Least Cost Path (LCP) assigns to each pixel a number between 0 and 250; a value of 250 indicates no wooded habitat in a neighborhood of pixels of 500 meter radius, while a value of 0 indicates that the pixel is completely surrounded forest trees in a radius of 500 meters. In this model we considered the pixels associated with slope values greater than 60° and values greater than 2200 meters of altitude, with a value of 250, because they are considered barriers for roe deer movement (Zanon et al., 2005).

The calculation of the value associated with each pixel has been placed through the "neighborhood statistics" implemented in the extension "Spatial Analyst" software ArcGIS® 10. The size of the surroundings has been chosen by extension average home range of roe deer, as determined in previous analysis conducted to determine the relationship between this species and the area under consideration (Ramanzin et al., 2007). This model therefore describes the degree of openness of the local landscape and can be interpreted as a measure of connectivity of the forest (Coulon et al., 2004).

According to FNM calculated for the area concerned and through the extension of ARCVIEW ™ PATHMATRIX ™, was then given the distance matrix of minimum cost. From all possible paths from one point to another, the software chooses the trajectory (not necessarily the straight line) that minimizes Σ FNM, the 'cost' of movement (sum of the values of FNM associated with each pixel the path goes through). This path is thus a compromise between the shortest path (the straight line)

between two individuals and the path that passes through only highly wooded local environments (with the lowest FNM values).

4.2.5.3 Average costs of Euclidean and Least cost Paths

For each pair of individuals we calculated the average cost of euclidean and least cost paths. For euclidean paths, the average cost of FNM and UNM were calculated as ratio between sum of cost and length of path. This permit to test a multiple regression between genetic and geographic distance taking into account the "weight" of landscape features of the pixels included in the path. The average cost on the base of FNM was calculated and tested also for the least cost paths.

4.2.6 Relationship between genetic and geographical distances: Mantel Test and Partial Mantel Test

To test for landscape structure effects on gene flow within this roe deer population, we compared the correlation between the matrix of pairwise genetic distances and the two different matrices of geographical distances. In order to test our hypothesis concerning the influence of the distribution of wooded habitat on dispersal, we compared the correlation between pairwise genetic and geographical distances using the Euclidean and the least cost distance. A better correlation using the least cost distance would indicate that connectivity is an important element determining dispersal pathways for roe deer in fragmented landscapes. Correlations between distance matrices were tested using Mantel tests. They were performed with ADE 4 software (Thioulouse *et al*., 1994), a R package, using the genetic distances and the logarithm of the geographical distances, as recommended by Rousset (1997) for tests performed on populations using a two dimensional landscape. The subsamples of females and males were tested separately. *P*-values were obtained using a permutation procedure (10.000 permutations).

While, we used multimodel approach to evaluate alternative hypotheses and identify the combination of environmental factors that appear to drive gene flow in this landscape. Partial Mantel test based on 10.000 permutations was run to calculate the percentage of variability explained by genetic distance of each variable of the landscape. This procedure, applied using the software FSTAT 2.9.3 (Goudet, 2001), is a modification of the multiple regression test and, such as the Mantel test, should bypass the problem of non-independence between points. The partial Mantel test calculates the partial correlations for each variable, adjusting simultaneously for all variables already introduced in the model. The independent variables considered in the latter analysis were: the logarithmic transformation of the Euclidean distance and the average costs of FNM and UNM for Euclidean paths. Then, another model was run with the following independent variables: the

logarithmic transformation of the Least Cost Path distance (Along least-cost path distance or APD), and the average cost of FNM for least-cost path.

4.3 Results and Discussion

The aim of this study was to determine whether the landscape features affect gene flow of roe deer. These elements are important to study the ecology of the species itself and once again can be determining the definition of management units.

Analysis of the structure of the population has already called attention to a correspondence between the different composition of the landscape to study area of the Trento and Belluno provinces and the genetic differentiation of the sample. Studies focused on understanding barriers to gene flow were the first to incorporate landscape data in genetic analyses (Manel et al., 2003). Initial applications were directed toward identification of major breaks in genetic structure and visual association with landscape features (Dupanloup et al. 2002). More recently, detection of barriers been extended to address more specific ecological and conservation questions, including: identifying linear features that act as barriers to gene flow, barriers in aquatic systems, cryptic barriers and barriers in relation to disease spread. Linear features, such as rivers, mountain ridges, roads and anthropogenic habitat fragmentation, are the most obvious testable barriers to gene flow and frequently considered in landscape genetics studies. The effects of putative barriers on gene flow varies by species, ranging from no discernable effect on genetic structure (Gauffre et al. 2008), to extreme effects (Funk et al. 2005). These natural features which vary in effect by taxonomic group, roads and other human development have been identified as barriers across several taxonomic groups, including carnivores (Riley et al. 2006; Millions & Swanson 2007), ungulates (Epps et al. 2005; Kuehn et al. 2007; Perez Espona et al. 2008) and amphibians (Manier & Arnold 2006; Murphy et al. 2010).

We performed analysis of “one – screen” to identify barriers to gene flow. In figures 1 and 2 are showed two sections of the study area where we identified hypothetical barriers, located in the south of Trento province, in Adige valley. The results show how the ridges (Figure 1) and urbanized areas (Figure 2) can cause a barrier to gene flow. In fact, the individual assigned to different populations are divided by high peak or area densely urbanized. The presence of urban settlements is strictly connected with the valley, so it is correlated with the morphology of the territory.

Using the correlation between the genetic distances between all possible pairs of individuals and geographic distances we wanted to first determine whether the area determines a gradient of genetic differentiation, or if there is an effect of "isolation by distance" (IBD).

The presence of "isolation by distance" is related to the existence of more pronounced genetic similarity between individuals or populations in space as compared to neighboring or more distant

populations. At equilibrium, under dispersal and genetic drift, IBD pattern is revealed by a positive and significant correlation between genetic differentiation and geographical distances (Garnier et al., 2004). Identification of IBD can help to show equilibrium between migration and genetic drift (contemporary processes), or to link limited dispersal ability and genetic differentiation. The identification of IBD may therefore be necessary to prove the existence of equilibrium between migration and genetic drift or to verify the link between the degree of genetic differentiation and dispersal capacity.

The IBD pattern is then integrated with other spatial model, the "barriers" to gene flow, which are not represented only by very obvious barriers such as rivers, valleys or mountain ranges, but can also correspond to subtle changes in habitat.

Then, calculating geographic distances using different spatial models able to incorporate different landscape features, we wanted to see if there is an environmental component that most affects the gene flow. For example, a higher correlation between genetic distances and distances FNM (which take account of forest cover of the area) than the simple Euclidean distances (Coulon et al., 2004), would indicate that the forest helps to determine the movements affecting the "connectivity" of the landscape and therefore also on the dispersion of the species.

Analyses were conducted in parallel for each of the six main subsets identified by GENELAND in order to assess whether the effect of a single component is the same in different geographic and genetic situations.

The results of the Mantel tests carried out on the matrices of genetic and geographical distances for the six subpopulations (subpopulation 7 is too small) are given in Table 3.

The value of correlation between genetic distances and Euclidean distances ranged from 0.014 (Sub-1) to 0.260 (Sub-6). The higher correlation was calculated in Fassa Fiemme (Sub-6); this is a small subsample, geographically "isolated" from the other subsamples. The result suggest the presence of IBD, but should be reconsider on the base of a larger sample. In the North of Belluno Province, IBD is higher with respect the South and the other population in Trento, and this confirm the result obtained by Targhetta (2006) with a reduced panel of microsatellites markers. To investigate the relationship between genetic variability and landscape features we calculated least cost paths, which takes into account the FNM corrected for high elevations (>2200 m asl) and slopes (>60°). In general, the correlations were higher, but the improvement were mild. The value of correlations between genetic distances and Along least cost path distance (APD) ranged from 0.036 for Sub-1 to 0.293 for Sub-6. Only the Sub-3 presented no significant correlation, and the Mantel test was significant when using the least cost distance in Sub-5. The absence of isolation by distance in some subpopulation can be attributed to various causes. First, gene flow may be very

low over the distances sampled such that populations are essentially isolated and allele frequencies are determined by drift. Second, gene flow may be very high over the entire distance range such that the sampled region functions effectively as one large population. Third, populations may not be at equilibrium with respect to the forces of gene flow and drift following some historical perturbation (e.g. large reductions in the population, or reintroduction of deer) (Keyghobadi et al., 2005).

Further diversification was introduced in each of the sub-populations by sex (we excluded sub-population 6 because the number of individuals is lower), table 4 and 5. In fact, there is an impact on the population of the landscape, it should be more relevant to animals that make more movements and which disperse farther. We tested if there were differences in dispersal between males and females (Coulon et al., 2006). For females, neither of the Mantel tests were significant, however, correlation coefficients with the least cost distance were slightly stronger than with the Euclidean distance. Unfortunately the small total number of females available for each sub-population prevents proper analysis of this subsample and its comparison with others, possibly resulting in the no significant of the correlation values. For males, only for Sub-2, 3, 5 the Mantel test were significant when we used least cost distance. Significant Mantel correlation between the genetic matrix and a cost matrix would indicate that the genetic structure of the population is correlated with a specific landscape-resistance hypothesis.

It should also be noted that the different geographical distances (Euclidean and APD) were highly correlated and this correspondence occurs for all subsets tested. High correlation values between different geographical distances were, also, obtained by Coulon et al. (2004) and in this study are shown a some improvement is the use of least cost distance.

Then, we decided to use one extension of Mantel test, Partial Mantel test. In this test a third (or more) matrix is held constant while the relationship of the first two is determined (Smouse et al., 1986). This test is done by regressing the elements of X and Y onto the additional matrix (multiple regression for more than one additional matrix), and using the residuals from the regressions as the input for the standard Mantel test. In this way we adjusted the effect of individual environmental parameters by autocorrelation. In this case, the approach based on multiple correlations can be identified, in addition to the simple geographic distance, other factors that play a role in influencing the dispersion of individuals and therefore gene flow. The tables 6.1 ÷ 6.6 show the results of Partial Mantel test for each subpopulations and for subsamples males and females. The r value indicates the correlation between genetic distance and landscape features tested equal for other variables included in the model. Probability values indicated the significance of the single variable within the model. The genetic differentiation are influenced by the presence of forests (average cost

of FMN). In fact, this confirms that the forest increases the connectivity or “the degree to which the landscape facilitates or impedes movement among resource patches" (Taylor et al., 1993). While, the development of urban areas represents a barriers to gene flow.

In particular, in subpopulation 1 can be seen as the UNM is slightly stronger than the Euclidean distance (0.036). This component is also increased in the sub-samples of male and female, 0.074 and 0.086, respectively. In subpopulation 2, the higher correlation is between genetic distance and logarithm of the Euclidean distance (0.079). Is subpopulation 3 all the factors included in the model for linear paths were significant, but the higher correlation was found for UNM (r=0.136). Significance was maintained for males, whereas for females the small number of samples reduced the statistical significance. The average cost of Euclidean distance is still a major component in the subpopulations 4, 5, 6. The values obtained are, respectively, 0.095, 0.112, 0.363.

In general, the Euclidean distance is the variable that gives the smaller contribution of all. The latter, however, remains the main component in the model obtained by sub population 2.

The approach based on multiple correlations is useful to identify, in addition to the simple geographic distance, other factors that play a role in influencing the dispersion of individuals and therefore gene flow. In the sub-1, 2 and 3 the presence of urban settlements is the landscape features more correlated with the genetic distances among animals, both males and females. Only in the sub-3 the females are affected by the average cost of Euclidean distance. In the north the predominant effect is that of simple geographical distance, which seems to be much more important other environmental parameters in determining the genetic differentiation.

4.4 Conclusion

In this study we tried to develop a contribute to explain the discipline “landscape genetics”, in particular to explain the interaction between landscape features and micro-evolutionary processes (Manel et al., 2003). We have shown the importance of landscape caractheristics for gene flow and how this may affect the structure of roe deer populations. We showed that genetic boundaries are overlapped with hypothetical barriers (natural or antrophic). Then, we attempted modeling of environmental factors that may affect the connectivity within the individual populations, leading to identify, in addition to the predictable and simple geographic distance, the relation of forest / open area, the slope and elevation, and presence of urban settlements, as factors which play a role in influencing the individual movements and therefore the gene flow. The results obtained in this study should be improved by testing other statistical approaches. It’s evident that the landscape features influence the genetic structure of roe deer, and the identification of ecological barriers or

corridor in the study area should be useful to define the future management of this species in the two provinces.

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FIGURES AND TABLES

Figure 1: Example of Euclidean Distance

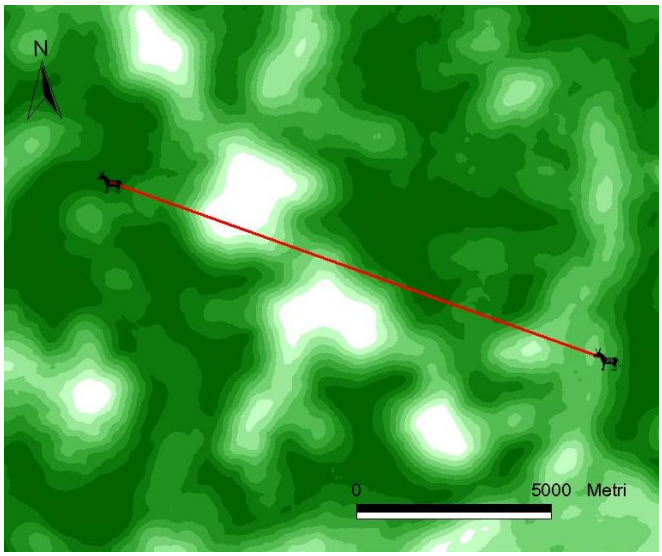

Figure 2: Example of Least Cost Distance calculated by Forest Numeric Model

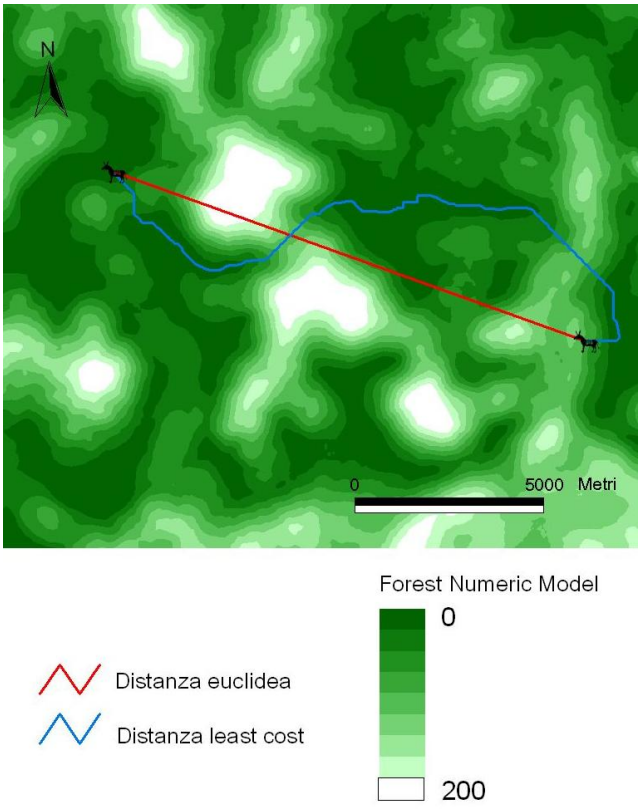

Figure 3: Section of the study area that represents a first hypothesis of a barrier to gene flow on the ridge line

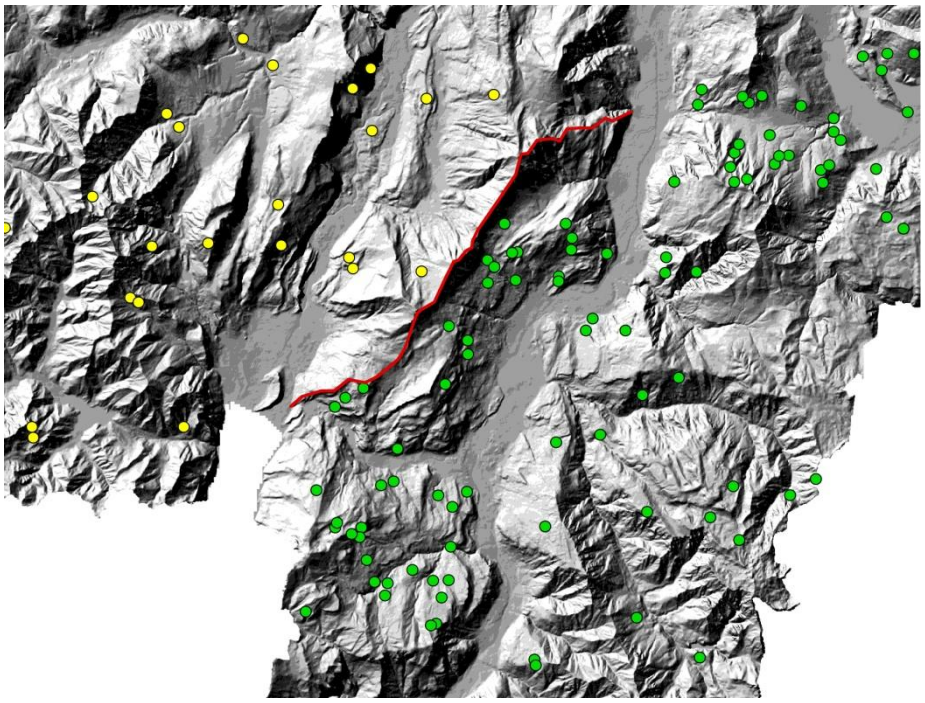

Figure 4: Section of the study area that represents a second hypothesis of a barrier to gene flow on the urban component

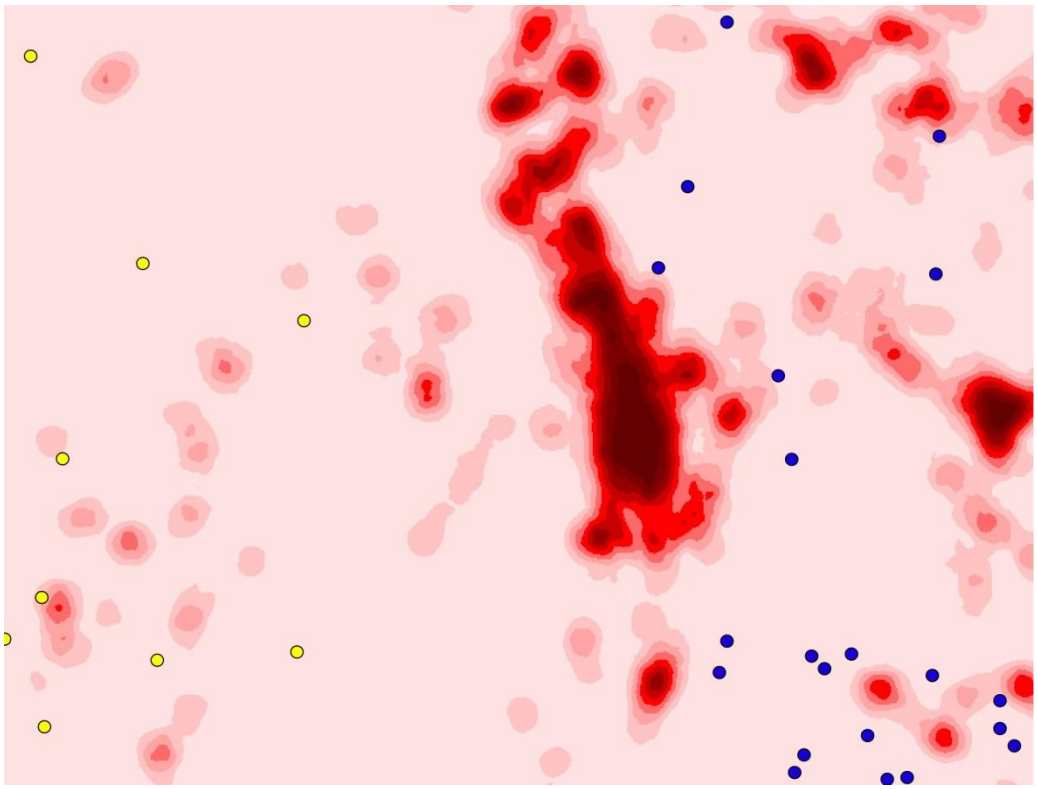

Table 1: Spatial genetic pattern

Isolation by distance (IBD): when genetic differentiation between individuals (or populations) increases with their geographical distance (because gene flow declines at larger distances)
Clines: a character gradient; continuous variation in a character through a series of contiguous or adjacent populations. In population genetics, the character could be multi-locus genotypes (at the individual level) or single locus allelic frequencies
Metapopulation: a group of subpopulations that exchange occasional migrants and that might be subject to local extinction and recolonization. In population genetics, it is commonly modeled using the island model (all subpopulations exchange migrants equally) or the stepping stone model (only adjacent subpopulations exchange migrants)
Random Pattern: spatial distribution pattern in which the presence of one individual has no influence on the distribution of other individuals
Genetic Boundaries: zones where genetic differences between pairs of populations are highest

Table 2: List of the 11 categories used for the creation of land use map

CATEGORIES
Unproductive areas
Open areas to share
Shrub share
Coniferous forests
Urbanized areas
Mixed woods
Rocky areas with scarce vegetation
Open areas in the valley
Deciduous forests
Scrub trough
Vineyards and orchards

Table 3: Correlations between genetic and (logarithmic) geographical distances for six sub populations of roe deer obtained by GENELAND. Values of the statistics r for Mantel tests are given for each relationship between genetic and geographical distances. Sub-1: Trento Sx; Sub-2: North Belluno; Sub-3: North Trento Dx; Sub-4: South Belluno; Sub 5: South Trento Dx; Sub 6: Fassa – Fiemme.

Model	Sub-populations											
	1 (N° 139)		2 (N° 176)		3 (N° 69)		4 (N° 175)		5 (N° 63)		6 (N° 17)	
	r	p	r	p	r	p	r	p	r	p	r	p
Euclidean Distance	0.014	0.002	0.096	< 0.001	0.044	0.232	0.06	0.0014	0.06	0.1081	0.260	< 0.001
Along least cost path distance	0.036	0.0015	0.103	< 0.001	0.051	0.222	0.07	0.0001	0.075	0.007	0.293	< 0.001

Table 4: Correlations between genetic and (logarithmic) geographical distances for males.

Model	Sub-populations (Males)									
	1 (N° 85)		2 (N° 144)		3 (N° 47)		4 (N° 121)		5 (N° 38)	
	r	p	r	p	r	p	r	p	r	p
Euclidean Distance	0.054	0.290	0.074	0.021	0.042	0.296	0.051	0.011	0.017	0.374
Along least cost path distance	0.063	0.249	0.075	0.011	0.097	0.003	0.028	0.3376	0.0828	0.005

Table 5: Correlations between genetic and (logarithmic) geographical distances for females

Model	Sub-populations (Females)									
	1 (N° 54)		2 (N° 32)		3 (N° 22)		4 (N° 54)		5 (N° 25)	
	r	p	r	p	r	p	r	p	r	p
Euclidean Distance	0.062	0.092	0.107	0.084	0.102	0.141	0.070	0.045	0.089	0.346
Along least cost path distance	0.073	0.073	0.117	0.027	0.191	0.038	0.090	0.025	0.092	0.258

Table 6.1: Results of multiple regression analysis using Partial Mantel test for sub-population 1 (a), male sub-sample (b) and female sub-sample (c). Values of the statistics r for Partial Mantel tests are given for each relationship between genetic and geographical distances expressed as the average of the costs along the linear trajectory Euclidean.

a) Sub 1: left side Trento, 139 deer				
Model	Correlation	Beta	p - VALUE	p-VALUE(%)
ln euclidean distance	0.01001	0.000211	37.55	0.3755
average cost euclidean distance	0.031415	0.002101	0.35	0.0035
R (%)			0.11	
ln apd	0.011582	0.000001	25.93	0.2593
least cost distance	0.044946	0.001047	0.01	0.0001
R(%)			0.22	
ln euclidean distance	0.014679	0.000038	72	0.72
average cost euclidean distance	0.023447	0.001318	18.8	0.188
urban numeric model (UNM)	0.035769	0.001325	0.4	0.004
R(%)			0.11	

b) Sub-1 :MALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.022856	0.000002	16.9	0.169
average cost euclidean distance	0.053118	0.00266	0.05	0.0005
R (%)			0.33	
ln apd	0.031834	0.005637	5.7	0.057
least cost distance	0.089278	0.010189	0.05	0.0005
R(%)			0.9	
ln euclidean distance	0.023118	0.000038	16.35	0.1635
average cost euclidean distance	0.052856	0.001318	0.05	0.0005
urban numeric model (UNM)	0.073608	0.001325	0.01	0.0001
R(%)			0.88	

c) Sub 1: FEMALES				
Model	Correlation	Beta	p - VALUE	p-VALUE(%)
ln euclidean distance	0.04554	0.000001	8	0.08
average cost euclidean distance	0.061942	0.008088	1.7	0.017
R (%)			0.59	
ln apd	0.033072	0.001628	21	0.21
least cost distance	0.067403	0.002265	1.55	0.0155
R(%)			0.56	
ln euclidean distance	0.04554	0.000003	8	0.08
average cost euclidean distance	0.061942	0.010752	1.7	0.017
urban numeric model (UNM)	0.086342	0.020659	0.2	0.002
R(%)			1.34	

Table 6.2: Results of multiple regression analysis using Partial Mantel test for sub-population 2 (a), male sub-sample (b) and female sub-sample (c). Values of the statistics r for Partial Mantel tests are given for each relationship between genetic and geographical distances expressed as the average of the costs along the linear trajectory Euclidean.

a) Sub 2: North of Belluno, 176 deer				
Model	Correlation	Beta	p - VALUE	p-VALUE(%)
ln euclidean distance	0.079644	0.004624	0.05	0.0005
average cost euclidean distance	0.022893	0.000001	0.45	0.0045
R (%)			0.69	
ln apd	0.076516	0.007627	0.05	0.0005
least cost distance	0.041644	0.000002	0.05	0.0005
R(%)			0.76	
ln euclidean distance	0.079644	0.00482	0.05	0.0005
average cost euclidean distance	0.022893	0.000001	0.45	0.0045
urban numeric model (UNM)	0.055847	0.024663	0.05	0.0005
R(%)			1	

b)Sub 2: MALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.079257	0.004023	0.05	0.0005
average cost euclidean distance	0.028734	0.000001	0.3	0.003
R (%)			0.71	
ln apd	0.075784	0.007201	0.05	0.0005
least cost distance	0.037184	0.000002	0.05	0.0005
R(%)			0.71	
ln euclidean distance	0.079257	0.004097	0.05	0.0005
average cost euclidean distance	0.028734	0.000001	0.5	0.005
urban numeric model (UNM)	0.059061	0.027549	0.05	0.0005
R(%)			1.06	

c)Sub 2: FEMALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.122686	0.009787	1.05	0.0105
average cost euclidean distance	0.04059	0.000001	36.75	0.3675
R (%)			1.67	
ln apd	0.132306	0.016526	0.05	0.0005
least cost distance	0.035144	0.000003	0.3	0.003
R(%)			3.58	
ln euclidean distance	0.122686	0.010047	0.55	0.0055
average cost euclidean distance	0.04059	0.000001	36.1	0.361
urban numeric model (UNM)	0.065232	0.02121	13.15	0.1315
R(%)			2.1	

Table 6.3: Results of multiple regression analysis using Partial Mantel test for sub-population 3 (a), male sub-sample (b) and female sub-sample (c). Values of the statistics r for Partial Mantel tests are given for each relationship between genetic and geographical distances expressed as the average of the costs along the linear trajectory Euclidean.

a) Sub 3: right side Trento, North, 69 deer				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.009694	0.000002	2.9	0.029
average cost euclidean distance	0.044724	0.008931	1.35	0.0135
R (%)			0.45	
ln apd	0.016571	0.000001	41.45	0.4145
least cost distance	0.051943	0.005495	0.95	0.0095
R(%)			0.3	
ln euclidean distance	0.024454	0.002504	3.4	0.034
average cost euclidean distance	0.059694	0.006501	0.95	0.0095
urban numeric model (UNM)	0.135712	0.045543	0.05	0.0005
R(%)			2.57	

b)Sub 3: MALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.066346	0.000003	3.35	0.0335
average cost euclidean distance	0.13664	0.019405	0.05	0.0005
R (%)			2.31	
ln apd	0.11568	0.000301	0.1	0.001
least cost distance	0.164747	0.022743	0.05	0.0005
R(%)			4.05	
ln euclidean distance	0.017295	0.000002	57.35	0.5735
average cost euclidean distance	0.042334	0.000382	16.15	0.1615
urban numeric model (UNM)	0.131982	0.047012	0.05	0.0005
R(%)			1.95	

c)Sub 3: FEMALES				
Model	Correlation	Beta	p - VALUE(%)	p-VALUE
ln euclidean distance	0.107827	0.00001	10.5	0.105
average cost euclidean distance	0.144232	0.026203	2.7	0.027
R (%)			3.24	
ln apd	0.086567	0.00003	20.6	0.206
least cost distance	0.191456	0.010619	0.35	0.0035
R(%)			4.41	
ln euclidean distance	0.107827	0.021482	9.2	0.092
average cost euclidean distance	0.144232	0.045773	2.85	0.0285
urban numeric model (UNM)	0.119518	0.035008	7.9	0.079
R(%)			4.67	

Table 6.4: Results of multiple regression analysis using Partial Mantel test for sub-population 4 (a), male sub-sample (b) and female sub-sample (c). Values of the statistics r for Partial Mantel tests are given for each relationship between genetic and geographical distances expressed as the average of the costs along the linear trajectory Euclidean.

a) Sub 4: South of Belluno, 175 deer				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.018198	0.000001	2.4	0.024
average cost euclidean distance	0.0946	0.00871	0.05	0.0005
R (%)			0.93	
ln apd	0.069971	0.000003	0.05	0.0005
least cost distance	0.079357	0.001004	0.03	0.0003
R(%)			1.12	
ln euclidean distance	0.016374	0.000001	4.3	0.043
average cost euclidean distance	0.018198	0.008534	0.05	0.0005
urban numeric model (UNM)	0.0946	0.003031	2.45	0.0245
R(%)			0.95	

b)Sub 4: MALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.008073	0.000001	49.95	0.4995
average cost euclidean distance	0.130028	0.008896	0.05	0.0005
R (%)			1.7	
ln apd	0.093696	0.00004	0.05	0.0005
least cost distance	0.142126	0.000418	0.01	0.0001
R(%)			2.9	
ln euclidean distance	0.005471	0.000006	64.85	0.6485
average cost euclidean distance	0.098073	0.001052	50.75	0.5075
urban numeric model (UNM)	0.130028	0.009146	0.05	0.0005
R(%)			1.7	

c)Sub 4: FEMALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.021121	0.000003	43.65	0.4365
average cost euclidean distance	0.087347	0.008983	0.3	0.003
R (%)			0.81	
ln apd	0.028571	0.000002	29.7	0.297
least cost distance	0.103184	0.009473	0.05	0.0005
R(%)			1.15	
ln euclidean distance	0.021121	0.000002	50.55	0.5055
average cost euclidean distance	0.087347	0.005234	42.05	0.4205
urban numeric model (UNM)	0.018018	0.008547	0.25	0.0025
R(%)			0.84	

Table 6.5: Results of multiple regression analysis using Partial Mantel test for sub-population 5 (a), male sub-sample (b) and female sub-sample (c). Values of the statistics r for Partial Mantel tests are given for each relationship between genetic and geographical distances expressed as the average of the costs along the linear trajectory Euclidean.

a) Sub 5: right side Trento, South, 63 deer				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.006166	0.000021	79.85	0.7985
average cost euclidean distance	0.112176	0.008343	0.05	0.0005
R (%)			1.26	
ln apd	0.022563	0.000003	32.95	0.3295
least cost distance	0.11254	0.006534	0.05	0.0005
R(%)			1.32	
ln euclidean distance	0.006166	0.00003	78.3	0.783
average cost euclidean distance	0.112176	0.008075	0.05	0.0005
urban numeric model (UNM)	0.037112	0.005212	10.05	0.1005
R(%)			1.4	

b) Sub 5: MALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.013201	0.000001	73.75	0.7375
average cost euclidean distance	0.085439	0.005258	2.9	0.029
R (%)			0.75	
ln apd	0.07286	0.000051	2.85	0.0285
least cost distance	0.080744	0.001551	2.7	0.027
R(%)			1.34	
ln euclidean distance	0.013201	0.000001	71.9	0.719
average cost euclidean distance	0.122201	0.047537	0.1	0.001
urban numeric model (UNM)	0.085439	0.005714	1.85	0.0185
R(%)			2.24	

c)Sub 5. FEMALES				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.046373	0.000003	41.65	0.4165
average cost euclidean distance	0.271124	0.022787	0.05	0.0005
R (%)			7.57	
ln apd	0.064477	0.00002	25.5	0.255
least cost distance	0.224711	0.018114	0.05	0.0005
R(%)			5.47	
ln euclidean distance	0.046373	0.000002	41.65	0.4165
average cost euclidean distance	0.27632	0.113812	0.03	0.0003
urban numeric model (UNM)	0.151124	0.020563	0.05	0.0005
R(%)			15.2	

Table 6.6: Results of multiple regression analysis using Partial Mantel test for sub-population 6. Values of the statistics r for Partial Mantel tests are given for each relationship between genetic and geographical distances expressed as the average of the costs along the linear trajectory Euclidean.

Sub 6: Fassa Fiemme, 17 deer				
Model	Correlation	Beta	p - VALUE	p-VALUE (%)
ln euclidean distance	0.116846	0.000008	18.35	0.1835
average cost euclidean distance	0.362684	0.011872	0.05	0.0005
R (%)			14.52	
ln apd	0.085509	0.000001	32.6	0.326
least cost distance	0.332824	0.011712	0.1	0.001
R(%)			11.81	
ln euclidean distance	0.090046	0.000091	18.6	0.186
average cost euclidean distance	0.152631	0.00141	0.05	0.0005
urban numeric model (UNM)	0.037965	0.000408	24.15	0.2415
R(%)			12.47	

ANNEXES

Genetic analysis reveals Roe deer (*Capreolus capreolus*) populations structure in North-Eastern Italian Alps

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Abstract: This preliminary study examined the population structure of 119 roe deer (*Capreolus capreolus*) sampled over the provinces of Belluno and Trento, in the north-eastern Italian Alps, using 11 microsatellite markers. The panel of microsatellites was highly informative, and the whole populations was subdivided into 2 distinct sub populations. The observed ecological population sub-units did not coincide with the administrative subdivision (the provinces borders) that are now in use for management. The results of this work provide useful indication for roe deer management in the provinces of Trento and Belluno, and confirm that molecular genetic approaches may give essential indications for wildlife management.

Key words: Roe deer, molecular genetic, microsatellite.

Introduction - The European Roe deer (*Capreolus capreolus*) is the most widely distributed and numerous cervid in Europe. Information on population sub-units is essential for a correct management, and approaches of molecular genetics may be very useful to this purpose (Zannèse *et al*., 2006). Aim of this preliminary work is analyse the genetic population structure of roe deer in the bordering provinces of Trento and Belluno (north-eastern Italian Alps).

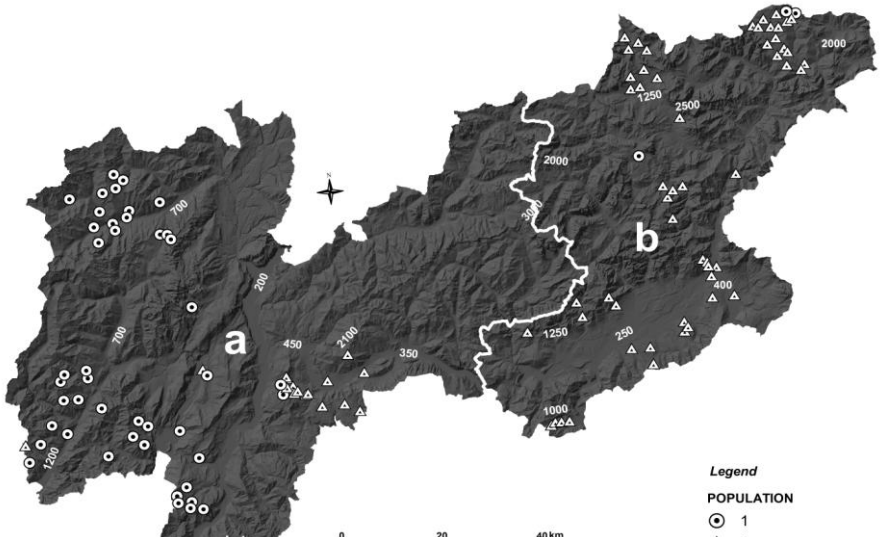
Materials and methods - A total of 119 roe deer (*Capreolus capreolus*) individuals of different ages and sex, harvested in the hunting season 2007-2008 in the hunting districts of the provinces of Trento (61 individuals) and Belluno (58 individuals) were used. Geographic coordinates of each shooting location were implemented in ArcGis (v. 9.2). Genomic DNA was extracted from ear punches using the PURGENE DNA tissue kit (Gentra System INC) protocol with modifications. Eleven microsatellite markers were chosen (Bonnet *et al*., 2002; Galan *et al*., 2003; Vial *et al*., 2003). Fifty ng of purified DNA were used in a singleplex "touch-down PCR" (Vial *et al*., 2003) for amplification of each microsatellite locus. Fragments were analyzed with Beckman Coulter CEQ 8000 automated sequencer using the GenomeLab Fragment Analysis protocol (Beckman Coulter). Amplicons were scored with the Fragment Analysis software (Genetic Analysis System v.9, Beckman Coulter). Summary statistics were obtained using Genepop v.4.0 (Raymond and Rousset, 1995), MSA (Microsatellite analyzer) v.4.05 (Dieringer *et al*., 2003), F-stat v.2.9.3 (Goudet *et al*., 2001) and Genetix v.4.01 (Belkir *et al*., 1998). Population structure was obtained using STRUCTURE version 2.2 (Pritchard *et al*., 2000).

Table 1. Number of alleles per locus (Na), expected (Het) and observed (Ho) heterozygosity, estimates of inbreeding coefficient (F_{IS}).

Locus	Totale			
	Na	Het	Ho	F_{IS}
BL4	10	0.591	0.433	0.257
BM1706	8	0.804	0.758	0.044
BM757	10	0.718	0.477	0.333
BM848	10	0.794	0.679	0.149
BMC1009	7	0.750	0.576	0.233
CSSM39	7	0.737	0.611	0.173
CSSM41	6	0.501	0.470	-0.036
CSSM43	6	0.580	0.628	-0.095
HUJ1177	10	0.766	0.710	0.070
IDVGA8	6	0.486	0.400	0.179
OARFCB304	13	0.861	0.710	0.167
Mean ± S.D.	8.45 ± 2.30	0.689 ± 0.128	0.586 ± 0.124	0.134

Results and conclusions - A total of 93 alleles were obtained in the whole population, ranging from 6 to 13 with an average of 8.45 alleles per locus (table 1). There was no significant linkage disequilibrium between pairs of loci using Bonferroni adjustments for multiple tests, and a significant deficit of heterozygosity was observed in the total population (results not shown). F_{IS} was low (0.13) but significantly different from zero; six loci showed a significant heterozygosity deficit. The roe deer population was not in Hardy Weinberg equilibrium (HWE), in agreement with Coulon et al. (2004) and Targhetta (2006). Explanations of the departure from HWE could be: the presence of null alleles, a significant level of inbreeding (less likely due to the small value of $F_{IS)}$, and the "Wahlund effect". The total population was structured in 2 sub-populations, one in the western part of the Trento province (Sub-1), and the other in the eastern part of the province and in the Belluno province (Sub-2) (Figure 1). Sub-1 showed a higher genetic variability than sub-2 (Ho = 0.596 and 0.577 and Het = 0.711 and 0.640, respectively). The F_{IS} estimates for each sub-population was 0.163 and 0.114 for sub-population 1 and 2 respectively. This results is important, because the observed ecological population sub-units do not coincide with the administrative subdivision (the provinces borders) that are now in use for management. In conclusion, the results of this work provide useful indication for roe deer management in the provinces of Trento and Belluno, and confirm that molecular genetic approaches may give essential indications for wildlife management.

Figure 1. Distribution of individuals belonging to the two populations identified by STRUCTURE on the Trento (a) and Belluno (b) provinces.



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