

Population Genetics and Phylogeography of European Red Deer (*Cervus elaphus*) and Roe Deer (*Capreolus capreolus*)



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I. GENERAL INTRODUCTION

I. I. RED DEER AND ROE DEER OCCURRENCE IN EUROPE

The genus *Cervus* is the major lineage in the subfamily Cervinae (Artiodactyla, Cervidae) and comprises about 15 species and numerous subspecies. The European red deer (*Cervus elaphus* L. 1758) is the most widespread and best known deer species in the world. It is widely distributed in the Palearctic and Nearctic. Today, there are up to 22 known subspecies based on coat colours and morphological characters of skulls and antlers (Mahmut *et al.*, 2002; Gyllenstein *et al.*, 1983; Ludt *et al.*, 2004; Geist, 1999; Trense, 1989, Whitehead, 1972). Red deer originated in the area between Kyrgyzstan and Northern India and basically form two distinct groups: a western group consisting of four subgroups in Eurasia and an eastern group consisting of three subgroups in Asia and America and additionally one or two primordial subspecies in Central Asia (Tarim group) (Ludt *et al.*, 2004). The European subspecies include *C. e. elaphus*, *C. e. hippelaphus*, *C. e. atlanticus*, *C. e. scoticus*, *C. e. corsicanus*, *C. e. hispanicus* and possibly *C. e. montanus* and *C. e. maral* distribution (Feulner *et al.* 2004, Groves and Grubb 1987, Pitra *et al.*, 2004). The present classification of the numerous species is mostly based on morphological characters such as body and antler sizes (Dolan 1988); antler shape or cranial measurements (Geist 1991; 1992); there are still some disagreements (Ludt *et al.*, 2004). Late Pleistocene red deer had complicated antlers that exhibited well-developed bez tines and crowns (Reynolds 1933).

The red deer is part of the cold-adapted Asian fauna which replaced the warm-climate European fauna in mid-Pleistocene times after a cold pulse between 900 000 and 700 000 years ago (Geist 1998). The biogeographic history of the European red deer has for many centuries been under human influences such as translocations, habitat fragmentation and selective hunting, potentially affecting their genetic structure (Hartl *et al.*, 2003; Lowe and Gardiner, 1974; Niethammer, 1963). Central European red deer (*Cervus elaphus hippelaphus* Erxleben, 1777) is a medium-sized subspecies standing approximately 120-125 cm at the shoulder and weighing from 100-350 kg. Females are 20-40% smaller than males. The distribution area comprises France, Holland, Belgium, Luxembourg, Denmark, Switzerland, East and West Germany, Austria, Czechoslovakia, Italy, the Balkan States, Poland and the western Soviet Union. It has been introduced to many countries, including the United States, New Zealand, Chile, and Argentina (Dolan 1988).



Figure I. Female and male red deer *Cervus elaphus* Linnaeus, 1758. Artiodactyla: Cervidae. (George Stubbs, 1792; Oil on canvas. Royal Collection, UK.)

The red deer is one of the biggest free-ranging mammals of Central Europe and, although not endangered in terms of population numbers, is the perfect model for studying the population genetic effects of a multitude of deliberate and unintentional anthropogenic influences on natural populations over a long period of time. Red deer gene pools are affected by habitat fragmentation, keeping of populations in enclosures, translocations, (re)introductions, and trophy hunting. Various schedules of population regulation by hunting are applied throughout Europe. Many autochthonous stocks have been hybridized with the introduced animals, thus blurring the historical boundaries between formerly natural populations (Hartl *et al.*, 2003; Zachos *et al.*, 2003).

The true roe deer *Capreolus capreolus* first appeared in the early Middle Pleistocene (Comorian stage) of Europe, with one presumably monotypic species (Lister 1984; Danilkin 1996). It is a small cervid with an adult live weight of typically 20-30 kg. It is grey-brown to pale grey in winter, red-brown or dark brown in summer. It is widely distributed and occurring from Scandinavia in the north to the Mediterranean in the south and from Spain and Portugal in the west to the Ural Mountains in the east. It is the most widespread and the most common ungulate in Europe. The European roe deer *Capreolus capreolus* (distributed in

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Western and Central Europe) and the larger Siberian roe deer *C. pygargus* (distributed in Asia and Eastern Europe) differ in body size, morphometric traits and karyotype. Siberian roe deer



Figure II. Roe deer (*Capreolus capreolus* Linnaeus, 1758; Artiodactyla: Cervidae)

have been subdivided into a west Siberian (Kurgan region) and an east Siberian (Amour region) group and European roe deer into an eastern and a western Alpine group. Both species separated at the Pliocene/Pleistocene boundary and evolved independently for about 2-3 million years according to the results of mitochondrial DNA (mtDNA) sequences and the available fossil records (Grubb 1993; Danilkin 1996; Vernesi *et al.*, 2002; Sokolov & Gromov 1990; Groves and Grubb 1987; Randi *et al.*, 1998). Roe deer are major hunting animals, and they have successfully colonized a wide area across Eurasia.

The objectives of the present studies were to gain insight into phylogeographic history; to characterize and quantify the genetic diversity within and among populations to implement conservation and management strategies and to compare different molecular marker systems with regard to their respective resolution power. For this purpose, a research programme was designed to characterize populations of two European deer species, red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*), using altogether three different molecular marker systems: allozymes, microsatellites and the mitochondrial control region (CR).

This research programme comprised four different studies: (1) an analysis of the two Italian red deer populations from Sardinia and Mesola in order to characterize their genetic variability and derive conservation and management suggestions and to gain a deeper insight

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into the phylogeography of the Sardinian population; (2) a genetic analysis of seven red deer populations from England and Scotland; (3) an analysis of three isolated red deer populations from Schleswig-Holstein as part of a more comprehensive study dealing with the effects of habitat fragmentation, low effective population size and inbreeding on red deer in northern Germany; and (4) an analysis of five roe deer populations from Schleswig-Holstein, among them two island stocks.

I. II. MARKER SYSTEMS IN MOLECULAR POPULATION GENETICS

Population genetic studies have been remarkably productive and successful in the last decade following the invention of PCR technology and the introduction of mitochondrial and microsatellite DNA markers. To obtain a fuller picture of the history and evolutionary potential of populations, genealogical data from nuclear loci are essential (Zhang and Hewitt 2003). Population genetic expertise for habitat improvement and population management can be provided. Over the past two decades, new molecular genetic techniques have had substantial impacts on the fields of ecology, evolution and conservation, e. g. the widespread use of mtDNA in the 1980s and microsatellites in 1990s (Morin *et al.*, 2004). Additionally, animal dispersal and gene flow have been measured with genetic markers for over 60 years (Berry *et al.*, 2004). Molecular markers in the DNA of all individuals provide highly accurate tools for assessing population size, sex ratio, mortality, reproduction, effective population size, inbreeding, and migration and are thus very useful in monitoring population growth or decline (Wan *et al.*, 2004). While mtDNA has proved to be powerful for genealogical and evolutionary studies of animal populations, microsatellite sequences are the most revealing DNA markers available so far for inferring population-genetic structure and dynamics.

Genetic diversity is required for populations to evolve in response to ubiquitous, natural environmental change and is usually assayed by molecular techniques (Frankham *et al.*, 2002; 2004). All different marker systems have been used to get deeper insight into population structure and genetic diversity for many conservation studies and they give great promise for estimating fundamental parameters or important characteristics in conservation, such as past effective population size (Garrigan *et al.*, 2002), past bottlenecks (Luikart & Cornuet, 1998), population origin of individuals (Cornuet *et al.*, 1999), individual inbreeding level (Ellegren, 1999; Lynch and Ritland 1999) and sex specific gene flow (Latta & Mitton, 1997) or founder contributions (Carvajal- Carmona *et al.*, 2000). Neutral markers are generally used for conservation applications and to estimate evolutionary parameters, and with highly variable loci, many more markers, or extensive sequence data, these approaches should become

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much more informative (Hedrick 2004). In the studies of the present thesis, three different marker systems were used: allozymes, microsatellites and sequences of the mitochondrial control region, which will briefly be characterized on the following pages.

I. II. I. Allozymes

Allozymes are electrophoretically different variants of an enzyme locus caused by multiple alleles (Harris and Hopkinson 1976). Before the introduction of PCR-based markers, allozymes were the most widely used markers in population genetics. The electrophoretic separation of enzymes can be carried by using starch gels, acrylamide gels, agarose gels and cellogels (Harris and Hopkinson 1976).

Protein electrophoresis was extensively used to study the phylogenetic relationships among populations or closely related species. The technique is inexpensive, but requires high-quality samples, often reveals little variation and gives limited genealogical information (Li and Graur 1991; Sunnucks 2000). Also, only a limited part of the genome, namely the enzyme-coding gene loci, can be studied. Allozyme electrophoresis is the “generalist” among molecular approaches. Allozyme data are suitable to address a broad spectrum of population genetic and systematic questions in a cheap and rapid way and may furthermore provide a basis for selecting an appropriate DNA technique for addressing a more specific problem (Hartl *et al.*, 1994; Hillis and Moritz 1990). In many studies allozymes have been implicitly or explicitly assumed to be a representative indicator of overall genomic variation. In spite of allozymis diversity cannot be considered representative for morphological variation within populations (Hartl *et al.*, 1994). The basic mutational mechanism for allozymes involves substitutions and insertions or deletions at the DNA level which occasionally change the amino acid sequence of the protein and hence possibly its steric conformation and electric charge (Avice 1994). However, only about 30% of these changes result in charge changes in the proteins, which are why this technique systematically underestimates the full extent of genetic diversity (Frankham *et al.*, 2004).

I. II. II. Microsatellite

Microsatellites are tandem-repetitive stretches of DNA with repeat units of 1-6 base pairs such as (CA/GT)_n or (AGC/TCG). They are widely present in eukaryotic genomes at high frequencies (Miesfeld *et al.*, 1981; Hamada *et al.*, 1982; Weber and May 1989; Litt and Luty 1989; Tautz. 1989; Economou *et al.*, 1990; Beckman and Weber 1992). In mammals, one of the most common motifs is (CA/GT)_n (Moore *et al.*, 1991). Microsatellites are rela-

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tively new powerful polymorphic markers for gene mapping and various aspects of population and evolutionary genetics and has been documented for many plant and animal species including several studies used in red deer and other cervids (e.g. Diethard 1993; Almeida and Penha-Gonçalves 2004; Tautz and Renz 1984; Tautz and Schlötterer 1994; Goldstein and Pollock 1997; Li *et al.*, 2002.; Metzgar, Bytof, and Wills 2000; Ashley and Dow 1994; Baron *et al.*, 1992; Feulner *et al.*, 2004; Goodman *et al.*, 2001; Hundertmark *et al.*, 2002, Kuehn *et al.*, 2003, Lorenzini, Lovari & Masseti, 2002, Lorenzini *et al.*, 2003, Nies, Zachos & Hartl, 2005, Zachos *et al.*, 2003; Schlötterer, 2000). They are inherited in a co-dominant Mendelian manner and are present in either heterozygous (with two different alleles at a locus) or homozygous (with two copies of the same allele at a gene locus) state in each individual. Alleles generally correspond to DNA fragments of different sizes as revealed by electrophoresis methods (Wan *et al.*, 2004). Between species, there are differences in structure, length and number of microsatellites (Bachtrog *et al.*, 1999; Chambers and MacAvoy 2000; Harr and Schlötterer 2000).

Microsatellites are amplified with specific PCR primers and the different alleles separated along an electrophoresis gradient in routine laboratory procedures. They play a significant role in genome evolution by creating and maintaining quantitative genetic variation (Jehle & Arntzen 2002; Tautz *et al.*, 1986; Kashi *et al.*, 1997). Addition or deletion of one or several repeat motifs due to slipped-strand mispairing during DNA replication is thought to be the predominant mutational mechanism by which new length alleles are generated at microsatellite loci (Schlötterer & Tautz 1992; Strand *et al.*, 1993). Compared with other loci, the mutation rate of microsatellite loci is very high: $10^{-2} - 10^{-6}$ per locus and generation (Bachmann *et al.*, 2004) with usually 10^{-3} - 10^{-5} in mammals (Schug *et al.*, 1997). Microsatellites are useful in paternity analyses (Wan *et al.*, 2004) and their high degree of polymorphism offers a good approach to a deeper understanding of population structures (Slatkin 1995; Vial *et al.*, 2003) and also a valuable tool in detecting genetic variability in conservation and management studies (Maudet *et al.*, 2002) and in assigning different individuals to their respective populations of origin (Cornuet *et al.*, 1999). Genetic evidence for historical bottlenecks can consist of size distributions of microsatellite alleles containing gaps (Côte *et al.*, 2002). The statistical power of microsatellite markers depends on the number of loci used, the degree of polymorphism of each locus and the sample size (Zane *et al.*, 2002). As regards geographic differentiation and phylogenetic relationships among deer populations microsatellites also turned out to be a powerful tool (Zachos *et al.*, 2003; Goodman *et al.*, 2001; Hartl *et al.*, 2003).

I. II. III. Mitochondrial control region

Mitochondria are present in all eukaryotic organisms. They are cellular organelles responsible for respiration and the production of energy. Contained within the mitochondria is a loop of DNA that is copied and transferred from generation to generation independently of the nuclear genomes (Appleton, 2003). And it is – apart from very rare exceptions - inherited maternally as the transmitted across generations as a maternal lineage. Vertebrate mtDNA is thought to evolve about 1-2%/10⁶ years although the rate may vary among taxa (Brown, 1985; Brown *et al.*, 1979). MtDNA is histone-free, has limited repair ability and a relatively high mutation-fixation rate and thus, evolves 5-10 times faster than the nuclear genome. The mitochondrial genome (mtDNA) of most vertebrates consists of a closed circular DNA molecule of 16,000-18,000 base pairs (Cantatore and Saccone 1987). The high frequency of deleterious mtDNA alleles within species is thought to result from four primary characteristics of the mitochondrial genomes: (i) high mutation rate; (ii) lack of recombination; (iii) reduced effective population size (due to its being haploid and maternally inherited); and (iv) maternal transmission (Gemmell *et al.*, 2004). The mtDNA gene pools of animals and plants are often subdivided into clines of phylogenetically related haplotypes that show no or little overlap in their geographical occurrence (Avise *et al.*, 1979; Avise 2000), a phenomenon that gave rise to the discipline of phylogeography (see below).

MtDNA sequence data are a powerful tool for determining population structure and also for the identification of subspecies and species but is of limited or little use in investigating recent loss of genetic variation and any individual-based approaches such as identity, individual dispersal, and mating systems (Brown *et al.*, 1979, 1982; Wan *et al.*, 2004; Jansen 2000). During mtDNA evolution, transitions occur much more frequently than do transversions. Investigation of higher taxonomic relationships must be restricted to coding sequences of mtDNA because the control region evolves too quickly to be useful (Brown 1983, 1985). For two decades now, the mitochondrial genome has been one of the cornerstones of modern evolutionary genetics and has been widely and successfully used in population and conservation genetic, phylogenetic and phylogeographic studies (Moritz *et al.*, 1987; Huchon *et al.*, 1999; Wan *et al.*, 2004) with the control region being among the most appropriate markers for analyses on the intraspecific level.

The genomes of mammalian mitochondria are duplex DNA circles. The two major transcriptional promoters and the origin of DNA replication for one DNA strand are located in a single region which contains no structural genes and copies about 6% of the genomes, is so called the mitochondrial control region (CR) sequence - the displacement loop (D-loop) re-

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gion, the main noncoding region of metazoan mitochondrial DNA (mtDNA), a central conserved section, is bounded by the transfer RNA genes t-RNA^{Pro} on the 5' side of the light (L)-strand and t-RNA^{Phe} on the 3' side of the heavy (H)-strand in mammals. Consequently the vertebrate CR is commonly subdivided three domains that differ from each other in base composition as well as in rate and mode of evolution (King and Low 1987; Douzery 1997; Brown *et al.*, 1986; Saunders and Edwards 2000). The CR contains sequences which control the replication of the H-strand and the transcription of mtDNA (Clayton 1991). The peripheral domains quickly accumulate point mutations, and variable numbers of tandem repeats (VNTRs). Extensive different length of the mtDNA molecule can be produced among species (Douzery 1997) as short repeats (Arnason and Johnsson 1991) and long repeats (Wilkinson and Chapman 1991). Control region length variation is a well-documented phenomenon and in most cases this variation arises due to slippage errors or false termination/elongation during mitochondrial DNA replication (Gemmell *et al.*, 1996)

The control region contains intervening sequences in the left and right domains that have high level of polymorphism are widely used for addressing evolutionary relationships among populations and species. And it is a powerful tool for determining the current status of population structure and identification of subspecies and species, rather than the individual identity and contemporary changes in genetic structure (Mortiz *et al.*, 1987.; Palumbi 1996; Douzery 1997; Wan *et al.*, 2004)

I. III. CONSERVATION GENETICS AND PHYLOGEOGRAPHY OF RED DEER

I. III. I. Conservation genetics

The biodiversity of the planet is being rapidly depleted as a direct and indirect consequence of human actions such as overexploitation, introduced species and pollution. Most of the world's species are threatened with extinction mainly by habitat loss as a result of increasing human activities and the most acute problems occur where the human population pressure is greatest. Large numbers of species are already extinct, while many others have reduced population sizes. Reduced numbers of populations are susceptible to stochastic effects such as environmental, demographic, or genetic (inbreeding depression and loss of genetic diversity) stochasticity and catastrophes (Frankham *et al.*, 2002; Frankham 2003).

Conservation genetics is the use of genetic theory and techniques to reduce the risk of extinction in threatened species, to preserve species as dynamic entities capable of coping with environmental change. It focuses on the effects of contemporary genetic structuring on

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long-term survival of a species or population. Conservation genetics deals with the effects of loss of genetic diversity and recent changes in genetic structure (Hedrick 2004; Wan *et al.*, 2004; Frankham *et al.*, 2002, 2004). Genetic considerations are probably most useful when incorporated early in a species' conservation plan (Hedrick 2001). Genetics contributes not only to the conservation of genetic diversity, but also to the conservation of species (Frankham 1999).

Conservation geneticists usually agree that genetic variability within and among populations are essential for the survival and adaptability of populations (Soulé, 1986). Therefore, the task of conservation genetics is not only to examine genetic variation in populations and species but to set guidelines drawn from genetic data for ensuring scarce financial resources (Wan *et al.*, 2004). The current extinction problem has been called the “sixth extinction”, as its magnitude compares with that of the other five mass extinctions revealed in geological records. They are 11 major genetic issues in conservation biology. They are derived from evolutionary genetics and from the quantitative genetic theory that underlies selective breeding of domesticated plants and animals (Frankham *et al.*, 2002: 2004):

- the deleterious effects of inbreeding on reproduction and survival (inbreeding depression)
- loss of genetic diversity and ability to evolve in response to environmental change (loss of evolutionary potential)
- fragmentation of populations and reduction in gene flow
- genetic drift overriding natural selection as the main evolutionary process
- accumulation and loss (purging) of deleterious mutations
- genetic adaptation to captivity and its adverse effects on reintroduction success
- resolving taxonomic uncertainties
- defining management units within species
- use of molecular genetic analyses in forensics
- use of molecular genetic analyses to understand aspects of species biology important to conservation, and
- deleterious effects on fitness that sometimes occur as a result of out crossing (out breeding depression)

Populations evolve through the actions of mutation, migration, selection and chance. Genetic diversity arises from mutation, or is added by migration, and is removed by selection, or lost by sampling in small populations (“genetic drift”, Frankham *et al.*, 2004). Genetic diversity is required for populations to adapt to environmental change. The World Conservation Union

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(IUCN) has recognised genetic diversity as one of three levels of diversity requiring conservation. It is typically described using polymorphism, average heterozygosity, and allelic diversity (McNeely *et al.*, 1990; Frankham *et al.*, 2004). Also, genetic variability is important consequences for population persistence, especially in species that have become fragmented, bottlenecked or that have rapidly lost genetic variability due to decreasing ability of populations in responding to environmental changes (Selander 1983; Saccheri *et al.*, 1998; Westemeier *et al.*, 1998). Populations with low genetic diversity i.e. without variation between individuals, population can not adapted to changing environments and are expected to suffer more seriously from diseases, pests and parasites, climatic changing, competitors, or changing food supplies than those with high genetic diversity (Lacy 1987; Frankham 2002, 2003). Endangered species generally have low effective population sizes and genetic variants are more likely to be effectively neutral in endangered than in common species (Hedrick 2004). Genetic depletion within populations and increased genetic differentiation among them should be quite obvious when populations are kept in enclosures over a number of generations (Hartl *et al.*, 1991). In general, variability is very slowly lost, usually over hundreds or thousands of generations, and the rate of loss is inversely proportional to the effective population size (N_e). In contrast to loss of variability, the effects of inbreeding depression will begin within a few generations of a decline and their strength will depend primarily on the magnitude of the drop in population size (Amos and Balmford, 2001). “Critically endangered” was defined as having at least a 50% probability of extinction within ten 10 years, or three generations, whichever is the longer. Effective population sizes of less than 50, 500 or 2000 were used to define “critically endangered”, “endangered” and “vulnerable”, respectively (Frankham 1999).

Evolutionary potential is predicted to depend not only on heterozygosity, but also on allele frequencies, so it may ultimately depend on allelic diversity. Allelic diversity, not high heterozygosity alone, is important for increasing the chances for long-term survival of a population (Hartl & Pucek 1994; Robertson 1960; James 1971). Still, the loss of heterozygosity is slow compared with the timescales over which conservation biology operates. For that why understanding gene flow is also fundamental for ascertaining factors that enable or prevent local adaptation, and for describing dynamics that facilitate the spread of new, beneficial mutations (Sork *et al.*, 1999; Reed and Frankham 2001). Indeed, genetic information is invaluable for wildlife managers to protect biodiversity by identifying a series of conservation units such as evolutionarily significant units (ESUs), management units (MUs), action units (Aus), and family nets (FNs) for the management of species (Wan *et al.*, 2004).

I. III. II. Phylogeography

Phylogeography which combines phylogenetics and biogeography is the examination of geographic distributions of evolutionary lineages to understand the evolutionary history of a taxon (Manel *et al.*, 2003). A comparison of the genealogy of lineages with the geographic distribution of the populations studied yields so-called phylogeographic patterns (Avice *et al.*, 1987). Under isolation-by-distance, genetic differences are inversely related to the amount of gene flow, which depend on geographical proximity between populations (Wright 1943; Morton *et al.*, 1968; Malécot 1973).

A large number of European species show strong phylogeographic patterns. At the beginning of the last glacial maximum, little phylogeographic patterns existed in European mammals over most of their geographical ranges (Avice 2000; Taberlet *et al.*, 1998). Phylogeographic patterns are blurred in species with high migration rates and the patterns currently observed are transient relics of the last glacial maximum and do not represent long-term adaptations to different environments (Hofreiter *et al.*, 2004). One of the most compelling results from comparative phylogeographic analyses is the occasional concordance of cline boundaries across diverse taxa (Remington 1968; Redenbach & Taylor 2002). A strong degree of phylogeographic structure both between subspecies and among populations within subspecies suggests that dispersal of individuals between populations has been very limited historically (Balakrishnan *et al.*, 2003).

Microsatellite loci and mitochondrial DNA sequences have been successfully used to reconstruct phylogenetic relationships among populations in a variety of species (Bowcock *et al.*, 1994; Machugh *et al.*, 1994, Wan *et al.*, 2004; Bulgin *et al.*, 2003; Van Den Bussche *et al.*, 2003; Hartl 1994, 1995; Avice *et al.*, 1979,2000; Hedrick 2004; Hofreiter *et al.*, 2004; Zachos *et al.*, 2003; Feulner *et al.*, 2004; Goodman *et al.*, 2001; Ludt *et al.*, 2004). In Europe, phylogeographic patterns have been found for several large mammals such as red deer and roe deer (Ludt *et al.*, 2004; Vernesi *et al.*, 2002). These patterns and phylogenetic relationships are also useful in determining conservation and management units which should consist of populations representing genetic entities different from their conspecifics (Frankham *et al.* 2002; for red deer, see Feulner *et al.* 2004 and Hmwe *et al.* in press).

I. IV. INBREEDING AND HABITAT FRAGMENTATION IN RED DEER

I. IV. I. Inbreeding

Inbreeding has been known to reduce reproduction and survival (inbreeding depression) since Darwin's classic work (Darwin 1876). Human activities such as selective hunting and deforestation directly or indirectly affect the structure and effective size of local populations. Inbreeding (mating between related individuals) is most serious and unavoidable in small populations. (Ralls *et al.*, 1970; O' Brien *et al.*, 1985; Frankham *et al.*, 2002) It increases the proportion of polymorphic loci at which an individual is homozygous (Wright 1921). Mating of closely related individuals increases the level of homozygosity on a genome-wide basis. Deleterious recessive alleles and loss of heterozygote advantage will eventually result in reduced fitness, which is called inbreeding depression (Slate *et al.*, 2000; Hansson & Westerberg 2002; East & Jones 1919). Inbreeding depression is nearly universal among species, both in captivity and in the wild (Wright 1977; Ralls and Ballou 1983; Falconer and Mackay 1996; Lynch and Walsh 1998; Crnokrak and Roff 1999; Frankham *et al.*, 2002; Keller and Waller 2002; Reed and Frankham 2003). Nearly all of the empirical evidence for inbreeding depression comes from laboratory or domestic species. The degree of inbreeding depression remains controversial in wild populations. It can be detected by correlating the multilocus marker heterozygosity of individuals with a trait related to overall fitness (Frankham, 1995; Slate *et al.*, 2000). Yet, it is problematic to demonstrate inbreeding depression for overall fitness in a wild population for two reasons. First, measuring fitness in the wild is difficult, especially if the study species is long-lived and has relatively long generation times. Second, detailed knowledge of familial relationships and pedigrees is needed to measure an individual's inbreeding coefficient (Endler 1986). Still, high levels of inbreeding have been observed in some populations of animals and plants (Thornhill, 1993; Husband & Schemske, 1996). Despite its practical application in conservation biology and evolutionary theory, the cost of inbreeding in natural populations of plants and animals remains to a large degree unknown (Crnokrak & Roff 1999).

The effects of inbreeding depression will begin within a few generations of a decline, and depend primarily on the magnitude of the drop in population size. Consequently, loss of fitness occurs when homozygosity is increased by inbreeding, not only individuals but entire populations can be affected (Amos and Balmford, 2001). The result is reduced fecundity and reduced survival and the population almost immediately decline due to overall increase in relatedness between individuals (Ralls *et al.*, 1970; Saccheri *et al.*, 1996; Newman & Pilson, 1997; Keller, 1998; Crnokrak & Roff, 1999; Hedrick & Kalinowski, 2000). Although fecun-

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dity drops to $\approx 20\%$ of butterfly *Bicyclus anynana* often returns to normal levels within a few generations of sustained inbreeding presumably as a result of the purging of deleterious alleles (Saccheri *et al.*, 1996).

Population bottlenecks (population size constrictions) are an important cause of losses of genetic variation and increased inbreeding. It is worth noticing that inbreeding in itself does not reduce genetic variability in terms of allelic diversity. It merely causes a change in the distribution of alleles by increasing homozygosity and decreasing heterozygosity. Alleles are not lost in this process, but since inbreeding is important only in small populations the effects of genetic drift and inbreeding often coincide. Small isolated populations are subject to genetic drift, which will affect their evolutionary potential, through fixation of deleterious mutations (Frankham 1995; Allendorf & Ryman 2002; Wright 1977; Madsen *et al.*, 1996; Frankham & Ralls 1998; Saccheri *et al.*, 1998; Higgins & Lynch 2001). Inbreeding caused by bottlenecks reduces fitness of domestic, captive and wild plants and animals. Furthermore, it increases the risk of extinction in captive populations, and there is now strong evidence that it is one of the factors causing extinctions of wild populations as well (Frankham 2003, Frankham *et al.*, 2002).

The immediate effect of intensive inbreeding is a loss of fitness through lowered fertility, higher juvenile mortality, depressed growth, etc. Based on experiences with breeding of domestic mammals, it has been suggested that a minimum of 50 effective breeding individuals is needed to keep inbreeding depression to a negligible level of below 1% inbreeding per generation (Franklin 1980). Estimating the large numbers of individuals in natural populations is difficult by the problems of establishing multigeneration pedigrees for the highly polygynous red deer (Coulson *et al.*, 1999). In a polygynous species, a single, highly successful male who carries a rare allele at a particular locus may produce large numbers of descendants, many of whom are both successful and heterozygous at this locus (Balloux *et al.*, 2004).

Within a subspecies, i.e. a genetically homogeneous group of populations, transfer of individuals between populations is recommended to reduce the degree of inbreeding (Balakrishnan *et al.*, 2003). There is, however, also a natural way by which the consequences of inbreeding may be alleviated: recessive deleterious alleles, once they are in a homozygous state, may be removed (purged) by natural selection in populations undergoing inbreeding. Inbreeding may potentially be reduced, or purged, by breeding related individuals. Purging may slow the rate of extinction but its effects are most likely to be relevant only when populations are extremely small or the difference between inbreeding rates very large (Reed *et al.*, 2003; Byers and Waller 1999).

I. IV. II. Habitat fragmentation

Habitat is defined “the resources & conceptions present in an area that produce occupancy including survival & reproduction by a given organism” (Hall, *et al.*, 1997). It consists of the effects of parental material, soil, slope, elevation, local hydrologic regime and events that have value as elements of habitat (Stromberg, 1995, Coulombe, 1977). A good habitat is mainly governed by the availability of abundant fodder and water sources in association with shelters for the survival of the species (Bhowmik, *et al.* 1999). Today, most of the forests are diminishing steadily and have become fragmented frequently throughout the world by the result of human actions. Additionally habitat fragmentation is one of the most serious environmental threats the plant and animal species.

Habitat quality and geographic location of the forests are the significant factors which influence the abundance of wild animal populations. In particular, wild populations of large mammals are exposed to the consequences of large-scale habitat fragmentation in the wake of human activities such as agricultural practices, industrial development, rural decline and extension of urban areas which are changing landscapes across the globe. Loss of habitat is currently the most important factor causing loss of biodiversity although global climate change due to pollution may overtake it in the future (Frankham 1999). Habitat fragmentation and loss results in small populations which are at risk of losing genetic variability because of stochastic demographic, environmental and genetic processes (drift effects) that may contribute to the endangering of fragmented populations (Shaffer 1981, 1987; Wan *et al.*, 2004; Brussard & Gilpin 1989; Srikwan & Woodruff 2000; Coulon *et al.*, 2004). Because the evolutionary potential is clearly related to population size the flexibility to react to environmental changes is reduced in small isolated populations (Frankham *et al.*, 2002; Frankham 1999).

Isolation of populations and small population sizes should chiefly result in increased genetic drift and inbreeding and a reduction of allelic variation within populations and in increased genetic differentiation among populations. Changes in allele frequencies are governed by genetic drift rather than by processes of local adaptation (e. g., Kimura 1983; Wright 1931; Nei 1975). Forest fragmentation can have a dramatic effect on landscape connectivity and the dispersal of animals, potentially reducing gene flow (i.e., dispersal). Since gene flow plays a major role in determining the genetic structure of populations and the level of genetic variability (Avisé 1994; Coulon *et al.*, 2004; Slatkin 1987) and since the impacts of population fragmentation on genetic diversity, differentiation, inbreeding and extinction risk depend on the level of gene flow among fragments (Frankham *et al.*, 2004) habitat fragmentation, if severe enough, is capable of causing a deleterious increase in genetic subdivision among popu-

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lations. The high density of human settlements in Europe, especially Central Europe, is a serious problem for the conservation of wild places, and for the use of natural renewable resources (Zunino 1995).

Deer are particularly dependent on forest and open woodland habitats. Many natural populations are threatened not only by a dramatic reduction in total area of available habitat but also by increasing habitat fragmentation. Reduction and fragmentation of habitat components may result in increasing intraspecific competition, which in turn may lower reproduction rates and eventually population's sizes. For this reason, the red deer has been classified as "near threatened" in the latest Red List of Schleswig-Holstein, northern Germany (see below). It is noteworthy that the genetic threats due to fragmentation, drift and inbreeding, and not census sizes of populations were the basis for this classification.

Although many wildlife species have been able to adapt to the drastic changes that man has caused in the environment (Klein, 1991) understanding genetic impacts of habitat fragmentation is important for managing long-term genetic variability of populations (Mech, 2000). Furthermore, knowledge and management of fragmented populations are important for the long-term survival of animal populations. Nucleotide diversity was lower in an isolated population of Siberian Jay (*Perisoreus infaustus*) than in populations within its contiguous area of distribution, which is a fine example of isolation by anthropogenic habitat fragmentation reducing gene flow (Uimaniemi *et al.*, 2000). Also, habitat fragmentation due to natural processes or human activities, together with restocking using distantly related source groups, usually results in divergence among local populations (Vernesi *et al.*, 2002).

Conserving genetic variation has been a major focus of recovery efforts for many endangered species. Retaining variation for adaptation to environmental change is of great concern, particularly because many taxa are in recently altered habitats and exposed to new biological threats, including predators, competitors and pathogens (Hedrick 2004). In an isolated population of moderate size, individual viability and fecundity are expected to decrease until population extinction because of the fixation of deleterious mutations (Lande, 1995). To maintain high levels of genetic variation in species in fragmented habitats, conservation should aim at securing connectivity between spatially distinct populations (Segelbacher *et al.*, 2003). Furthermore, small and isolated populations are prone to extinction due to environmental stochasticity (e.g., natural catastrophes and diseases), demographic stochasticity (i.e., random variations in sex ratio, mortality or reproduction), genetic effects (e.g., mutation accumulation and inbreeding), and too little immigration (Dale 2001).

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Since the study of the genetic consequences of population fragmentation is a major tenet of conservation biology; our study on red deer from Schleswig-Holstein in northern Germany was designed to examine genetic variability and genetic distances within and among populations that were isolated from each other recently by habitat fragmentation in the wake of motorway constructions. These populations are very similar to populations on islands and many aspects of island biology and ecology hold for them as well. Island populations of vertebrates are more prone to extinction and typically have less genetic diversity and are more inbred than mainland population (Frankham *et al.*, 2002; Frankham 1997; 1998). Among all extant European ungulate species, the red deer (*Cervus elaphus*) is probably the one most strongly affected by anthropogenic influences (Hartl *et al.*, 1995, 2003).

I. V. COMPARISON OF DIFFERENT MARKERS AND GENETIC DIFFERENTIATION IN ROE DEER

Genetics may contribute to the extinction proneness of island populations. And the level of genetic polymorphism tends to be lower in island populations than in mainland populations (Frankham 1997), which is probably a result of genetic drift (Côte *et al.*, 2002). Notably, endemic island populations that are more prone to extinction than non-endemic populations have lower genetic diversity and higher inbreeding levels than non-endemic populations (Frankham 1999). The knowledge of how genetic variation is partitioned among populations may have important implications not only for evolutionary biology and ecology, but also for conservation biology (Balloux and Lugon-Moulin 2002). The extent and pattern of molecular variation within a population is generally consistent with neutrality, i.e. there is a balance predicted by a reduction in variation from genetic drift and an increase in variation from mutation (Kimura, 1983; Nei 1987). Roe deer are common throughout Europe and among the most important game species in Germany. Management of German roe deer (*Capreolus capreolus*) populations is a challenge for wildlife managers and foresters because population densities are difficult to estimate in forests and forest regeneration can be negatively affected when roe deer density high (Radeloff *et al.*, 1999).

The following study is aimed at uncovering genetic variability within small-scale differentiation among roe deer populations from northern Germany. At the same time, since these questions are addressed using three different marker systems, the resolving capacity of allozymes, microsatellites and sequences of the mitochondrial control region are compared. This is important because in most studies it is only one or two markers that are used, and conclusions drawn from one marker do not a priori have to match those drawn from others. In

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this respect, the goal of the present roe deer study is not only zoological but also methodological in that it contributes to our understanding of the dependence of population genetic studies upon the markers chosen.

STUDY A :

Conservation genetics of the endangered red deer from Sardinia and Mesola with further remarks on the phylogeography of *Cervus elaphus corsicanus*

1. 1. INTRODUCTION

The European red deer (*Cervus elaphus* Linnaeus, 1758) is not an endangered species in terms of absolute census size of its stocks - there are still about one million free-living head in Europe (Bützler, 1986). However, due to human activities, red deer populations have locally become increasingly isolated and mixed with foreign stocks as a consequence of translocations (Hartl, Zachos & Nadlinger, 2003). The red deer from Sardinia and Mesola are special in that they represent endangered unique populations. The Sardinian, or Corsican, red deer (*Cervus elaphus corsicanus* Erxleben, 1777), inhabits the Tyrrhenian islands Sardinia and Corsica and, with temporarily only 100-150 individuals remaining (Krumbiegel, 1982, Lovari, pers. comm.), was regarded as one of the most threatened mammal taxa in the world (Dolan, 1988). It is listed as endangered by the IUCN (Baille & Groombridge, 1996), but fortunately, it has recovered over the last decade in terms of population size, numbering 2000-2300 individuals in 1998 (Mattioli *et al.*, 2001, Lovari, pers. comm.). It is also the smallest of red deer with a maximum weight of 70-80 kg in females and 105-120 kg in males and a shoulder height of 80-100 cm (Beccu, 1989, Dolan, 1988). The Corsican population became extinct in 1970 and was refounded with nine individuals from Sardinia (Dolan, 1988).

The population from the „Gran Bosco della Mesola“ reserve, located in the Po delta area in Italy, is even smaller with only 67 animals remaining in 1999 (Mattioli *et al.*, 2003). It is the only truly autochthonous red deer population of the Italian peninsula (cf. Mattioli, 1990, who gives a detailed historical review of the Mesola population) – all other Italian populations were either founded by humans through reintroduction or by natural immigration from neighbouring countries after indigenous stocks had died out. The census size of the Mesola population dropped from 250-300 in the 1930s to about 10 after the Second World War (Mattioli, 1990). In 1980, there were again 120 individuals, but then population size continuously declined to 59 animals in 1998 with an average effective population size (N_e) of about 15 individuals (Lorenzini, Mattioli & Fico, 1998). Accordingly, Lorenzini *et al.* calculated an increase in inbreeding of 3.3% per generation - a value more than three times higher than the threshold of 1% up to which natural selection can overpower the fixation of deleterious alleles

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(Franklin, 1980, Soulé, 1980). A previous study based on allozymes (Lorenzini *et al.*, 1998) found genetic variability of the Mesola population to be low but still within the range reported for other European red deer populations. Results from an analysis of mitochondrial restriction fragment length polymorphisms (RFLPs, Lorenzini, Fico & Mattioli, 2005) yielded no variation at all for the Mesola population. The Mesola red deer exhibits a simplified antler structure, low birth rates (about 50% of the species average) and high juvenile mortality (Mattioli, 1990, 1993). It represents a „maintenance phenotype“ (sensu Geist, 1987) due to environmental constraints of an unproductive habitat with scarce food supply (Lorenzini *et al.*, 1998).

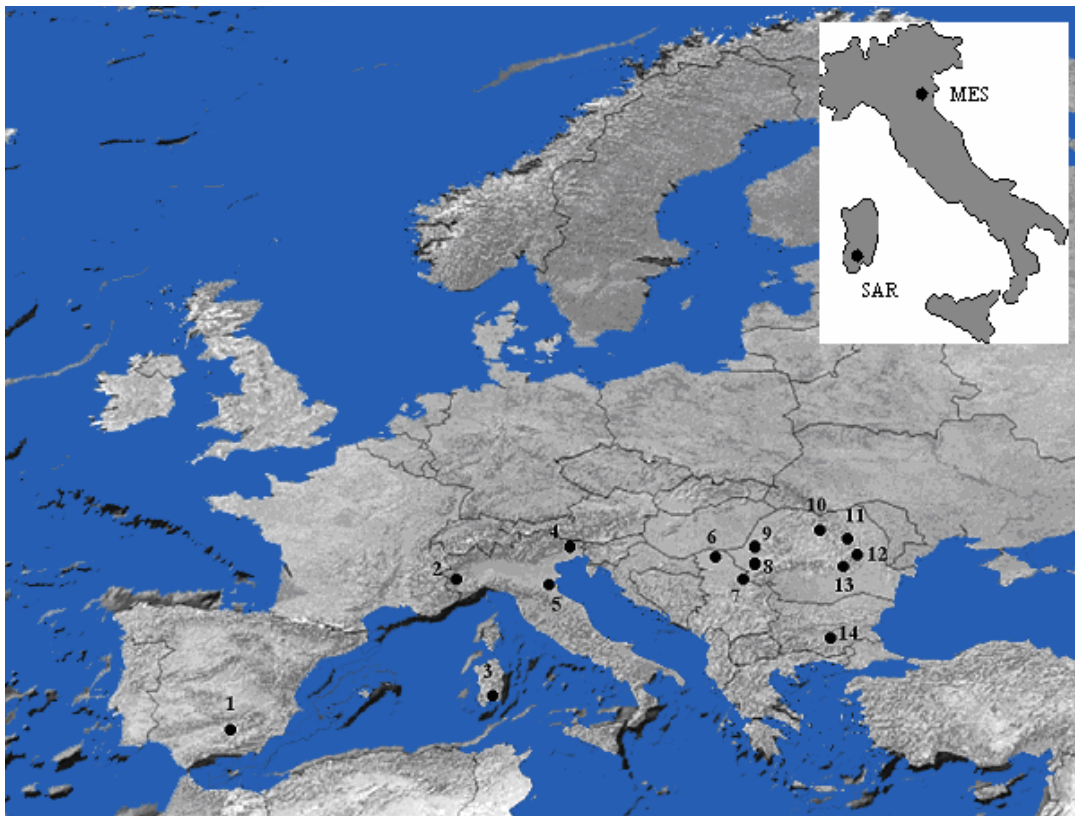


Figure 1. 1. Geographic map with the location of the red deer populations studied. 1 = SSP, 2 = VDS, 3 = Sardinia, 4 = TAR, 5 = Mesola, 6 = BAC, 7 = BAN, 8 = TEM, 9 = ARA, 10 = REG, 11 = HAR, 12 = COV, 13 = BRA, 14 = BUL. For abbreviations, see text.

A previous analysis of *C. e. corsicanus* yielded low genetic variability but was unable to resolve the phylogeographic origin of this island population (Zachos *et al.* 2003). Lorenzini *et al.* (2005) found two mitochondrial RFLP haplotypes. It is still an open question if red deer reached Corsica and Sardinia naturally in the Upper Pleistocene or, later, through human introduction. The latter hypothesis was already mentioned by Lydekker (1898), and Groves holds that the Corsican red deer are derived from African Barbary stags (*C. e. barbarus*)

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which are supposed to have been shipped to the Tyrrhenian islands by the Phoenicians (pers. comm. to Geist, 1998). Flerov (1952) regarded Spanish red deer (*C. e. hispanicus*), Barbary stag and Corsican red deer as closely related. The corsicanus-barbarus relationship has previously been corroborated by a cytochrome b analysis (Ludt *et al.*, 2004). Hartl *et al.* (1995), on the other hand, found mitochondrial RFLP similarities between Sardinian and Bulgarian red deer, possibly indicating an origin from the Near East, which would be in line with Hilzheimer's view that *C. e. corsicanus* is closely related with the Caucasian red deer or maral (*C. e. maral*) (cf. Krumbiegel, 1982). This latter assumption, however, is based on antler traits, which are of dubious value in phylogenetic inference due to their susceptibility to environmental influences (Geist, 1998).

Finally, Zachos *et al.* (2003), in an analysis of south European red deer populations, found the closest microsatellite relationships between the Sardinian population and deer from northern Italy belonging to the central European *C. e. hippelaphus*. This result, although unconfirmed by sequence data of the mitochondrial control region (which interestingly showed close relationships between *C. e. corsicanus* and *C. e. hispanicus*), suggested that red deer might have reached Corsica and Sardinia via mainland Italy during the maximum of the last glaciations (22-18,000 years before present) when the sea level was considerably lower. However, since no indigenous Italian red deer, which should be closer to the Sardinian red deer than the introduced deer from northern Italy, were included in the analysis, the results were only preliminary. In the present study, we analysed the genetic variability of the two endangered populations from Sardinia and Mesola at twelve microsatellite loci and 531 bp of the mitochondrial control region and also addressed the question of the phylogeographic origin of *C. e. corsicanus* by comparing Sardinia with Mesola and with other European populations analysed in two previous studies (Feulner *et al.*, 2004, Zachos *et al.*, 2003). Microsatellites are highly variable repetitive DNA sequences with repeat units of up to 6 base pairs. Different alleles differ in the number of repeat units and hence in length. The mitochondrial control region, also known as the D-loop, is a hypervariable part of the mitochondrial genome. Both marker systems are known to be powerful tools in population genetics and have already been widely used in red deer and other cervids (e. g. Feulner *et al.*, 2004, Goodman *et al.*, 2001, Hundertmark *et al.*, 2002, Kuehn *et al.*, 2003, Lorenzini, Lovari & Masseti, 2002, Lorenzini *et al.*, 2003, Nies, Zachos & Hartl, 2005, Zachos *et al.*, 2003).

1. 2. MATERIAL & METHODS

1. 2. 1. Sampling and DNA extraction

The study is based on 29 red deer from Sardinia and 28 from Mesola (Figure 1.1). Blood samples were collected by jugular puncture from live-caught animals between 1995 and 1998 (Mesola) and between 2000 and 2003 (Sardinia). DNA was extracted by standard methods of proteinase-K digestion, phenol-chloroform extraction and precipitation in ethanol (Sambrook and Russel, 2001). Apart from the conservation genetic analysis of the deer from Sardinia and Mesola the question of the phylogeographic origin of *C. e. corsicanus* was addressed by including data from all the populations studied by Zachos *et al.* (2003) and Feulner *et al.* (2004) except for one population from a fenced area in Banat, Serbia, which was excluded due to its semi-captive status. The combined data set then comprised 231 red deer from Sardinia (SAR), Mesola (MES), L. Navas in southern Spain (SSP), non-indigenous Italian mainland stocks (Val di Susa = VDS and Tarvis = TAR), Bulgaria (BUL), Serbia (Bachka = BAC and Banat = BAN) and Romania (Arad = ARA, Temishvar = TEM and four Carpathian populations: Reghin = REG, Harghita = HAR, Covasna = COV and Brashov = BRA). For sample sizes, see Table 1. 3. The mitochondrial control region of one single specimen from Karansebes, Romania, was also sequenced by Feulner *et al.*, but since its haplotype was identical to one found in Arad, Karansebes was not further considered in the present study. For the phylogenetic haplotype analysis, the sequences taken from Randi *et al.* (2001, accession numbers AF291885-9) that had already been used in the calculations of Feulner *et al.* were also included in the combined data set.

1. 2. 2. Mitochondrial DNA

PCR amplification of the mitochondrial control region was performed with the primers Pro-L and Phe-Hb (cf. Zachos *et al.*, 2003) under the following conditions: 40 cycles of 75s at 94°C, 90s at 59°C and 75s at 72°C, preceded by a 5 min initial denaturation at 95°C and followed by a 7 min terminal elongation at 72°C. PCR products were sequenced with an automatic sequencer (ABI 377) using the forward primer Pro-L. Sequences were aligned with BioEdit version 5.0.9 (Hall, 1999). Haplotype and nucleotide diversities within as well as net nucleotide diversities among populations (Nei, 1987) were calculated using DnaSP3.51 (Rozas & Rozas, 1999). The combined phylogenetic analysis was conducted on the basis of 332 bp because this was the length of the sequences analysed by Zachos *et al.* (2003). Trees were

reconstructed with the neighbor-joining (based on Kimura-2-parameter distances), maximum likelihood and maximum parsimony programmes of the PHYLIP package (Felsenstein, 1993) with *Cervus alfredi*, a close relative of *C. elaphus*, as outgroup (<http://www.ncbi.nlm.nih.gov/>, accession number AF291891, Randi *et al.*, 2001). Node support was assessed by bootstrap resampling with 100 (maximum likelihood) or 1000 (neighbor-joining and maximum parsimony) replicates. The overall fixation index F_{ST} , yielding the proportion of the total genetic variation accounted for by the differentiation among (as opposed to variation within) populations, and pairwise F_{ST} -values as a measure of population differentiation were calculated with Arlequin (Schneider, Roessli & Excoffier, 2000) taking into account not only haplotype frequencies but also the differences between them (estimated by Kimura-2-parameters). According to the intrinsic transition-transversion ratio of the combined data set, transversions were weighted 4.86-fold over transitions. Since some of the statistical programmes do not consider deletions they were treated as transversions in all analyses in order not to lose informative sites.

1. 2. 3. Microsatellites

Microsatellite amplification was carried out as described in Zachos *et al.* (2003). The twelve loci studied were: OarCP26 (Ede, Pierson & Crawford, 1995), OarFCB304 (Buchanan & Crawford, 1993), MAF35 (Swarbrick, Buchanan & Crawford, 1991), MAF109 (Swarbrick & Crawford, 1992), INRA11 (Vaiman *et al.*, 1992), BM888, BM4208, BM4513 (Bishop *et al.*, 1994), NVHRT16, NVHRT21 (Røed & Midthjell, 1998), RT1 and RT7 (Wilson *et al.*, 1997). The loci were tested for linkage disequilibrium with GENEPOP (Raymond & Rousset 1995). Observed and expected heterozygosities were calculated with Arlequin. Significance of deviations from Hardy-Weinberg-frequencies was calculated with Arlequin (single loci) and FSTAT (Goudet, 1995, over all loci). Allelic diversity was calculated as the mean number of alleles per locus for Sardinia and Mesola. In the combined study, which also comprised the populations studied by Zachos *et al.* (2003) and Feulner *et al.* (2004), we computed values of allelic richness with the FSTAT software instead of allelic diversity because sample sizes varied considerably among the populations studied. Contrary to allelic diversity, allelic richness calculations yield a diversity measure independent of sample sizes. Since FSTAT adjusts measures of alleles per locus to the smallest number of individuals typed for a locus in a sample, the Harghita population from Feulner *et al.* (2004) was excluded from this analysis because one locus failed to be amplified in many of its individuals. The number of alleles shared by Sardinia and only one other population was counted, and the proportions of Sardinian al-

leles present in the other populations were calculated. F_{ST} -values (overall and pairwise) for the combined analysis were calculated using Arlequin. Population differentiation was further quantified with Nei's (1972) standard distance D , Cavalli-Sforza & Edwards' (1967) chord distance (both computed with the PHYLIP software) and with the proportion-of-shared-alleles-distance D_{ps} (Bowcock *et al.*, 1994), calculated as $(1-D_{ps})$ with the programme MICROSAT (Minch *et al.*, 1995). D_{ps} is based on the sums of allele frequencies shared between populations and has been shown to be especially capable of resolving relationships of closely related taxa with microsatellite data (Cornuet *et al.*, 1999). The distance values were used for tree reconstruction with the neighbor-joining algorithm as implemented in PHYLIP. Support for nodes was assessed by 1000 bootstrap replicates. All calculations including the data from Zachos *et al.* (2003) and Feulner *et al.* (2004) were carried out on the basis of the seven loci analysed by Zachos *et al.* (OarCP26, OarFCB304, MAF35, MAF109, INRA11, BM888 and BM4513).

1. 3. RESULTS

1. 3. 1. Sardinia and Mesola

Sequencing of 531 bp of the mitochondrial control region of 27 Sardinian and 28 Mesola red deer yielded 24 variable sites (21 transitions, one transversion and two deletions) defining nine putative haplotypes (Table 1. 1). One was confined to Mesola and was exhibited by all analysed specimens, the other eight were Sardinian haplotypes and occurred between one and six times (Table 1. 1). The according values of haplotype and nucleotide diversity were 0.872 and 0.473, respectively, for Sardinia and zero for Mesola (Table 1. 2). The Sardinian haplotypes differed in one to five sites among each other with haplotype SAR 8 being the most divergent one (separated from the remaining haplotypes by three to five mutations).

The twelve microsatellite loci analysed were all polymorphic and yielded between 6 and 15 alleles for the 57 individuals genotyped (29 from Sardinia and 28 from Mesola). Bonferroni-corrected p-values showed no linkage disequilibrium between any two of them at the 5%-level and consequently, they were all included in the multi-locus analysis. Both populations showed similar variability in terms of heterozygosity and allelic diversity (Table 1. 2). Even after Bonferroni-correction, both populations exhibited significant (5%-level) deviations from Hardy-Weinberg equilibrium at five single loci (Sardinia at OarCP26, BM4208,

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NVHRT21, RT1 and RT7, Mesola at INRA11, BM4208, NVHRT16, NVHRT21 and RT7) due to an excess of homozygotes as well as over all loci combined.

Table 1. 1. Mitochondrial haplotypes found in this study. Numbers (vertical) refer to aligned sites in the 531 bp data set (only variable sites are shown). Dots indicate identity with the haplotype from Mesola (MES 1), dashes denote deletions. Numbers in parentheses refer to the absolute number of individuals showing the respective haplotype.

Haplotype	0	0	0	0	0	0	0	1	1	1	1	2	2	2	2	2	3	3	3	3	3	3	3	4	4
	2	3	4	4	6	6	8	0	2	7	7	2	4	4	4	8	5	5	5	5	7	7	7	7	7
	8	0	5	9	0	7	3	4	5	7	9	6	2	4	6	6	1	2	4	5	2	3	3	8	8
MES 1 (28)	C	T	-	T	C	A	C	G	C	T	C	G	A	T	C	A	G	A	C	T	A	A	A	A	A
SAR 1 (2)	T	C	C	C	T	G	T	A	T	C	•	A	G	C	T	G	A	G	T	C	•	•	-	C	
SAR 2 (6)	T	C	C	C	T	G	T	A	T	C	•	A	G	C	T	G	A	G	T	C	•	•	•	•	
SAR 3 (5)	T	C	C	C	T	G	T	•	T	C	•	A	G	C	T	•	A	G	T	C	•	•	•	•	
SAR 4 (5)	T	C	C	C	T	G	T	A	T	C	T	A	G	C	T	G	A	G	T	C	•	•	-	•	
SAR 5 (4)	T	C	C	C	T	G	T	A	T	C	T	A	G	C	T	G	A	G	T	C	•	•	-	C	
SAR 6 (3)	T	C	C	C	T	G	T	•	T	C	•	A	G	C	T	•	A	G	T	C	•	•	-	C	
SAR 7 (1)	T	C	C	C	T	G	T	A	T	C	•	A	G	C	T	•	A	G	T	C	•	•	-	C	
SAR 8 (1)	T	C	C	C	T	G	T	A	T	C	•	A	G	C	T	•	A	G	T	C	G	G	-	•	

1. 3. 2. Combined study

The data on genetic variability of the combined population set are summarized in Table 1. 3. Mesola, together with Banat in Serbia, which also exhibited only one mitochondrial haplotype, showed the lowest mitochondrial variation, i. e. none at all. Sardinia, on the other hand, showed comparatively high haplotype diversity but a rather low value for nucleotide diversity. This latter parameter takes into account not only the number of different haplotypes (as does haplotype diversity) but also their degree of similarity. The data thus reflect the fact that there is a relatively high number of similar haplotypes present in the Sardinian population.

In terms of microsatellite variation Sardinia and Mesola exhibited the lowest values of all populations studied – regarding both allelic richness and heterozygosity. Allelic richness of Sardinia and Mesola was 3.6 and 3.3, respectively, while the other populations showed

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values between 4.8 and 6.2. Expected heterozygosity, which is usually preferred to observed heterozygosity as a measure of variability due to its being less sensitive to sample size, was much lower for Sardinia and Mesola than for any other population including Banat (0.60 and 0.53 vs. values between 0.80 and 0.90).

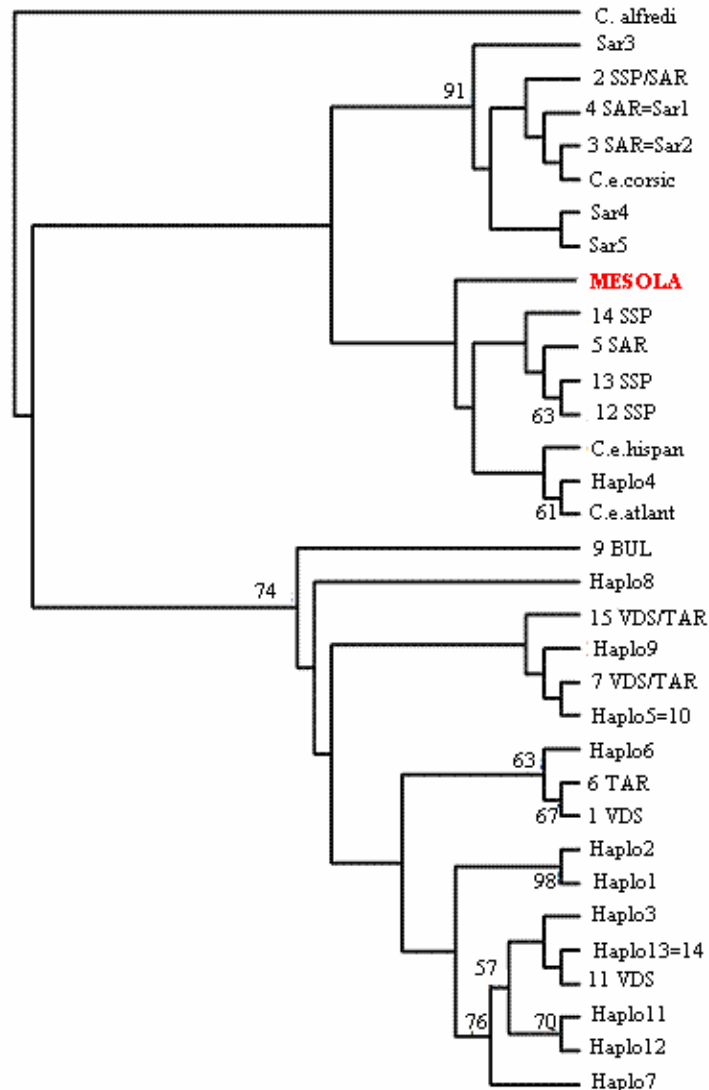


Figure 1. 2. Maximum-likelihood consensus tree of the haplotypes found in this study (Sar 1-5, MESOLA, reduced to 332 bp) combined with red deer sequences from the literature. Haplotypes represented by three capital letters are taken from Zachos *et al.* (2003). The sequences denoted *Cervus alfredi* (outgroup), *C. e. corsic [anus]* (Sardinia), *C. e. hispan[icus]* (Spain) and *C. e. atlant[icus]* (Norway) are taken from Randi *et al.* (2001, *C. hippelaphus* identical to Haplo5=10). Numbers at nodes denote support from 100 bootstrap replications (only values above 50% are shown). Apart from the position of the Mesola haplotype the topology of this tree is consistent with the results gained from other tree reconstruction algorithms (neighbor-joining, maximum parsimony). For details, see text.

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Overall F_{ST} -values were 0.852 for mtDNA and 0.147 for microsatellites (both being significantly different from zero, $p < 0.00001$), indicating that 85% of the total variation were among populations for mitochondrial DNA sequences and 15% for microsatellites. Pairwise F_{ST} -values (not shown in detail) showed both Sardinia and Mesola to be significantly differentiated from all other populations even after Bonferroni correction ($p < 0.00001$ for both mtDNA and microsatellites). Further estimates of pairwise population differentiation are presented in Tables 1. 4 and 1. 5. Net nucleotide diversities (Table 1. 4) are highest for the Sardinian population (mean 0.044, SD 0.00632) and apart from the Spanish deer, with which they share one haplotype (cf. Zachos *et al.*, 2003), the Sardinian red deer are closest to the Mesola population. This result is in line with the genetic distance measures based on microsatellites shown in Table 1. 5 and the standard distances (not shown in detail): the distances between Sardinia and Mesola are lower than for Sardinia or Mesola and any other population studied including Spain. When we counted the alleles exclusively shared by Sardinia and only one other population we found that Temishvar, Bachka and Val di Susa each had one such allele in common with Sardinia while Mesola had three and all other populations had none.

Table 1. 2. Parameters of genetic variability for red deer from Sardinia and Mesola. N is the sample size for mitochondrial and (in parentheses) microsatellite analyses. H_E , H_O and AD are expected and observed heterozygosity and allelic diversity averaged over all twelve microsatellite loci. HD and π are haplotype and nucleotide diversity based upon 531 bp of the mitochondrial control region.

Pop	N	H_E	H_O	AD	HD	$\pi(\%)$
SAR	27(29)	0.66	0.48	6.4	0.872	0.473
MES	28(28)	0.62	0.51	7	0	0

Twenty-five of the 51 alleles found in Sardinia were also present in Mesola, which, together with Bachka, was the highest proportion of Sardinian alleles (49%) found in the study area. Most of the other populations showed values between 31% and 37%. Spain and Tarvis had the smallest proportions (24% and 25%, respectively). The only population with a proportion higher than 37%, apart from Mesola and Bachka, was Arad with 43%.

Due to the reduction to 332 bp in the combined data set, the number of Sardinian haplotypes was reduced from eight to five in this study. The phylogenetic haplotype analysis

yielded three different clades (Fig. 1. 2): one comprising haplotypes from Sardinia and Spain only, one combining haplotypes from Sardinia, Spain and Norway as well as the divergent haplotype 4 from the Carpathians (for details, see Feulner *et al.*, 2004) and a third one comprising all the other haplotypes. While the first and the third clade were supported by high bootstrap values in all trees (between 74% and 97%), the second one only showed values of 32% to 42%. As to the position of the Mesola haplotype, our results were not consistent: maximum-parsimony and neighbor-joining algorithms (not shown) paired it with the haplotypes from Serbia, Romania, Bulgaria and the other Italian mainland haplotypes in clade three whereas the maximum likelihood analysis placed it in the Sardinia-Spain-Norway clade (Fig.1. 2). However, in both cases bootstrap support was below 50%.

Table 1. 3. Parameters of genetic variability for the extended set of populations. N is the sample size for mitochondrial and (in parentheses) microsatellite analyses. H_E , H_O and AR are expected and observed heterozygosity and allelic richness averaged over seven microsatellite loci. HD and π are haplotype and nucleotide diversity based upon 332 bp of the mitochondrial control region. Harghita (HAR) was excluded from the calculation of allelic richness (see text for explanation).

Pop	N	H_E	H_O	AR	HD	$\pi(\%)$
SAR	44(38)	0.60	0.48	3.6	0.719	0.541
MES	28(28)	0.53	0.45	3.3	0.000	0.000
BAC	33(30)	0.82	0.53	5.5	0.061	0.081
BAN	13(13)	0.80	0.52	5.1	0.000	0.000
TEM	10(10)	0.90	0.57	6.2	0.511	0.937
ARA	15(12)	0.89	0.5	6.0	0.79	1.228
BRA	12(10)	0.86	0.54	5.6	0.667	1.187
COV	8(8)	0.87	0.55	5.2	0.464	0.656
HAR	11(11)	0.88	0.59	----	0.327	0.493
REG	9(9)	0.87	0.51	5.7	0.222	0.803
SSP	12(13)	0.81	0.65	4.8	0.652	1.292
TAR	8(9)	0.83	0.71	5.4	0.75	0.645
BUL	7(7)	0.85	0.76	5.3	0.667	0.975
VDS	19(16)	0.81	0.69	5.2	0.591	0.726

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Table 1. 4. Net nucleotide diversities between populations as a pairwise distance measure based on 332 bp of the mitochondrial control region.

	SAR	MES	BAC	BAN	TEM	ARA	BRA	COV	HAR	REG	SSP	TAR	BUL	VDS
SAR	---													
MES	0.036	---												
BAC	0.053	0.042	---											
BAN	0.053	0.042	0.000	---										
TEM	0.049	0.032	0.014	0.014	---									
ARA	0.045	0.029	0.008	0.008	0.005	---								
BRA	0.043	0.028	0.008	0.008	0.006	0.003	---							
COV	0.045	0.028	0.013	0.013	0.007	0.005	0.000	---						
HAR	0.047	0.029	0.016	0.016	0.005	0.006	0.002	0.000	---					
REG	0.044	0.026	0.017	0.017	0.009	0.007	0.002	0.000	0.000	---				
SSP	0.030	0.030	0.045	0.046	0.037	0.035	0.033	0.036	0.037	0.033	---			
TAR	0.045	0.027	0.015	0.015	0.006	0.003	0.004	0.003	0.004	0.004	0.035	---		
BUL	0.040	0.025	0.008	0.008	0.005	0.003	0.002	0.003	0.004	0.005	0.035	0.004	---	
VDS	0.046	0.030	0.011	0.011	0.007	0.004	0.003	0.003	0.005	0.005	0.037	0.001	0.003	---

Sardinian and Mesola red deer

Table 1. 5. 1-Dps (below diagonal) and Cavalli-Sforza and Edwards' chord (above diagonal) distances between populations based upon allele frequencies at seven polymorphic microsatellite loci.

	SAR	MES	BAC	BAN	TEM	ARA	BRA	COV	HAR	REG	SSP	TAR	BUL	VDS
SAR	---	0.082	0.095	0.106	0.098	0.097	0.102	0.115	0.096	0.111	0.125	0.106	0.102	0.114
MES	0.988	---	0.095	0.112	0.105	0.096	0.090	0.111	0.107	0.111	0.129	0.115	0.122	0.121
BAC	1.276	1.303	---	0.065	0.060	0.060	0.065	0.080	0.074	0.069	0.085	0.069	0.064	0.065
BAN	1.649	1.426	0.777	---	0.072	0.083	0.079	0.082	0.074	0.080	0.100	0.073	0.076	0.066
TEM	1.314	1.614	0.818	0.804	---	0.053	0.069	0.086	0.073	0.079	0.092	0.068	0.078	0.083
ARA	1.439	1.570	0.844	0.893	0.615	---	0.068	0.086	0.087	0.070	0.101	0.065	0.073	0.082
BRA	1.456	1.254	0.862	0.990	0.873	0.839	---	0.051	0.053	0.046	0.092	0.077	0.081	0.091
COV	1.694	1.678	1.121	1.045	1.187	1.139	0.705	---	0.054	0.062	0.100	0.093	0.085	0.095
HAR	1.269	1.520	0.958	1.070	0.848	1.014	0.711	0.664	---	0.046	0.104	0.075	0.071	0.085
REG	1.623	1.687	0.892	0.984	0.901	0.818	0.607	0.670	0.579	---	0.091	0.067	0.081	0.088
SSP	2.061	2.403	1.133	1.277	1.293	1.403	1.176	1.300	1.501	1.335	---	0.091	0.091	0.085
TAR	1.412	1.669	0.807	0.772	0.820	0.811	0.897	1.050	0.907	0.813	1.306	---	0.081	0.069
BUL	1.493	2.029	0.888	0.901	1.006	0.914	1.021	0.978	0.895	1.005	1.115	1.023	---	0.067
VDS	1.864	1.740	0.964	0.614	1.035	1.058	1.223	1.217	1.189	1.127	1.135	0.862	0.917	-----

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The neighbor-joining trees (standard, chord and shared alleles distances) based on seven microsatellite loci all yielded very similar topologies. Most importantly, they all favoured a Sardinia-Mesola clade with high bootstrap support (Nei's D 85%, Dps 91% and chord distances 99%, cf. Fig. 1. 3). Contrary to the mitochondrial trees but in accordance with the microsatellite results of Zachos *et al.* (2003), the Spanish red deer are clustered with Val di Susa and Bulgaria in the Nei and chord distance trees, but they form a clade with Sardinia and Mesola in the Dps tree. The four Carpathian populations (Reghin, Harghita, Covasna, and Brashov) clustered together in all trees as did the two populations from the Romanian lowland (Temesvar and Arad). The only tree to reflect the close relationship of the Serbian red deer from Bachka and Banat found by Feulner *et al.* (2004) was the one based on the chord distances.

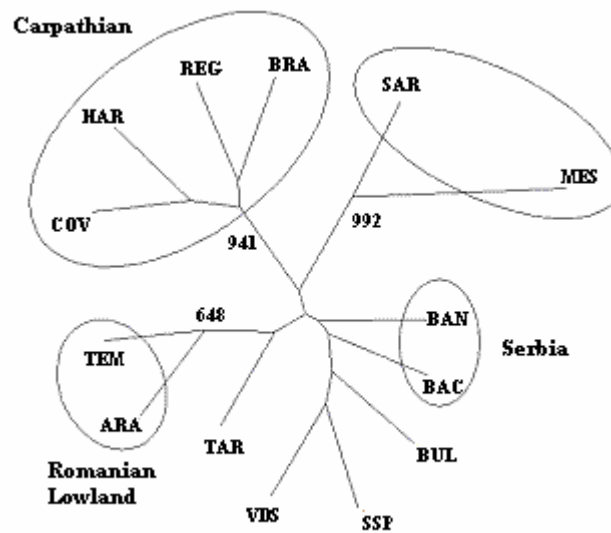


Figure 1. 3. Neighbor-joining tree based on chord distances derived from allele frequencies at seven microsatellite loci. Numbers at nodes denote support from 1000 bootstrap replications (only values above 50% are shown). Sardinia and Mesola are paired with high statistical support. Corresponding trees based on Nei's standard and shared alleles distances yielded similar topologies. For details, see text.

1. 4. DISCUSSION

1. 4. 1. Conservation genetics

Both the Sardinian and the Mesola red deer showed very low microsatellite variation as compared to other European populations. The striking deviations from Hardy Weinberg expectations are difficult to ascribe to a particular cause but given that both populations ex-

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perienced a severe bottleneck and that the Mesola population comprises only between 60 and 70 specimens, Hardy-Weinberg equilibrium at all loci is a priori not very likely.

Sardinia exhibited surprisingly high variability at the mitochondrial control region: haplotype and nucleotide diversity were within the range of the values for other red deer populations with haplotype diversity being comparatively high and nucleotide diversity being comparatively low. As mentioned above, this is explained by the fact that nucleotide, but not haplotype diversity takes into account the degree of similarity among the different haplotypes occurring in a population. This discrepancy between microsatellite and mtDNA variability in the Sardinian population had been found before by Zachos *et al.* (2003) in a previous study. One possible, although admittedly purely speculative explanation for the high mitochondrial variation in Sardinian red deer is introduction of female animals by men. To our knowledge, no such introduction has occurred in historic times but of course, this does not mean that it did not happen, and only a few females would be sufficient to introduce new mitochondrial lineages in the population without significantly raising the number of microsatellite alleles. Alternatively, the eight Sardinian haplotypes, which differ in seven sites, could have been derived from one or a few founder haplotypes by mutation, which is more likely if the red deer has been present on the island since the Upper Pleistocene (see below). As the Sardinian population has considerably grown during the last decade (from about 100 to about 2000 individuals) its extinction in the near future seems unlikely. Management should concentrate on keeping the effective population size as big as possible and on reducing poaching which is the major present threat to the animals. Unfortunately, we did not have samples from the Corsican population, which was refounded some thirty years ago with nine Sardinian specimens (Dolan, 1988), but due to the small number of founders this population is expected to show high levels of inbreeding and only little genetic variation.

The Mesola red deer, on the other hand, yielded no mitochondrial variation at all, which is explained by the small effective population size and the repeated bottlenecks and is in accordance with recent findings based on mitochondrial RFLPs (Lorenzini *et al.*, 2005). Similarly low mitochondrial variation was found in Serbian red deer, where 53 out of 54 individuals from Banat and Bachka showed the same haplotype (Feulner *et al.*, 2004), and for an isolated red deer population from a highly fragmented habitat in northern Germany where all 27 individuals studied yielded the same haplotype (Zachos *et al.*, unpublished data). Some populations of the endangered Eld's deer (*Cervus eldi*) did not show any variation in the mitochondrial control region either (Balakrishnan *et al.*, 2003, Pang *et al.*, 2003). This species is the target of considerable conservation and management action. Contrary, the red deer has

been widely neglected by conservation biologists (but see Kuehn *et al.*, 2003). This is probably due largely to the view obviously still held by many biologists that it is absolute numbers of individuals which make species worthy of attention.

However, red deer are suffering a variety of anthropogenic influences such as translocations, selective hunting and, above all, habitat fragmentation (cf. e. g. Hartl *et al.*, 2003, Niethammer, 1963), which result in populations being isolated from one another, reduction of effective population sizes, increase in inbreeding and loss of genetic variability due to random drift. As a reaction to this threat, the red deer has already been included as “near threatened” in the Red List of Schleswig-Holstein, northern Germany. Inbreeding depression in red deer has been reported for the Isle of Rum population in Scotland (Slate *et al.*, 2000), and morphological aberrations (jaw deformations and anophthalmia) have occurred in an isolated population in northern Germany numbering about 50 specimens and exhibiting low genetic variation (Zachos *et al.*, unpublished). For the Mesola population, low birth rates and high juvenile mortality have been reported (Mattioli, 1990), which may be signs of inbreeding depression. Management plans for this population should focus on the increase of effective population size, e. g. by artificially raising the number of reproducing males and trying to equalize family sizes, i. e. lifetime production of offspring per individual (cf. Frankham, Ballou & Briscoe, 2002). Artificial splitting of the population into two or more separate populations would reduce the risk of extinction through catastrophic events such as diseases while at the same time further loss of genetic variability and increase in inbreeding could be avoided by an occasional exchange of individuals among the stocks. The introduction of unrelated individuals from other populations to reduce inbreeding (as has been carried out, e. g., in the Florida panther and the Pyrenean brown bear, cf. O’Brien *et al.*, 1996, and Chapron *et al.*, 2003) should be avoided because this would lead to the pollution of the Mesola gene pool and thus to the genetic extinction of the last indigenous Italian red deer.

1. 4. 2. Phylogeography of *Cervus elaphus corsicanus*

As to the origin of the Sardinian or Corsican red deer there are two open questions: where did it come from and how did it reach the Tyrrhenian islands – naturally or through human introduction? As outlined above, red deer from northern Africa and the Near East have been hypothesized to be the ancestors of *C. e. corsicanus*, which would imply human introduction. The only way of reaching Sardinia naturally would have been via mainland Italy and Corsica during the last glacial regression in the Upper Pleistocene when the sea-level was up to 120-130 m lower than today (Shackleton, 1987).

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Still, the deer would have had to cross the Canale di Corsica between Corsica and the Tuscan Archipelago (from where Pleistocene red deer fossils are known, cf. Zachos *et al.*, 2003). Red deer are excellent swimmers - according to Whitehead (1993) distances of at least 7 km are verified – and are known to have reached various islands (Cyprus, Crete, Sicily, Malta) in the Mediterranean during the Pleistocene, often along with elephants and hippopotami as the only other big mammal species (Søndaar, 1977). This hypothesis of an Italian origin of *C. e. corsicanus* was put forward by Zachos *et al.* (2003) on the basis of microsatellite relationships between red deer from Sardinia and northern Italy (Tarvis). However, these were not indigenous Italian deer, and an inclusion of Mesola red deer was indispensable for this hypothesis to be either corroborated or refuted. The present study confirms the microsatellite results of the previous analysis in that Sardinia indeed shows the closest affinity to Mesola and is unequivocally paired with Mesola in the resulting trees with high bootstrap support. If this reflects the true genealogical relationships, then it seems most likely that Upper Pleistocene red deer from mainland Italy reached Corsica and, via Corsica, Sardinia. The Street of Bonifacio, today separating Corsica and Sardinia, repeatedly lay dry in the Pleistocene (Charlesworth, 1957), rendering the two islands a biogeographic unit with free faunal exchange (Vigne, 1992).

Palaeontological data could play a pivotal role in evaluating this scenario. The oldest undisputed red deer remains on the Tyrrhenian islands were found in the Corbeddu cave on Sardinia with an approximate age of 8000 years (Sanges, 1987). This age coincides with the beginning of Neolithic culture on Sardinia (Masseti, 1993, Masseti & Vianello, 1991), which would fit the hypothesis of human introduction of red deer on the island - by that time; the sea-level was too high for deer to cross the sea. Yet, red deer on Sardinia might be considerably older than 8000 years: according to Di Stefano and Petronio (2002 and references therein), red deer were already present as *Cervus elaphus rossii* on Corsica during the late Middle and Late Pleistocene! This is in accordance with the geological finding that during the Biharian (Middle and, possibly, late Early Pleistocene), Corsica and Sardinia, then a single island, were probably separated by only 13 km from Elba which in turn could have functioned as a stepping-stone between Tuscany and the Cirno-Sardinian massif (see Alcover *et al.*, 1999 and references therein). However, red deer on Sardinia might be considerably older than 8000 years because the allegedly endemic species of giant deer, *Megaloceros cazioti*, also found in the Corbeddu cave (Klein Hofmeijer, 1997, Søndaar *et al.*, 1986) is, according to the palaeontologist Th. Pfeiffer quite certainly a red deer (pers. comm.). The oldest remains of this deer have been carbon-dated to about 13,500 years.

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Further evidence of a close relationship between the corsicanus-subspecies and west-European red deer (*C. e. hippelaphus*) is given by Geist (1998) who lists similarities in exterior markings, the shape of the stag's mouth when roaring (which is a good diagnostic feature) and possibly the voice. Moreover, the tree based on the complete mitochondrial control region presented by Randi *et al.* (2001) pairs corsicanus with a hippelaphus-specimen but because the origin of the latter specimen is not given and may in fact be unknown, because of the small sample sizes ($n = 1$ for Spain and Sardinia) and because of possibly general limitations of mitochondrial DNA for this kind of analysis (see below) this result must be viewed with caution. Of course, the fact that the Sardinian red deer might have reached the island naturally in the Upper Pleistocene does not rule out the possibility that in later times deer from Italy were introduced to the island again: Schüle (1993) holds that all extant wild ungulates on the Mediterranean islands were introduced there by man during the Neolithic or later after Pleistocene populations had been exterminated by human invasions. For Sardinia, this introduction hypothesis is probably true for the mouflon (*Ovis ammon musimon*) and the red fox (*Vulpes vulpes*) (Fрати *et al.*, 1998, Geddes, 1985, Vigne, 1992).

The results yielded by the analysis of the mitochondrial control region confirm the results of Zachos *et al.* (2003): Sardinia and Spain appear to be most closely related in terms of net nucleotide diversity and haplotype distribution. Mesola, however, comes second and is indeed clustered together in one clade with the Spanish and Sardinian haplotypes in one (maximum-likelihood) of the three trees. The similarity between Sardinia and Spain is in clear contrast to the microsatellite data according to which Sardinia and Spain exhibit the highest distance values and Spain shows the smallest proportion of Sardinian alleles among all populations studied. One reason for the striking differences between the two marker systems may be the different mode of inheritance: due to the maternal inheritance of mitochondria the number of female founders, a bias in migration rates between the sexes or bottlenecks in female effective population size affect mitochondrial variability and relationships much more strongly than those for nuclear markers. But perhaps the differences found in this and the previous study (Zachos *et al.*, 2003) are explained best by referring to the different levels in time that the two marker systems are able to resolve. Generally, gene trees depict the evolutionary history of molecules and this history need not necessarily be the same as that of the populations or species these molecules are taken from (e. g. Avise, 1989, Pamilo & Nei, 1988). Depending on their mutation rates, different markers can uncover demographic and biogeographic processes that occurred at different time levels in the past. The mitochondrial control region might simply be too conservative to correctly depict the relationships among popu-

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lations that separated within the last 20,000 years, which is the time frame for the colonisation of Sardinia and Corsica. Taberlet *et al.* (1998: 462) even conclude that “intraspecific divergence amongst mtDNA lineages appears to often largely predate the Quaternary cold periods”! To be fair, it should be added that microsatellites may have mutational constraints and can also be misleading if size homoplasies (i. e. alleles of the same length are identical in state through convergence rather than identical by descent) have accumulated in different populations. Even though much of the data analysed by Taberlet *et al.* were based on cytochrome b sequences, the general tendency may also be correct for the control region. There is a considerable amount of evidence that sometimes mitochondrial haplotypes are of limited use in analyses below the species level (e. g. Cronin, 1993, Funk & Omland, 2003, Taberlet *et al.*, 1998). In the light of these findings, trees showing *Cervus elaphus* to be paraphyletic (e. g. Kuwayama & Ozawa, 2000, Ludt *et al.*, 2004, Polziehn & Strobeck, 1998, Randi *et al.*, 2001) may have to be viewed with caution. Lineage sorting, the stochastic distribution of haplotypes from a polymorphic ancestral population to its descendant populations, can result in patterns where one of two sister taxa shares its haplotype with an outgroup taxon while the two sister taxa show different haplotypes. This blurs the true genealogical relationships (for a possible case of lineage sorting in bison see Verkaar *et al.*, 2004, for a review see Funk & Omland, 2003). Both the red deer from southern Spain, which have been regarded as a relic population of the Holstein interglacial 200,000 to 300,000 years before the present (Meunier, 1984), and *Cervus elaphus corsicanus* are probably free from introductions that so many other European populations underwent. Thus, they could represent ancient mitochondrial gene pools far too old to find in them traces of recent demographic and phylogeographic developments. The clustering of Sardinian with North African red deer in the tree by Ludt *et al.* (2004) based on cytochrome b sequences may also be a consequence of lineage sorting rather than true phylogenetic relationship. Alternatively, red deer could have colonized North Africa via Sardinia or Italy (through human introduction), a possibility which seems to have occurred to Ludt *et al.* judging from the arrows in their figure 5. This scenario would reconcile their results (and thus the longheld view of a close relationship between *C. e. corsicanus* and the African Barbary stag, cf. Flerov, 1952 and Lydekker, 1898, 1915) with our conclusion based on microsatellites that the red deer from Sardinia and Corsica are descendants of deer from mainland Italy which reached the Tyrrhenian islands either naturally during the Upper Pleistocene or later in Holocene times in a boat with man.

STUDY B:

Genetic variability and differentiation within and among red deer (*Cervus elaphus*) populations from Great Britain

2. 1. INTRODUCTION

The red deer (*Cervus elaphus*) is one of two – the other one being the roe deer (*Capreolus capreolus*) – cervid species indigenous to the British Isles. The British or Scottish red deer (*Cervus elaphus scoticus* Lönnberg 1906) is a small subspecies, stags standing approximately 110 cm at the shoulder, females being somewhat smaller (Dolan 1988). Still, it is Britain's largest terrestrial mammal and has a very long history on the British Isles. It was already present some 120,000 years BP during the last interglacial (Currant and Jacobi 2001), and the first postglacial records date from approximately 12,500 BP (Hedges *et al.* 1986). The loss of suitable habitat in the course of the growing human population eventually resulted in the species being confined to the Scottish Highland, southwest England and a few more small scattered populations. In the lowlands, red deer are either absent or have been greatly reduced (Mitchell-Jones *et al.* 1996). Nonetheless, where they still occur red deer thrive in Britain which has the largest European population (e. g., Bützler 1986). Apart from stocks on islands off the mainland – among them the famous Scottish Isle of Rum population - there are today five more or less isolated populations. The largest one is in Scotland, lesser populations occur in the Lake District of northwest England, Exmoor in the southwest, the New Forest on the south coast and Norfolk in East Anglia.

The genetic structure of the red deer has been under human influences for centuries (e. g. Hartl *et al.* 2003, 2005, Lowe and Gardiner 1974), and especially in Britain, the long-established practice of enclosing deer in parks for recreational purposes including hunting and translocations of such deer to other parts of the country have blurred the natural genetic patterns. Consequently, most British stocks are allochthonous, native stocks are probably confined to some parts of Scotland and northwest England (Lowe and Gardiner 1974, Niethammer 1963). In Scotland, deforestation of native woodlands and persecution led to the probable extinction of all native stocks in the lowland by the 17th century. At the end of the 18th century, red deer numbers were probably lowest, but they increased again in the 19th century (Ritchie 1920, Staines and Ratcliffe 1987). From a peak around 1914, numbers are thought to have declined during the First World War. The situation in the 1920s and 1930s is unclear, but the population may still have been 200,000 at the start of the Second World War. By 1950, how-

British red deer

ever, the population had declined to approximately 100,000 (Callander and MacKenzie 1991). Subsequently, red deer numbers in the Scottish highlands have increased from 150,000 in the early 1960s to about 450,000 today.



Figure 2. 1. Red deer distribution in Scotland (inset) and map of Britain showing the location of the red deer populations studied (Ac = Achnacarry; Ar = Arran; D = Dunachton; M = Mar; G = Glenartney; E = Euston; I = Islay)

While the red deer has been the subject of a variety of genetic studies dealing with, among others, population structure (e. g., Nussey *et al.* 2005), phylogeography (e. g. Hmwe *et al.* in press, Ludt *et al.* 2004, Zachos *et al.* 2003), conservation and management (e. g. Hartl *et al.* 2005, Hmwe *et al.* in press, Kuehn *et al.* 2003, Lorenzini *et al.* 2005), among British red deer, the focus has mainly been on the famous population on the Scottish Isle of Rum (recent publications include e. g. Nussey *et al.* 2005, Slate and Pemberton 2002, Slate *et al.* 2000).

Here, we present a population genetic analysis of seven red deer populations from Scotland and England. The questions of variability within and differentiation among the populations were addressed using two different molecular marker systems, nuclear microsatellite loci and sequences of the mitochondrial control region. Microsatellites are highly variable repetitive DNA sequences with repeat units of up to 6 base pairs. Different alleles differ in the number of repeat units and hence in length. The mitochondrial control region, also known as the D-loop, is a hypervariable part of the mitochondrial genome. Both marker systems are known to be powerful tools in population genetics and have already been widely used in red deer and other cervids (e. g. Feulner *et al.* 2004, Goodman *et al.* 2001, Hundertmark *et al.* 2002, Kuehn *et al.* 2003, Nies *et al.* 2005, Zachos *et al.*, 2003).

2. 2. MATERIAL AND METHODS

2. 2. 1. Sampling

The study is based on 69 red deer from one English (Euston / Thetford, n = 10) and six Scottish populations (Figure 2.1): Achnacarry (n = 10), Dunachton (n = 10), Mar (n = 10), Glenartney (n = 10), Dunlossit / Islay (n = 10) and Arran (n = 9). Except for the deer from Islay and Arran, which are island populations, all animals analysed come from mainland stocks. As to the history of these populations, Whitehead (1960) gives the following information: Dunachton and Achnacarry are probably free from introductions (i. e., no such records can be found). On the island of Arran, all red deer had been wiped out by the middle of the 19th century but in 1859, 20 specimens (six males and 14 females) were introduced from Knowsley Park (10 km east of Liverpool), and around 1900, two more stags from Nottinghamshire and Windsor Park, respectively, as well as a wapiti hind were released (see also Whitehead 1964). Six stags and three hinds, all English park deer from Warnham in Sussex, are known to have been introduced to Kildalton south of Dunlossit on the Isle of Islay between 1908 and 1913 (plus one possible but unconfirmed further introduction of a stag from Warnham between 1968-70). From Mar, the introduction of stags from Warnham and unknown localities in 1884 and 1903 has been recorded. In Glenartney, some deer of unknown origin were probably introduced before 1914, and in 1923, 4 male and 8 female animals from Grimsthorpe Park in Lincolnshire were brought north and released. As to the English sample from Euston / Thetford), Whitehead (1960) suggests that the indigenous red deer of East Anglia were killed out by 1860. From the early 1800s until the middle of the 20th century, how-

ever, red deer were repeatedly introduced as game animals for hunting events. These deer came from Gunton (Suffolk), Woburn, Warnham, Richmond Park (Surrey), Ashridge Park (Hertfordshire) and Blickling Park (Norfolk). The extant red deer of Euston are thought to be descendants of these introduced animals.

2. 2. 2. Microsatellites

DNA extraction, PCR amplification and length analysis were carried out as described in Zachos *et al.* (2003). The following eleven polymorphic microsatellite loci were analysed: OarCP26 (Ede *et al.* 1995), OarFCB304 (Buchanan and Crawford 1993), MAF35 (Swarbrick *et al.* 1991), INRA11 (Vaiman *et al.* 1992), BM888, BM4208, BM4513 (Bishop *et al.* 1994), NVHRT16, NVHRT21 (Røed and Midthjell 1998), RT1 and RT7 (Wilson *et al.* 1997).

The loci were tested for linkage disequilibrium with GENEPOP (Raymond and Rousset 1995). Observed and expected heterozygosities as well as the significance of deviations from Hardy-Weinberg frequencies were calculated with the Arlequin software (Schneider *et al.* 2000). Values of allelic richness were calculated with the Fstat programme (Goudet 1995). This parameter yields a measure of allelic diversity independent of sample size by adjusting measures of alleles per locus to the smallest number of individuals typed for a locus in a sample. Fstat was also used to test for significant differences in expected heterozygosity and allelic richness between the island group (Islay and Arran) and the mainland group of populations (Achnacarry, Dunachton, Mar, Glenartney, Euston). Arlequin was used for calculating the overall fixation index F_{ST} and R_{ST} (a microsatellite-specific analogue of F_{ST}), yielding the proportion of the total genetic diversity accounted for by the differentiation among (as opposed to variation within) populations, as well as for testing population differentiation on the basis of pairwise F_{ST} values. Population differentiation was further quantified with Nei's (1972) genetic standard distances and chord distances (Cavalli-Sforza and Edwards 1967), both calculated with the software Genetix (Belkhir 2000), and by counting private alleles (alleles exclusive to only one of the populations studied, Slatkin 1985). Isolation by distance was tested for with a Mantel test as implemented in Arlequin on pairwise geographical and genetic distances, the latter given as $F_{ST}/(1-F_{ST})$ (Rousset 1997). Pairwise geographical distances were calculated with the Geomedia 5.2 software. The two island populations were not included in this analysis because their geographic distance to the mainland populations is theoretically infinite. An assignment test based on individual genotypes was carried out with the Bayesian method as implemented in GeneClass (Piry *et al.* 2004). All individuals are assigned to the

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population of which their genotype is most typical so that incorrect assignments indicate little genetic differentiation between the respective populations.

Tests for recent bottlenecks in the populations studied were carried out with the Bottleneck software (Cornuet and Luikart 1996) using, as recommended for microsatellite data by the authors, the Wilcoxon test of deviation from mutation-drift equilibrium under the Two-Phase-Model (TPM) of microsatellite evolution with 70% of the mutations following the strict Stepwise Mutation Model and 30% being multistep changes. This approach is based on the fact that recently bottlenecked populations often show a heterozygosity excess at selectively neutral loci relative to expectations under mutation-drift equilibrium. The reason for this excess is that allelic diversity is reduced faster than heterozygosity during a bottleneck leading to a relative deficiency in allele number.

Pairwise chord and Nei distances were used to create neighbor-joining trees with the software package PHYLIP (Felsenstein 1993). Support for nodes was assessed by bootstrap resampling using 1000 replicates.

2. 2. 3. Mitochondrial DNA

PCR amplification of the control region was performed with the primers Pro-L and Phe-Hb (cf. Zachos *et al.* 2003). Sequences were aligned with BioEdit version 5.0.9 (Hall 1999). Since some of the statistical programmes do not consider deletions they were treated as transversions in all analyses in order not to lose informative sites. According to the intrinsic transition-transversion ratio, transversions were weighted 4.5-fold over transitions.

Haplotype and nucleotide diversities within as well as net nucleotide diversities among populations (Nei 1987) were calculated using DnaSP3.51 (Rozas and Rozas 1999). Overall and pairwise F_{ST} -values were calculated with Arlequin (Schneider *et al.* 2000) taking into account not only haplotype frequencies but also the differences between them (estimated by Kimura-2-parameters). Relationships between variability indices (expected heterozygosity, allelic richness, haplotype and nucleotide diversity) were tested by means of Spearman rank correlations using the SPSS software version 10.0. Isolation by distance was tested for as described for microsatellites (see above). Mantel tests were also used for tests of pairwise correlations among genetic distances based on microsatellite and sequence data.

Genealogical relationships among the haplotypes were reconstructed using neighbour-joining (based on Kimura-2-parameters), parsimony and maximum-likelihood approaches as implemented in PHYLIP. Support for nodes was estimated by bootstrapping using 100 (maximum-likelihood) or 1000 replicates. Additionally, since it has been found that it might

be haplotype distribution rather than haplotype phylogeny which reflects population genealogy (e. g., Feulner *et al.* 2004), a neighbor-joining tree was constructed with Nei (1972) distances based on haplotype frequencies (PHYLIP, 1000 bootstrap replicates).

As for the microsatellite analyses, Bonferroni corrections were applied whenever multiple tests were carried out.

2. 3. RESULTS

2. 3. 1. Microsatellites

There was no significant linkage disequilibrium between any two of the eleven loci analysed. They were all polymorphic yielding between six (NVHRT16) and 23 (BM888, BM4513) alleles. Interestingly, NVHRT16 was monomorphic for the same allele (157 bp) in three populations (Islay, Dunachton and Achnacarry). Deviations from Hardy-Weinberg-Equilibrium frequencies occurred in all populations although never for all loci.

Table 2. 1. Parameters of genetic variability for the extended set of populations. N is the sample size for microsatellite and (in parentheses) mitochondrial analyses. H_E , H_O , AR and PA are expected and observed heterozygosity, allelic richness and proportions of private alleles averaged over eleven microsatellite loci. HD and π are haplotype and nucleotide diversity based upon 439 bp of the mitochondrial control region.

Pop.	N	H_E	H_O	AR	PA (%)	HD	π [%]
GLE	10(10)	0.78	0.48	5.6	13.2	0.533	0.729
MAR	10 (8)	0.80	0.39	5.4	11.1	0.643	0.504
DUN	10 (10)	0.78	0.49	5.3	7.6	0.733	0.772
ACH	10 (10)	0.84	0.40	5.7	5.7	0.711	0.810
ARR	9 (8)	0.84	0.48	6.0	16.9	0.250	0.399
ISL	10 (10)	0.81	0.39	5.3	13.6	0.000	0.000
EUS	10 (10)	0.76	0.50	4.8	6.8	0.356	0.729

Likewise, none of the loci showed deviations in all populations. The stocks with the highest rate of deviations (five loci) were Islay, Mar and Achnacarry. Altogether, the study

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Table 2. 2. Nei's 1972 (above diagonal) and Cavalli-Sforza and Edwards' chord (below diagonal) distances between red deer populations based upon allele frequencies of eleven polymorphic microsatellite loci.

	GLE	ISL	MAR	EUS	DUN	ARR	ACH
GLE		0.453	0.596	0.752	0.544	0.768	0.505
ISL	0.106		0.564	0.747	0.505	0.612	0.544
MAR	0.148	0.136		0.51	0.425	0.563	0.388
EUS	0.142	0.152	0.104		0.732	0.682	0.653
DUN	0.129	0.14	0.098	0.135		0.4	0.184
ARR	0.175	0.158	0.146	0.145	0.114		0.357
ACH	0.134	0.149	0.112	0.13	0.069	0.099	

yielded 156 alleles 50 of which (32%) were confined to only one of the populations studied (private alleles). Values of heterozygosity (expected and observed) and allelic richness as well as the proportions of private alleles for each population are given in Table 2. 1. There were no

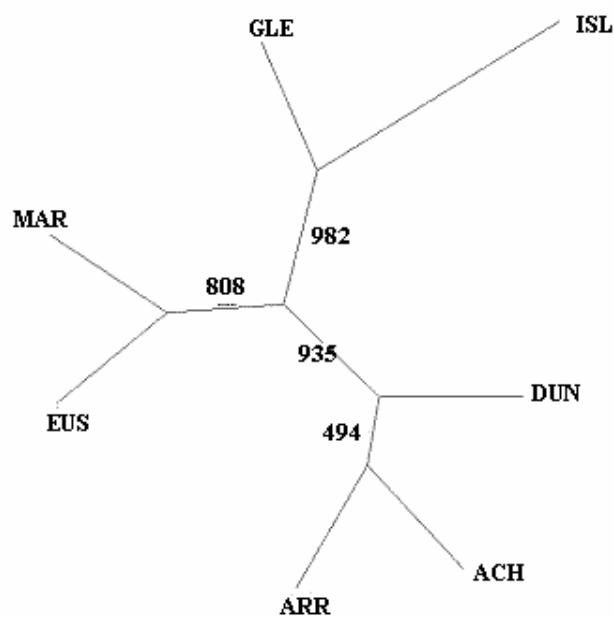


Figure 2. 2. Neighbor-joining tree based on chord distances derived from allele frequencies at eleven microsatellite loci. Numbers at nodes denote support from 1000 bootstrap replications (only values above 40% are shown).

significant differences in expected heterozygosity and allelic richness between the island and the mainland populations (p values being 0.5799 and 0.3658, respectively). Expected heterozygosity and allelic richness values of the seven populations showed a Spearman correlation coefficient of 0.761 but the p -value of 0.23 was not significant after Bonferroni-correction for multiple tests.

Table 2. 3. Mitochondrial haplotypes (HT) found in this study. Vertical numbers refer to the aligned sites in the 439 bp data set (only variable sites are shown). Dots indicate identity with HT 1, dashes denote deletions.

Haplotype	0	0	0	0	0	1	1	1	1	1	2	2	2	2	2	2	2	3	3	3	4	4
	0	4	5	6	8	1	7	7	8	9	0	1	2	4	4	7	8	1	5	5	3	3
	6	5	4	3	4	9	2	6	5	8	1	4	6	7	8	1	9	9	2	6	3	9
HT1	A	T	T	G	A	T	T	C	A	A	A	T	C	T	T	C	T	G	G	T	T	T
HT2	T	.	-	A	-	G	C
HT3	T	G	-	A	-	G	G	C	T	.	.	T	.	A	A	.	.	.
HT4	T	G	-	A	-	G	.	.	G	.	.	C	.	C	.	.	C	C
HT5	T	G	-	A	-	G	.	T	.	G	G	C	.	.	.	T	.	A	A	C	.	C
HT6	T	G	-	A	-	G	.	T	.	.	G	C	.	.	.	T	.	A	A	C	.	C
HT7	T	G	-	A	-	G	.	T	.	.	G	C	C
HT8	T	G	-	A	-	G	G	C	C
HT9	T	G	-	A	-	G	.	T	.	.	G	C	.	.	C	.	.	A	.	.	C	C
HT10	T	G	-	A	-	G	C	C	C
HT11	T	G	-	A	-	G	C	C	.	C	.	.	C	C
HT12	T	G	-	A	-	G	G	C	T	.	.	T	.	.	A	.	.	C
HT13	T	G	-	A	-	G	.	T	.	.	G	C	A	.	C	.	C

Overall differentiation was 0.1077 (F_{ST}) and 0.1489 (R_{ST}), meaning that between 10.8% and 14.9% of the total genetic variation was among populations (as opposed to variability within them). Pairwise F_{ST} -values were all significantly different from zero except for the ones between Achnacarry and Dunachton, Achnacarry and Mar and Achnacarry and Arran. Standard and chord distances are given in Table 2. 2. The Mantel test of isolation by distance yielded no significant correlation between geographic distances and pairwise $F_{ST}/(1-F_{ST})$. In the assignment test, 48 out of 69 individuals (69.57%) were correctly assigned to their population of origin. For Glenartney and Euston, there were no incorrect assignments while for Ach-

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nacarry, seven out of ten individuals were not assigned to its origin. Among the total of 21 wrong assignment, eight occurred between Dunachton and Achnacarry.

The tests of heterozygosity excess did not produce evidence for a recent bottleneck in any of the populations. The neighbour-joining trees based on standard (not shown) and chord distances (Figure 2. 2) showed nearly identical topologies in producing three clades comprising Glenartney and Islay, Euston and Mar and Arran, Achnacarry and Dunachton, respectively. Bootstrap support was higher in the chord distance tree, and taken together, only Glenartney and Islay are paired with statistical confidence.

Table 2. 4. Distribution of mitochondrial haplotypes (HT) among the seven red deer populations studied.

Haplotype	GLE	ISL	EUS	ARR	MAR	ACH	DUN	Σ
HT1	6							6
HT2	4	10						14
HT3			2					2
HT4			8	7				15
HT5					1			1
HT6					5		2	7
HT7					1			1
HT8					1		2	3
HT9						4		4
HT10						4	1	5
HT11						2		2
HT12				1				1
HT13							5	5
Σ	10	10	10	8	8	10	10	66

2. 3. 2. Mitochondrial DNA

Sequencing of 439 base pairs revealed 22 variable sites (18 transitions, 2 transversions and 2 deletions) and resulted in the detection of 13 putative haplotypes (Table 2. 3). The haplotypes occurred between one and 15 times, their distribution is given in Table 2. 4. Eight of them were confined to only one population (private haplotypes), and one population (Islay) showed no variation at all yielding only haplotype 2. The other populations exhibited between

two and four different haplotypes. Haplotype and nucleotide diversities are shown in Table 2. 1.

Overall F_{ST} was 0.6222, indicating that 62.2% of the variation were due to differentiation among and only 37.8% due to variability within populations. Again, three out of the 21 pairwise F_{ST} -values were not significantly different from zero (Euston-Arran, Mar-Dunachton and Islay-Glenartney). As in the case of our microsatellite data, there was no significant correlation of pairwise geographic and genetic ($F_{ST}/(1-F_{ST})$) distances. Net nucleotide diversities (Table 2. 5) were not significantly correlated with chord or standard distances either. Haplotype and nucleotide diversities of the seven populations were significantly correlated (Spearman correlation coefficient $r_s = 0.847$, $p = 0.008$, which is almost exactly at the Bonferroni-corrected 5%-level of 0.0083), but neither haplotype nor nucleotide diversity showed any such correlation with the expected heterozygosity or allelic richness values.

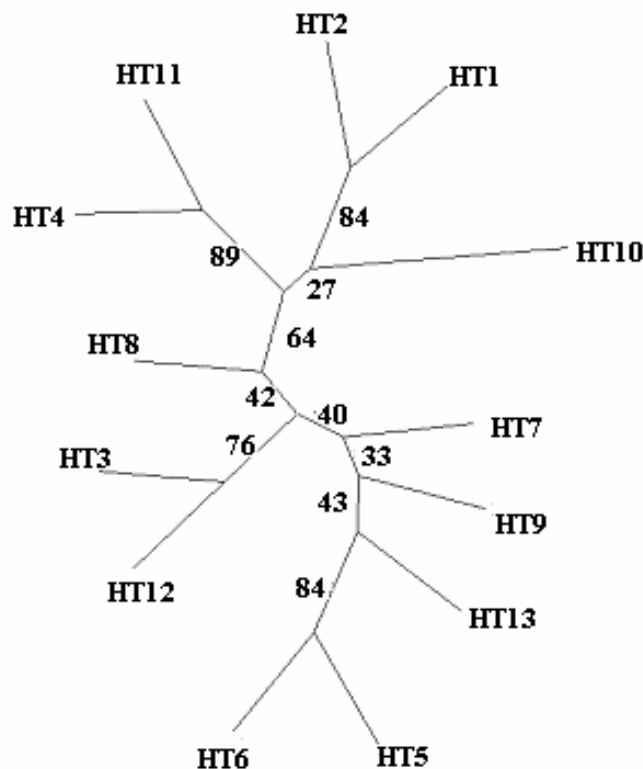


Figure 2. 3. Maximum-likelihood consensus tree of the haplotypes found in this study. Numbers at nodes denote support from 100 bootstrap replications.

The phylogenetic analyses of the 13 haplotypes (parsimony, maximum likelihood and neighbor-joining algorithms, Figure 2. 3) yielded consensus trees with almost identical to-

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pologies. In particular, they all showed the pairing of haplotypes 1 and 2, haplotypes 4 and 11, haplotypes 3 and 12 and haplotypes 5 and 6. The haplotype frequency tree yielded a topology similar to the microsatellite trees in that Glenartney and Islay were paired but differed in that Euston formed a clade with Arran instead of Mar and that Mar (instead of Arran) was part of the Achnacarry-Dunachton clade.

Table 2. 5. Pairwise F_{ST} -values (given in %) calculated from microsatellite (above diagonal) and mitochondrial sequence (below diagonal) data and net nucleotide diversities given in percent (in parentheses below diagonal). The asterisk (*) denotes non-significant F_{ST} -values.

	GLE	ISL	MAR	EUS	DUN	ARR	ACH
GLE		10.03	11.24	14.2	12.08	12.01	9.86
ISL	55.56* (1.84)		12.20	15.79	12.48	11.18	12.21
MAR	74.31 (1.84)	86.02 (0.06)		9.93	9.41	8.81	7.29
EUS	64.36 (1.34)	71.16 (1.06)	62.39 (1.10)		15.66	10.90	13.05
DUN	68.9 (1.45)	77.23 (0.10)	14.64* (0.10)	54.67 (0.79)		7.33	2.55
ARR	70.99 (1.49)	84.71 (1.28)	73.34 (1.28)	7.08* (0.03)	65.76 (0.96)		4.93
ACH	60.97 (1.21)	64.38 (0.74)	50.4 (0.70)	40.03 (0.52)	32.65 (0.34)	48.95 (0.62)	

2. 4. DISCUSSION

Genetic variability of the populations studied as revealed by haplotype and nucleotide diversities and expected heterozygosities are within the range of values previously found for red deer (cf. Feulner *et al.* 2004, Kuehn *et al.* 2003, Martinez *et al.* 2002, Zachos *et al.* 2003). Islay is special in that there is no mitochondrial variation at all (only one haplotype found). This lack of control region variability has also been found for other European populations, e. g. Serbian red deer from Banat (Feulner *et al.* 2004) and red deer from places in northern Germany (Zachos *et al.* unpublished). Interestingly, Islay does not show any reduced variability in the microsatellite-based parameters: both allelic richness and expected heterozygosity

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are average when compared to the other populations. The observed heterozygosity is at the lower end of the range but due to its being more susceptible to sample size than expected heterozygosity the latter is to be preferred as a measure of variability.

The deviations from the Hardy-Weinberg-Equilibrium of the microsatellite genotypes are probably best explained by the rather small sample sizes. With 10 specimens per population and hypervariable markers yielding high allele numbers it is a priori unlikely to match Hardy-Weinberg expectations for every locus. Other possible explanations are the translocation of animals into many of the populations studied which has the same effect as immigration (whose absence is one of the premises of the Hardy-Weinberg-Equilibrium) and null alleles.

These are alleles that are not detected due to a mutation in the primer binding-site. They can lead to an underestimation of heterozygotes in a population because individuals with one null allele will falsely appear homozygous. Null alleles are known to occur in red deer (see Pemberton *et al.* 1995), but in our case their impact (if there are null alleles) is very probably irrelevant because we experienced only very few total PCR failures out of a total of $69 \times 11 = 759$ amplifications. Yet, total failures (as a consequence of animals being homozygous for null alleles) are to be expected regularly if these alleles occur in relevant frequencies.

The locus NVHRT16 is interesting in that it proved to be monomorphic in three populations (Islay, Dunachton and Achnacarry), a rare case with microsatellites but it might also be a locus-taxon-specific phenomenon since a small isolated red deer population in northern Germany has also been found to be monomorphic at this locus (Zachos *et al.* unpublished). The overall proportion of private alleles (32%) is relatively high when compared to an analysis of Serbian and Romanian red deer including the genetically distinct Carpathian subspecies where the overall proportion only amounted to 10.4% (Feulner *et al.* 2004). This, along with the overall and pairwise F_{ST} -values, indicates a considerable differentiation among the populations studied.

The only two populations which repeatedly show little pairwise differentiation are Dunachton and Achnacarry. Pairwise distance values between them are low or even lowest (see particularly the Nei and chord distances as well as the pairwise F_{ST} -values) for both nuclear and mitochondrial data, and eight of the 21 incorrect assignments are between these two populations. The only other population exhibiting one of the haplotypes (haplotype 10) found in Achnacarry is Dunachton, and in the phylogenetic trees both populations are either paired in one clade (microsatellite-based neighbor-joining tree with Nei distances, not shown) or at least belong to the same cluster (haplotype frequency tree, not shown, and chord distance tree, Figure 2. 2). Glenartney and Islay are also paired with high bootstrap support in the microsa-

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tellite trees (and also in the haplotype frequency tree) but the pairwise distance values and the assignment results (only two incorrect assignments between them) are not nearly as unequivocal as the ones for Dunachton and Achnacarry. Still, haplotypes 1 and 2 which only occur in Glenartney and Islay are consistently paired in all three phylogenetic haplotype analyses.

The reason for the genetic proximity of Dunachton and Achnacarry is very probably the combination of their being geographically adjacent and, contrary to the other five populations, their being free from introductions which are, just like isolation through habitat fragmentation, likely to blur natural relationships (cf. Hartl *et al.* 2003, 2005 for similar examples in French red deer). In line with this, the overall test of isolation by distance was negative but given the history of the stocks as outlined above, this is hardly surprising. The fact that there was not statistically significant difference in variability parameters between the island populations and the mainland group is possibly also an effect of the translocations, particularly in the case of Arran where repeated introductions from different localities are likely to have mitigated any reduction in variability through founder effects, genetic drift or inbreeding.

As expected from the distribution of the different haplotypes the phylogenetic haplotype trees do not mirror the geographical pattern of the populations. This vertical approach (based on sequence coalescence as opposed to the horizontal analysis of sequence distribution) has been shown to be inferior to distribution analyses in red deer before (Feulner *et al.* 2004) which might be due to general shortcomings of mitochondrial sequence data in studies of the present kind. The mitochondrial lineages might simply be too old and therefore, not be capable of resolving population affinities that result from recent demographic processes (cf. Cronin 1993, Funk and Omland 2003, Hmwe *et al.* in press, Taberlet *et al.* 1998). After all, sequence trees must not be mistaken for population (or species) trees (Avice 1989, Pamilo and Nei 1988).

This line of argumentation is further supported by the fact that, although there is a significant correlation between haplotype and nucleotide diversities and at least a tendency for such a relationship between allelic richness and heterozygosity, such a correlation neither exists between sequence-based and microsatellite-based parameters of variability nor between genetic distance measures derived from the two marker systems. This is indicative of different processes affecting the nuclear and mitochondrial gene pool. One relevant aspect is certainly the difference in effective population size which is four times higher for nuclear markers than for mitochondrial ones but another important point is the generally higher variability in microsatellites which makes them more suitable markers for uncovering recent demographic

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processes. The present study, however, has again shown that a better fit between the two marker systems can be achieved when the horizontal approach is chosen for mitochondrial sequences, and taken together, microsatellite and sequence data show the discrepancy between the two natural populations of Dunachton and Achnacarry and the populations deeply affected by human influences. From a management point of view the results strongly suggest that Dunachton and Achnacarry be kept free from introductions to conserve at least the remnants of the original gene pool of *Cervus elaphus scoticus*. The question of the extent to which the British, and in particular the Scottish, population shows introgression from mainland populations or subspecies will be addressed in a future, more comprehensive analysis which is likely to yield interesting results since, for example, a mitochondrial haplotype very similar to one found in the Sardinian population of *C. e. corsicanus* has been found on the Isle of Rum (D. Nussey, pers. comm.). Given that the British red deer population is the biggest in Europe and that already some populations seem to be endangered by introgression of sika deer (Abernethy 1994) the genetic status and demographic history of *C. e. scoticus* are an integral and important part of future management and conservation regimes and phylogeographic analyses of the red deer in Europe.

STUDY C:

Microsatellite variability and differentiation within and among red deer (*Cervus elaphus*) populations from northern Germany

3. 1. INTRODUCTION

It has often been emphasized recently that red deer populations are affected by a variety of anthropogenic influences such as habitat fragmentation, keeping of populations in enclosures, translocations and (re-)introductions (Hartl *et al.*, 1995, 2003, Niethammer, 1963). Still, species which are not threatened in terms of census size – such as the red deer - have been neglected by conservation biologists. Red deer populations, however, are often threatened by genetic depletion through drift and inbreeding as a consequence of habitat fragmentation, small population sizes and founder effects (e. g., Hmwe *et al.* in press, Kuehn *et al.*, 2003). This also holds for the red deer populations in Schleswig-Holstein which are being analysed at the moment with regard to their genetic variability and differentiation. The present study is one part of this more comprehensive project (see below).

After being widely distributed in Schleswig-Holstein, red deer already were reduced in the 16th century (Jessen 1988). As a consequence of an enormous increase in land use in the course of the growing agriculture red deer were deprived of their habitat and, in the emerging conflict between humans and wild animals, hunted by farmers and foresters. Thus, by about 1730, red deer were only patchily distributed throughout Schleswig-Holstein (Jessen 1988). Ironically, it was the hunting passion among the aristocracy that saved the deer, which were kept in high densities in restricted areas or enclosures where they were safe from poaching (Beninde 1937, Jessen 1988, Schloeth 1988). Nevertheless, hunting pressure increased steadily, and after the revolution of 1848, hunting legislation was liberalized which led to the red deer's extinction in many parts of the country (Jessen 1988, Tillmann and Reck 2004). After stricter hunting laws were reintroduced the Schleswig-Holstein stock slowly recovered again but was again reduced during the wars of the 20th century (Jessen 1988, Wagenknecht 2000).

Occurrence of red deer in Schleswig-Holstein is restricted to forest areas (Meissner *et al.* 2004). At present, there are at least 1500 red deer in Schleswig-Holstein which belong to some eight populations (one of which was recently founded in the north of the country by immigrating deer from Denmark) (Jahresbericht 2004: Jagd und Artenschutz 2005). While not being endangered in terms of its census size, the species has been included in the regional Red List as “near threatened” (Borkenhagen 2001) due to the danger of inbreeding depression and

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loss of genetic variability through random drift in the mostly small and disjunctive populations which are separated by motorways and settlements. In 1980, to avoid agricultural and forest damages through red deer, authorities agreed on a restricted area for red deer to occur outside of which the animals are supposed to be shot. Thus, recolonisation of formerly occupied territory is rendered impossible (Peters 2000, Schmäser pers. comm., Tillmann and Reck 2004).



Figure 3. 1. Geographic map with the location of the red deer populations studied.

BA = Barlohe; DB = Dovenstedter Brook; LB = Lauenburg; SE = Segeberger Forst; HB = Hasselbusch and EW = Elsdorf-Westermühlen (for SE, HB and EW cf. Althoff 2005)

This study is part of a more comprehensive analysis of the red deer in the federal country of Schleswig-Holstein. Six populations – Elsdorf-Westermühlen, Barlohe, Hasselbusch, Segeberger Forst, Dovenstedter Brook and Lauenburg (Figure.3.1.)– are being analysed with regard to genetic variability and differentiation as revealed by microsatellite loci and sequences of the mitochondrial control region. In the present study, three populations, Barlohe, Dovenstedter Brook and Lauenburg, are screened for microsatellite diversity.

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While the (fenced!) motorway A7 separates the Segeberg from the Hasselbusch population, a non-fenced stretch of it allows for the migration of deer between Segeberg and Barlohe, and indeed migrating deer have been observed in this area (Meissner in press, Peters 2000). In former times, gene flow between Segeberg and the Duvenstedter Brook (and Lauenburg as well) occurred but the last individual known to migrate from Duvenstedt to Segeberg was a male in 1986 (Meissner in press). However, until the mid-1970ies, there was little but regular exchange between the two populations (Peters 2000). The federal street B206 and several settlements and small towns cut off the Barlohe population from Hasselbusch. Consequently, migration events are scarce, the last one dating from 1987 when a stag from Hasselbusch reached Barlohe (Meissner in press).

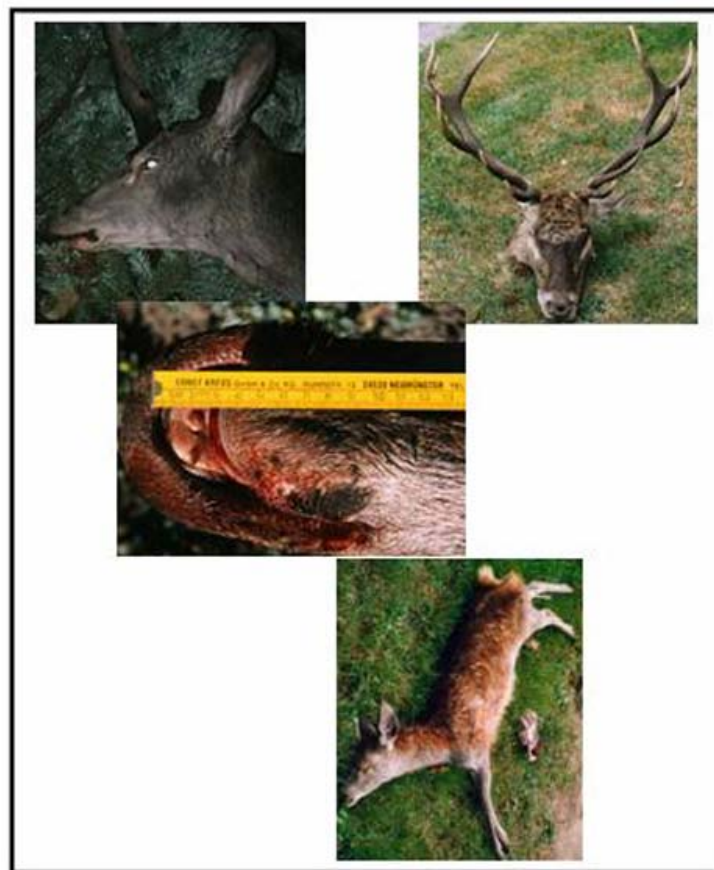


Figure 3. 2. Shortened lower jaw (brachygnathia) and fawn born without eyes in the Hasselbusch population. Particularly brachygnathia is likely to be a sign of inbreeding depression (pictures: Hewicker).

Although the population of Elsdorf-Westermühlen was founded by immigrating red deer from Barlohe, the Kiel Canal is an effective barrier between the two. Before it was built,

the narrow Eider river did not prevent an exchange but especially after the 1970ies when the Kiel Canal was widened to 162 m and, even more important, its banks were steepened, gene flow was rendered nearly impossible although in some cases red deer have been seen crossing the Canal nonetheless (for details see Althoff 2005). In the future, the motorway A20 (currently under construction) will inevitably lead to further fragmentation of the red deer's habitat. This motorway will run directly through the Segeberger Forst and completely separate Hasselbusch and Barlohe from one another.

As to a detailed description of the three populations not analysed in this study (Elsdorf-Westermühlen, Hasselbusch and Segeberger Forst), see Althoff (2005). It is worth mentioning, however, that in Hasselbusch, there have been rather obvious signs of inbreeding depression in that, apart from a red deer fawn born without eyes, at least six cases of brachygnathia have occurred where the lower jaw of the respective specimens was considerably shorter than the upper jaw (Hewicker, pers. comm., Figure 3. 2.; see also Althoff 2005). Morphological abnormalities (malformation characteristics) are known to have a genetic basis and are therefore inherited in a variety of species such as red and white-tailed deer to name but a few (Frieden *et al.*, 1996; Walker *et al.*, 1985; White *et al.*, 1978; Kierdorf *et al.*, 1994; Kuiken *et al.*, 1997; Rollor 1993; Marburger *et al.*, 1972; Chapman and Chapman 1969).

3. 2. MATERIALS AND METHODS

3. 2. 1. Sampling

A total of 66 individuals from three different populations in Schleswig-Holstein, northern Germany, was analysed regarding variability and differentiation at twelve microsatellite loci. The populations were (sampling size in parentheses) Barlohe (n=7), Duvenstedter Brook (n=13) and Lauenburg (n= 46) (Figure 3. 1.). Tissue samples were taken from animals culled during the hunting seasons in 2003 and 2004.

3. 2. 2. Microsatellite analysis

DNA extraction, PCR amplification and length analysis were carried out as described by Zachos *et al.*, 2003. The twelve loci analysed were OarCP26 (Ede *et al.* 1995), OarFCB304 (Buchanan and Crawford 1993), MAF35 (Swarbrick *et al.* 1991), INRA11 (Vaiman *et al.* 1992), MAF109 (Swarbrick & Crawford, 1992), BM888, BM4208, BM4513 (Bishop *et al.* 1994), NVHRT16, NVHRT21 (Røed and Midthjell 1998), RT1 and RT7 (Wilson *et al.* 1997).

The loci were tested for linkage disequilibrium with GENEPOP (Raymond and Rousset 1995). Observed and expected heterozygosities as well as the significance of deviations from Hardy-Weinberg frequencies were calculated with the Arlequin software (Schneider *et al.* 2000). The probability of null alleles being present in frequencies relevant to the analyses was considered to be low since there were only three (0.5%) complete failures out of 552 PCR amplifications. This failure rate would be higher if null alleles occurred more often because then the probability of individuals homozygous for null alleles would increase.

Because of considerable differences in sample sizes, we calculated values of allelic diversity (number of alleles per locus in a population) as so-called allelic richness values with the Fstat programme (Goudet 1995). This parameter yields a diversity measure independent of sample size by adjusting measures of alleles per locus to the smallest number of individuals typed for a locus in a sample. Arlequin was used for calculating the overall fixation indices F_{ST} and R_{ST} (a microsatellite-specific analogue of F_{ST}), yielding the proportion of the total genetic diversity accounted for by the differentiation among (as opposed to variation within) populations, as well as for testing population differentiation on the basis of pairwise F_{ST} values.

Population differentiation was further quantified with Nei's (1978) genetic distances adjusted to small sample sizes and chord distances after Cavalli-Sforza and Edwards (1967), both calculated with the software Genetix (Belkhir 2000), and by counting private alleles (alleles exclusive to only one of the populations studied, Slatkin 1985). The inbreeding coefficient F_{IS} for each of the three populations was calculated with Fstat. Tests for recent bottlenecks in the populations studied were carried out with the Bottleneck software (Cornuet and Luikart 1996) using, as recommended for microsatellite data by the authors, the Wilcoxon test of deviation from mutation-drift equilibrium under the Two-Phase-Model (TPM) of microsatellite evolution with 70% of the mutations following the strict Stepwise Mutation Model and 30% being multistep changes. This approach makes use of the fact that recently bottlenecked populations often show a heterozygosity excess at selectively neutral loci relative to expectations under mutation-drift equilibrium. The reason for this excess is that allelic diversity is reduced faster than is heterozygosity during a bottleneck leading to a relative deficiency in allele number.

Individual genotypes were further analysed by means of Analysis Factorial of the Correspondences (AFC) with the programme GENETIX (Belkhir 2000). It is an analysis type canonical particularly well adapted to describe the associations between two quality variables. The analyzed objects are seen as a cloud of points and defined by the clean vectors of the ma-

trix, determine a series of axes factoriels. By convention, the first axis is the one that has the strongest contribution to total inertia.

The Bayesian approach of the population assignment test implemented in the GENECLASS 1.0.02 program (Cornuet *et al.*, 1999) was used to estimate the likelihood of an individual's multilocus genotype to occur in a given population. Correct assignments (whose significance was tested in χ^2 -tests) indicate that the individual's genotype is typical of the population's gene pool and thus, high rates of correct assignments suggest genetic distinctiveness of the populations. For all calculations Bonferroni corrections were applied whenever multiple tests were carried out.

3. 3. RESULTS

The pairwise tests of linkage disequilibrium for the 12 microsatellite loci did not show any significant results at the Bonferroni-corrected 5%-level. Therefore, all loci were used in the multilocus-analysis. The microsatellites were all polymorphic yielding between 5 and 21 alleles.

Table 3. 1. Parameters of genetic variability for the populations studied. N= sample size, H_E = expected heterozygosity, H_O = observed heterozygosity, F_{IS} = inbreeding coefficient, AR = allelic richness, PA = private alleles (given as the percentage of all alleles found in the population). H_E , H_O and AR are calculated as the mean over all twelve loci. The respective values for the three populations analysed by Althoff (2005) are also shown. They are, however, based on only nine loci.

Pop.	N	H_E	H_O	AD	F_{IS}	AR	PA (%)
BA	7	0.77	0.63	4.8	0.135	4.8	17.24
DB	13	0.75	0.55	6.0	0.239	4.8	16.67
LB	46	0.79	0.49	10.3	0.369	5.9	25.20
SE	27	0.75	0.49	6.56	0.342	5.8	29.31
HB	18	0.66	0.59	4.89	0.124	4.7	2.86
EW	13	0.61	0.44	3.89	0.290	3.9	15.91

The total allele number was 148, the single populations showed 58 (BA, 2-8 alleles per locus), 72 (DB, 1-10 alleles per locus) and 123 (LB, 4-16 alleles per locus) alleles, respectively. Intrapopulation indices are shown in Table 3. 1. LB exhibited both the highest expected and the lowest observed heterozygosity. Consequently, the inbreeding coefficient (which is based upon the ratio between these two parameters) is also the highest of the three values. On the other hand, allelic richness is also highest for LB. 53 (36%) out of a total of 148 alleles over the three populations were private alleles. BA and DB showed very similar proportions of private alleles whereas the respective value for LB was considerably higher (Table 3.1.).

Table 3. 2. Observed (H_O) and expected heterozygosity (H_E) at the twelve microsatellite loci. *= significant deviation from HWE. The locus NVHRT16 was monomorphic in population DB.

Locus	BA		DB		LB	
	H_O	H_E	H_O	H_E	H_O	H_E
OarCP26	0.714	0.802	0.846	0.790	0.543*	0.849
OarFCB304	0.571	0.747	0.231	0.415	0.543*	0.847
MAF35	1.000	0.791	0.769	0.794	0.500*	0.787
INRA11	1.000	0.868	0.231*	0.825	0.422*	0.837
BM4513	0.714	0.692	0.615	0.880	0.717*	0.893
NVHRT16	0.143	0.495	-----	-----	0.109*	0.321
NVHRT21	0.571	0.901	0.385*	0.794	0.130*	0.914
RT1	0.857	0.769	0.308	0.508	0.500	0.695
RT7	0.714	0.923	0.769	0.840	0.870	0.832
BM888	0.286*	0.923	0.692	0.905	0.391*	0.859
BM4208	0.571	0.626	0.462	0.712	0.587*	0.851
MAF109	0.429	0.659	0.769	0.782	0.614	0.768

After Bonferroni-correction, there was one significant (5%-level) deviation from Hardy-Weinberg expectations in the BA population (at locus BM888) and two in DB (loci INRA11 and NVHRT21). DB was special in that it was found to be monomorphic at the locus NVHRT16 and in LB that only three loci (RT1, RT7 and MAF109) were in accordance with Hardy-Weinberg expectations (the locus NVHRT16, being monomorphic, was not included in these calculations) (Table 3.2.).

Overall genetic differentiation was 0.118 (F_{ST}) and 0.154 (R_{ST}), respectively. Both values were significantly different from zero ($p < 0.00001$), indicating that about 12-15% of the total variation is due to differences among populations. Even after Bonferroni-correction, pair-

Schleswig-Holstein red deer

wise F_{ST} -values showed the populations to be significantly differentiated from one another ($p < 0.00001$) (Table.3.3).

The pairwise genetic Nei and chord distances are shown in Table 3.4. Interestingly, BA and LB showed the highest Nei distance although in terms of the chord distance, the separation of two populations was practically non-existent. The results of the bottleneck tests did not show any significant heterozygosity excess for any of the populations (p -values ranged from 0.11670 to 0.38232).

Table 3. 3. Pairwise F_{ST} -values (given in %) calculated from twelve microsatellite loci (all three values are significantly different from zero).

	BA	DB	LB
BA			
DB	13.03		
LB	11.73	11.55	

The assignment test assigned 100% of the individuals to the correct population, i. e. its population of origin, showing that each specimen's genotypic information based on the twelve loci was sufficient to genetically distinguish the three populations.

Table 3. 4. Nei's 1978 (above diagonal) and chord (below diagonal) distances between red deer populations based upon allele frequencies of twelve polymorphic microsatellite loci.

	BA	DB	LB
BA		0.388	0.531
DB	0.388		0.414
LB	0	0.531	

The result of the Analysis Factorial of the Correspondences (AFC) showed a different pattern (Figure 3. 3). Scores of populations were plotted on three factorial axes (Axe1, Axe2 and Axe 3), which cumulatively demonstrated between the correspondences genetic diversity as range as 61.18%, 39.82% and 0.00% , respectively.

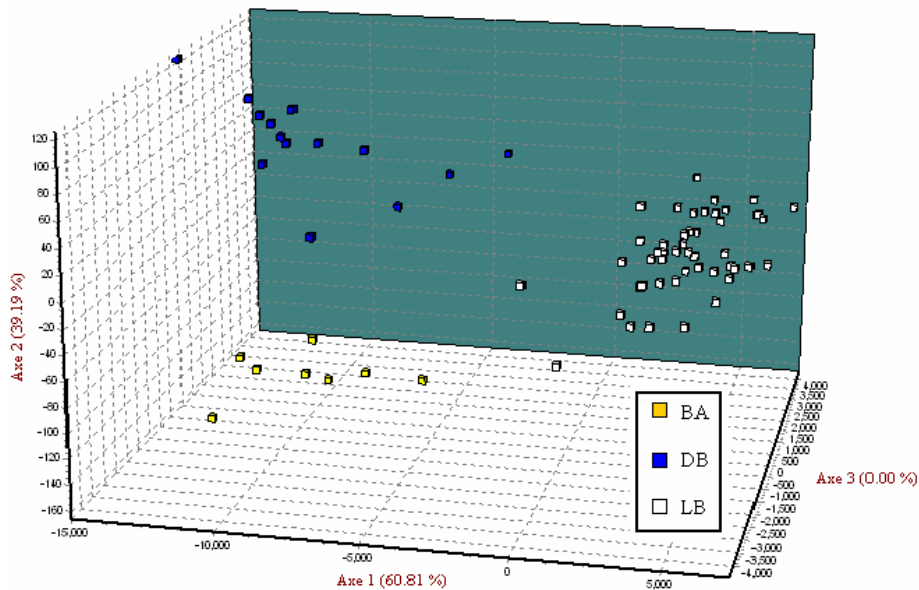


Figure 3. 3. Factorial analysis of the correspondence scores of the three red deer populations in Schleswig-Holstein as microsatellite genotype frequencies of individuals plotted on the three factorial axes (Axe1, Axe2 & Axe 3).

3. 4. DISCUSSION

Conservation geneticists usually agree that genetic variability within and among populations is a prerequisite for the survival and adaptability of populations (see Soulé 1986). In the present study, we observed that microsatellite variabilities in three north German red deer populations as measured by of expected heterozygosity were lower (0.75-0.79) than in 12 European red deer populations (0.80-0.90) and higher than in 2 indigenous red deer populations in Mesola and Sardinia (0.53 and 0.60, respectively) (Hmwe *et al.*, inpress; Zachos *et al.*, 2003 and Feulner *et al.*, 2004).

Interestingly, while showing the highest expected heterozygosity among the three populations studied, LB also exhibited the lowest observed heterozygosity. However, since the latter is more susceptible to sample size, the relatively high H_O values of BA and DB may in fact be not too reliable, which would fit the fact that their allelic richness is considerably lower than the respective value for LB. The high frequency of private alleles shows considerable differentiation between the populations as do the significant pairwise F_{ST} -values, the assignment test results and the analysis factorial of the correspondence. The very high number of private alleles in the LB population is, however, almost certainly due to the much bigger

sample size (46 vs. 13 and 7, respectively). The fact that the chord distance between LB and BA is zero is surprising but given that the Nei distance is much bigger and in light of all the other results a considerable genetic differentiation of these two populations seems beyond reasonable doubt.

The fixation indices (F_{ST} and R_{ST}) are relatively high given the geographically rather small scale of the study. Feulner *et al.* (2004), in an analysis of Serbian and Romanian red deer, found an F_{ST} -value (based on nine microsatellite loci eight of which were also used in the present study) of only 3.6%, and Zachos (2001), in an analysis of red deer comprising such distant and isolated populations as southern Spain, Sardinia and Bulgaria, found an overall genetic differentiation of 17% (F_{ST}) and 14% (R_{ST}), respectively.

It is worth mentioning that, just like in the analysis of roe deer from Schleswig-Holstein (see study four and Zachos *et al.* in press b); the bottleneck dating from the mid-nineteenth century after a liberalization of huntig legislation almost resulted in the extinction of the species was not detected by the bottleneck test. This may indicate that either reintroduction measures blurred the drift and inbreeding effects of the bottleneck or that the test is not strong enough for the data of this study. However, two of the other three red deer populations from Schleswig-Holstein analysed by Althoff (2005) on the basis of nine microsatellite loci showed a significant test result for bottlenecks, which at least partly confirms the test's validity for the populations from northern Germany.

3. 4. 1. COMPARISON WITH OTHER STUDIES ON RED DEER FROM SCHLESWIG-HOLSTEIN AND CONCLUSION

As mentioned before, this study is part of a more comprehensive analysis combining data from six red deer populations and different molecular marker systems (microsatellites and mitochondrial DNA sequences). The mitochondrial data for three populations (Hasselbusch, Segeberg and Elsdorf-Westermühlen; the other three are being analysed at the moment) show very low variability (v. Steynitz 2004). There were altogether only four haplotypes, and out of the 52 specimens studied, 46 yielded the same haplotype. This is, to my knowledge, the lowest mitochondrial variability in red deer uncovered so far (except for two analyses of the single Mesola population in Italy which both showed the Mesola deer to be completely monomorphic for mitochondrial markers; see study one, Hmwe *et al.* in press and Lorenzini *et al.* 2005), even lower than the one for the severely bottlenecked and threatened population from Sardinia (see study one, Hmwe *et al.* in press and Zachos *et al.* 2003). A similar (i. e. non-existent) variability at the mitochondrial control region has been found in two popula-

Schleswig-Holstein red deer

tions of the endangered Eld's deer (*Cervus eldi*) from southeast Asia (Balakrishnan *et al.* 2003).

The microsatellite analysis of the three remaining populations from Schleswig-Holstein (Althoff 2005) yielded similar results in terms of variability (see Table 3.1.) although overall genetic differentiation was lower (between 9% and 10% as opposed to 14-17% in this study). Interestingly, both the present analysis and the one carried out by Althoff (2005) showed that one population was completely monomorphic at the locus NVHRT16, DB in the present study and Elsdorf-Westermühlen in the analysis of Althoff (2005). In both studies, the locus yielded a total of only five alleles showing that variability at this locus, at least in northern Germany, is generally low. The fixation of one allele in two populations, however, still is convincing evidence of severe genetic depletion in these stocks.

Taken together, the analyses on the red deer from Schleswig-Holstein conducted so far show that there is differentiation among the stocks which is indicative of an increasing isolation of the populations. This is in line with the findings of Meissner (in press) and historical reports on migration behaviour of the animals. Genetic variability is very low for both marker systems confirming the results of v. Steynitz (2004) and Althoff (2005) and underlining the need for conservation actions to avoid further inbreeding and genetic drift. Migration corridors and translocations may be appropriate measures in future management regimes. The fourth analysis of the red deer from Schleswig-Holstein is being carried out at the moment (mitochondrial control region sequencing for the populations LB, DB and BA) and when all the data have been taken there will be a final total analysis combining all six populations for both marker systems and, additionally, the data from Meissner (in preparation) based on satellite imagery. This interdisciplinary synthesis is likely to shed further light on the genetic status of the red deer populations in Schleswig-Holstein and to yield a solid basis for further recommendations of wildlife management in northern Germany.

STUDY D :

Biochemical and DNA markers yield strikingly different results regarding variability and differentiation of roe deer (*Capreolus capreolus*) populations from northern Germany

4. 1. INTRODUCTION

The European roe deer (*Capreolus capreolus*) is the most common and widespread ungulate species in Europe with about 15 million head in central Europe alone (Mitchell-Jones *et al.* 1999). Being a popular game species of the man-shaped cultural landscape – yearly bags in Germany amount to about one million (Mitchell-Jones *et al.* 1999) -, the roe deer is suffering a variety of anthropogenic influences such as selective hunting, translocations and habitat fragmentation.

Roe deer genetics has been extensively analysed with regard to genetic variability and population differentiation (e. g., Hartl *et al.* 1991, 1993, Lorenzini *et al.* 1997, Milosevic-Zlatanovic *et al.* 1997, Wang *et al.* 2002), phylogeography (e. g., Lorenzini *et al.* 2003, Randi *et al.* 2004, Vernesi *et al.* 2002), conservation and management (e. g., Lorenzini 2002, Randi *et al.* 2004), behavioural aspects (e. g., Nies *et al.* 2005, Wang and Schreiber 2001) and habitat fragmentation (e. g., Coulon *et al.* 2004, Wang and Schreiber 2001). The molecular markers most frequently used in these studies were allozymes, microsatellites and sequences of the mitochondrial control region, all known to be powerful tools of comparisons at the population level. While in a couple of roe deer studies two of these markers were analysed (allozymes and microsatellites: Wang and Schreiber 2001, allozymes and sequences: Nies *et al.* 2005, microsatellites and sequences: Randi *et al.* 2004), to our knowledge, there is as yet no analysis of roe deer populations (and only relatively few dealing with other taxa, for a study on red and sika deer see Abernethy 1994) using both nuclear allozymes and microsatellites and mitochondrial markers. However, this would be a useful approach possibly uncovering the different capacities of the three marker systems with regard to detecting genetic variability within and differentiation among populations. The general comparability of nuclear and mitochondrial data has been questioned by studies yielding contradictory results for these markers (e. g., Cathey *et al.* 1998, Feulner *et al.* 2004, Zachos *et al.* 2003). Game species like cervids could be affected particularly strongly by these differences because, apart from selective hunting of males, translocations aiming at trophy improvement may have been male-biased, which

would have led to an even greater gap between diploid nuclear and maternally inherited haploid mitochondrial markers.

In species or populations relevant for conservation or (economical) management, it is of high importance to correctly assess the genetic variability and similarity of management units. Variability, however, may be strongly marker-dependent as shown by Feulner *et al.* (2004) for Serbian red deer where 53 out of 54 individuals showed the same mitochondrial control region haplotype whereas in terms of microsatellites, variability was not or only slightly reduced relative to the other populations studied.

These results show the value of comparative approaches based on the various marker systems frequently used in population genetic research. In the present study, we analysed 105 roe deer from five German populations with regard to their variability and differentiation at eight allozyme and eight microsatellite loci as well as 404 base pairs of the mitochondrial control region and carried out correlation analyses of genetic distances and variability indices based on nuclear and mitochondrial data to document the extent to which the different methodological approaches yield matching results.

4. 2. MATERIAL AND METHODS

4. 2. 1. Populations studied

Altogether 105 roe deer specimens from five different populations in Schleswig-Holstein, northern Germany, were analysed (Fig. 1, for sample sizes see Table 4. 1). Two of the populations were island stocks (Foehr, FO, and Fehmarn, FM) while the other three were mainland populations (Nordfriesland, NF, Schleswig, SL, and Rantzau, RA). The two island populations were artificially founded in the 1930s with five specimens from an area near Cologne and Dueren in western Germany on Foehr and with eight Danish founders (three males and five females) on Fehmarn. On Foehr, however, one roe buck and a doe died shortly after their arrival, leaving a founder population of only two bucks and one doe (Niethammer 1963, Rieck 1956, see also Zachos *et al.* in press a.). According to local authorities and hunters, there have never been any additional introductions to Fehmarn while on Foehr, 15 Danish roe deer (seven males and eight females) were released in the 1950s and 1970s two of which, however, died shortly after their introduction to the island.

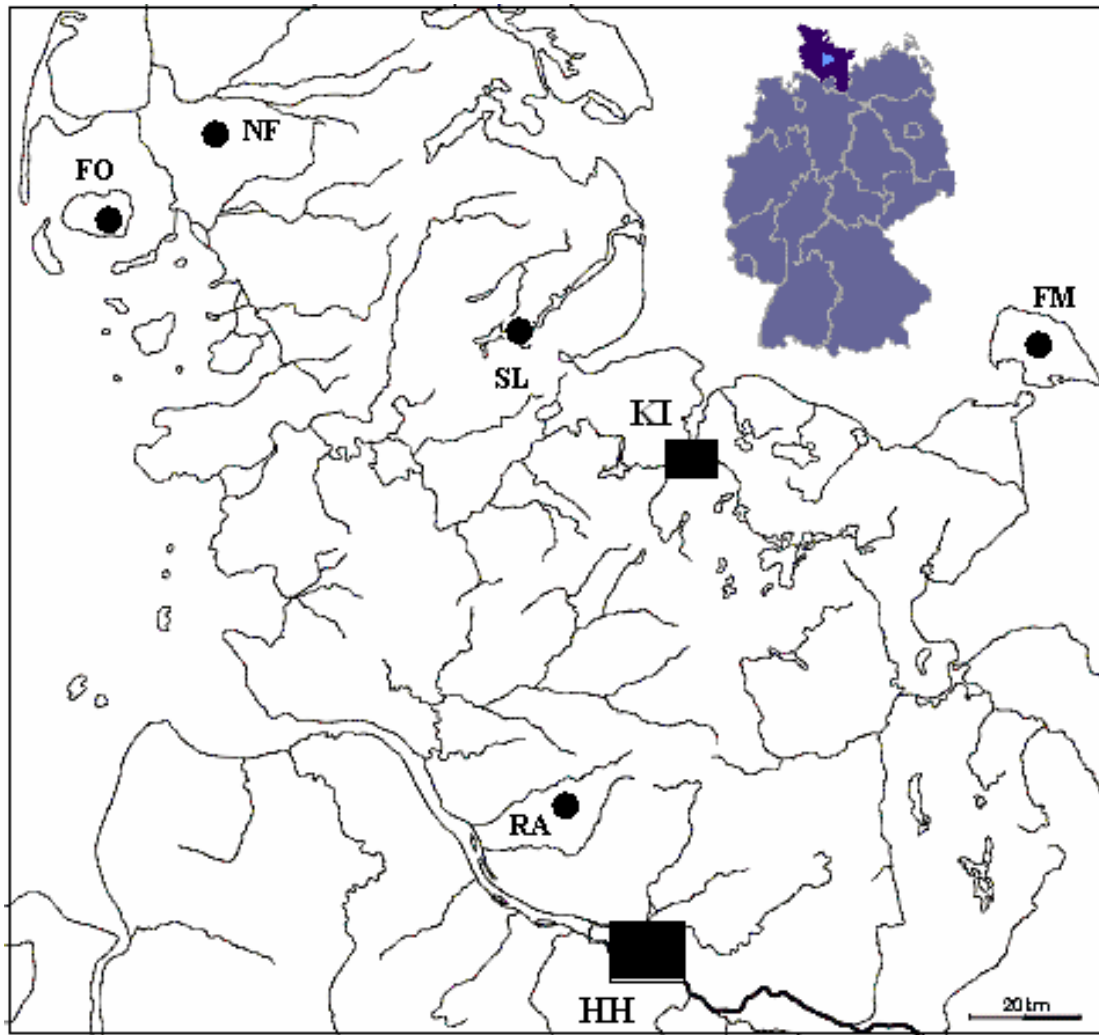


Figure 4. 1. Map of northern Germany showing the geographical location of the populations studied. FO: Foehr, NF: Nordfriesland, SL: Schleswig, FM: Fehmarn, RA: Rantzau. HH: Hamburg, KI: Kiel.

Altogether 105 roe deer specimens from five different populations in Schleswig-Holstein, northern Germany, were analysed (Fig. 4. 1, for sample sizes see Table 4. 1). Two of the populations were island stocks (Foehr, FO, and Fehmarn, FM) while the other three were mainland populations (Nordfriesland, NF, Schleswig, SL, and Rantzau, RA). The two island populations were artificially founded in the 1930s with five specimens from an area near Cologne and Dueren in western Germany on Foehr and with eight Danish founders (three males and five females) on Fehmarn. On Foehr, however, one roe buck and a doe died shortly after their arrival, leaving a founder population of only two bucks and one doe (Niethammer 1963, Rieck 1956, see also Zachos *et al.* in press a.). According to local authorities and hunters, there have never been any additional introductions to Fehmarn while on

Foehr, 15 Danish roe deer (seven males and eight females) were released in the 1950s and 1970s two of which, however, died shortly after their introduction to the island.

4. 2. 2. Allozymes

Allozymes are enzyme variants encoded by different alleles at the same locus (Harris 1980). Eight loci known to be polymorphic in roe deer from earlier studies (e. g., Hartl *et al.* 1991, 1993, Hewison 1995, Lorenzini *et al.* 1997, Wang and Schreiber 2001) were analysed. Preparation of tissue extracts, horizontal starch gel electrophoresis and enzyme-specific staining procedures were performed as described in Hartl and Höger (1986) and Grillitsch *et al.* (1992). The eight loci studied were (abbreviation and EC number given in parentheses): acid phosphatase (*Acp*, 3.1.3.2), adenylate kinase (*Ak*, 2.7.4.3), phosphoglucose isomerase (*Pgi* or *Gpi*, 5.3.1.9), isocitrate dehydrogenase (*Idh*, 1.1.1.42), lactate dehydrogenase (*Ldh*, 1.1.1.27), malate dehydrogenase (*Mdh*, 1.1.1.37), mannose-6-phosphate isomerase (*Mpi*, 5.3.1.8) and phosphoglucomutase (*Pgm*, 2.7.5.1). Due to the complete lack of any polymorphisms (see results section) no further analysis of the data was carried out.

4. 2. 3. Microsatellites

Microsatellites are short tandem-repetitive DNA units with repeat lengths of 1-6 base pairs. Mutation rates are very high leading to high allele numbers with the various alleles differing in repeat unit number and hence in length (see Goldstein and Schlötterer 1999). The 105 roe deer were genotyped for eight polymorphic microsatellite loci: OarFCB304 (Buchanan and Crawford 1993), RT1, RT7 (Wilson *et al.* 1997), ILSTS008, ILSTS058 (Kemp *et al.* 1995), NVHRT16, NVHRT21 and NVHRT24 (Røed and Midthjell 1998). DNA extraction, PCR amplification and length analysis were conducted as described in Zachos *et al.* (2003). Annealing temperatures for the PCR were as given in the cited literature but were reduced by 2°C in case of initial PCR failure. The lowered temperature results in an increased probability of primer annealing even in the case of mutations in the binding site sequence. The allele data were tested for linkage disequilibria between any two of the loci studied using the programme Fstat (Goudet 1995). The probability of null alleles being present in frequencies deleterious to our analyses was considered to be low because of the lack of complete PCR failures. Such failures occur in individuals homozygous for a null allele, and their absence suggests low null allele frequencies if there are any at all. Expected (H_E) and observed (H_O) heterozygosities as well as significant deviations from Hardy-Weinberg equilibrium (HWE) for single loci were calculated and tested with Arlequin (Schneider *et al.* 2000). Arlequin was also used for calcu-

lating the overall fixation index F_{ST} and R_{ST} (a microsatellite-specific analogue of F_{ST}), yielding the proportion of the total genetic diversity accounted for by the differentiation among (as opposed to variation within) populations, as well as for testing population differentiation on the basis of pairwise F_{ST} values. Population differentiation was further quantified with Nei's (1978) genetic distances adjusted to small sample sizes and chord distances (Cavalli-Sforza and Edwards 1967), both calculated with the software Genetix (Belkhir 2000), and by counting private alleles (alleles exclusive to only one of the populations studied, Slatkin 1985). In order to obtain a measure of allelic diversity (mean number of alleles per locus) independent of sample sizes, allelic richness values were calculated with Fstat by adjusting measures of alleles per locus to the smallest number of individuals typed for a locus in a sample (in our case, 16). Fstat was also used to calculate the inbreeding coefficient F_{IS} for each of the five populations and to test for significant differences in F_{IS} , H_E and allelic richness between the island group (FO and FM) and the mainland group of populations (NF, SL and RA). Tests for recent bottlenecks in the populations studied were carried out with the Bottleneck software (Cornuet and Luikart 1996) using, as recommended for microsatellite data by the authors, the Wilcoxon test of deviation from mutation-drift equilibrium under the Two-Phase-Model (TPM) of microsatellite evolution with 70% of the mutations following the strict Stepwise Mutation Model and 30% being multistep changes. This approach is based on the fact that recently bottlenecked populations often show a heterozygosity excess at selectively neutral loci relative to expectations under mutation-drift equilibrium. The reason for this excess is that allelic diversity is reduced faster than heterozygosity during a bottleneck leading to a relative deficiency in allele number.

As for the mtDNA and correlation analyses, Bonferroni corrections were applied whenever multiple tests were carried out.

4. 2. 4. Mitochondrial DNA

The mitochondrial control region, also known as the D-loop, is a part of the mitochondrial DNA that comprises a conserved central region flanked by two highly variable peripheral domains which yield sufficiently high numbers of different haplotypes for studies at the intraspecific level (cf. Douzery and Randi 1997). 404 bp of the control region were sequenced in 104 individuals with an ABI 377 automatic sequencer using a modified ProL (5'-CAGTCTCACCATCAACCCCAAAGC-3') and a DLH primer (5'-CCTGAAGTAAGAACCAGATG-3') (cf. Nies *et al.* 2005). PCR amplification was conducted as described in Zachos *et al.* (2003) with an annealing temperature of 58°C. Sequences

were aligned using BioEdit version 5.0.9 (Hall 1999). Haplotype and Jukes-Cantor corrected nucleotide diversities (the latter taking into account not only the number of different haplotypes but also the amount of difference between them) were calculated with the DnaSP software (Rozas and Rozas 1999). In addition, Jukes-Cantor corrected net nucleotide diversities were calculated, also with DnaSP, as a pairwise measure of genetic distance between populations. This parameter is mathematically similar to the nucleotide diversity and can be regarded as a distance between two populations corrected for the variation within these populations. Overall and pairwise F_{ST} -values were calculated with Arlequin considering not only haplotype frequencies but also the differences between them as estimated by Kimura-2-parameters. According to the intrinsic transition-transversion ratio of the combined data set, transversions were weighted 7.66-fold over transitions in these calculations. Since some of the statistical programmes do not consider deletions they were treated as transversions in all analyses in order not to lose informative sites.

4. 2. 5. Correlation analyses

Relationships between variability indices (expected heterozygosity, inbreeding coefficient, allelic richness, haplotype and nucleotide diversity) were tested by means of Spearman rank correlations using the SPSS software version 10.0. Mantel tests for pairwise correlations among genetic distances based on microsatellite and sequence data were performed with the programme NTSYS (Rohlf 1993).

4. 3. RESULTS

4. 3. 1. Allozymes

All scorable allozymes of the eight loci (PGM-2 was not scorable due to unequivocal zymogrammes) were completely monomorphic in the roe deer studied. Any further quantitative comparisons using variability parameters were thus rendered impossible.

4. 3. 2. Microsatellites

All loci were polymorphic yielding between nine and 18 alleles. There was no significant linkage disequilibrium between the loci. Significant deviations from HWE occurred in each population for single loci. The values of expected and observed heterozygosity and allelic richness as well as the inbreeding coefficients are shown in Table 4. 1.

The total numbers of alleles ranged from 50 (FO) to 68 (NF), but of course, this number is dependent upon sample size. The number of private alleles was high: 39 (34.2%) out of a total of 114 alleles over all populations. SL showed the lowest proportion of private alleles (5.9% of all its alleles were exclusive), the respective values for the other four populations were as follows: FO 12.0%, NF 10.3%, FM 19.7% and RA 16.9%.

Table 4. 1. Genetic variability of the roe deer populations studied. H_E and H_O : expected and observed heterozygosity, F_{IS} : inbreeding coefficient, AR: allelic richness, HD: haplotype diversity, π : nucleotide diversity. H_E , H_O , F_{IS} and AR refer to microsatellite loci, HD and π to the mitochondrial sequences. H_E , H_O and AR are calculated over all eight loci. N: sample size for microsatellite and (in parentheses) mitochondrial sequence analyses.

Pop. N	H_E	H_O	F_{IS}	AR	HD	π [%]
FO 18 (16)	0.74	0.55	0.239	6.1	0.892	1.533
NF 22 (23)	0.78	0.64	0.180	7.8	0.834	0.547
SL 16 (16)	0.76	0.55	0.271	6.4	0.650	0.223
FM 26 (26)	0.74	0.58	0.213	6.5	0.729	0.723
RA 23 (23)	0.79	0.64	0.187	7.9	0.636	0.253

There was no statistically significant difference between the island and the mainland populations with regard to expected heterozygosity, allelic richness or inbreeding coefficient. Overall genetic differentiation was 7.04% (F_{ST}) and 7.68% (R_{ST}), respectively, meaning that 7-8% of the total genetic variation was accounted for by differences among populations. All populations were significantly differentiated from one another as shown by pairwise F_{ST} -values significantly different from zero (Table 4. 3). Nei and chord distances are shown in Table 4. 2. The results of the bottleneck tests did not show any significant heterozygosity excess for the five populations (p-values ranged from 0.125 to 0.727).

4. 3. 3. Mitochondrial DNA

The 104 roe deer analysed yielded 27 different haplotypes with 26 variable sites: 23 transitions, one transversion and two deletions (Table 4. 4). Haplotypes 1 and 3 were the most common, both present in four of five populations, at least partly in high numbers (Table 4. 5).

Table 4. 2. Nei's 1978 (above diagonal) and chord (below diagonal) distances between roe deer populations based upon allele frequencies of eight polymorphic microsatellite loci.

	FO	NF	SL	FM	RA
FO		0.081	0.298	0.247	0.345
NF	0.078		0.414	0.134	0.298
SL	0.082	0.06		0.219	0.29
FM	0.079	0.056	0.075		0.254
RA	0.087	0.074	0.079	0.07	

None of the haplotypes found occurred in all populations but 23 of the 27 haplotypes (85.2%) were exclusive to one of the five populations (private haplotypes). In the FO population, nine haplotypes were found eight of which (88.9%) were private haplotypes.

Table 4. 3. Pairwise F_{ST} -values (given in %) calculated from microsatellite (above diagonal) and mitochondrial sequence (below diagonal) data and net nucleotide diversities given in percent (in parentheses below diagonal). The asterisk (*) denotes the only non-significant F_{ST} -value.

	FO	NF	SL	FM	RA
FO		9.868	8.757	7.713	9.064
NF	40.408 (8.876)		4.044	4.116	6.944
SL	54.521 (1.051)	8.775* (0.043)		6.605	7.324
FM	42.615 (0.750)	24.913 (0.212)	37.454 (0.326)		6.954
RA	57.942 (1.049)	25.742 (0.139)	21.221 (0.065)	41.899 (0.363)	

Table 4. 4. Mitochondrial haplotypes (HT) found in this study. Vertical numbers refer to the aligned sites in the 404 bp data set (only variable sites are shown). Dots indicate identity with HT 1, dashes denote deletions.

Haplo-type	0	0	0	0	0	0	0	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	
Haplo-type	1	1		5	6	8	8	1	5	5	6	1	1	2	2	2	3	3	4	4	5	5	6	8	1	3	
Haplo-type	0	1	2	9	2	1	6	9	0	9	2	5	8	2	3	4	1	7	1	3	3	7	9	5	0	2	
HT 1	A	G	A	A	A	A	A	G	G	T	G	G	C	G	A	A	A	A	A	T	T	G	C	T	T	C	
HT 2	A	
HT 3	A	
HT 4	A	T	A	.	.	C	.	
HT 5	A	.	T	C	.	.	.	C	.	
HT 6	A	.	.	A	T	.	G	.	G	.	.	.	C	.	.	C	.	T	.	
HT 7	.	.	G	A	.	T	C	.	.	.	C	.	.	
HT 8	.	.	G	.	.	G	.	A	.	.	A	.	.	G	.	G	.	.	.	C	.	.	C	.	T	.	
HT 9	-	-	A	.	.	A	T	.	G	.	G	.	.	.	C	.	.	C	.	T	.	
HT 10	.	.	G	.	.	G	.	A	.	.	A	T	.	G	.	G	.	.	.	C	.	.	C	.	T	.	
HT 11	G	.	A	.	.	A	T	.	G	.	G	.	.	.	C	.	.	C	.	T	.	
HT 12	.	.	G	.	G	G	G	.	A	.	.	A	T	.	G	.	G	.	.	.	C	.	.	C	.	T	.
HT 13	A	.	.	.	A	
HT 14	A	
HT 15	G	G	C	.	.	
HT 16	C	.	.	T	C	.	.	
HT 17	G	.	.	C	.	.	T	T	C	.	.	
HT 18	A	T	C	.	.	
HT 19	A	A	.	C	.	.	T	C	.	.	
HT 20	A	G	
HT 21	-	-	
HT 22	A	G	A	.	.	.	G	C	C	.	
HT 23	A	.	G	G	.	G	A	.	.	.	G	C	C	.	
HT 24	A	A	.	.	.	G	C	C	.	
HT 25	A	A	.	.	.	A	.	.	.	G	C	C	.	
HT 26	A	.	G	.	.	G	G	A	.	.	.	G	.	G	C	.	.	
HT 27	A	.	G	G	.	G	G	A	.	.	.	G	C	C	.	

Table 4. 5. Distribution of mitochondrial haplotypes (HT) among the five roe deer populations studied.

Haplotype	FO	NF	SL	FM	RA	Σ
HT1	1	7	9		9	26
HT2					11	11
HT3		1	1	13	2	17
HT4					1	1
HT5	2					2
HT6	5					5
HT7	2					2
HT8	1					1
HT9	1					1
HT10	1					1
HT11	2					2
HT12	1					1
HT13		6		3		9
HT14		4	4			8
HT15		1				1
HT16		1				1
HT17		1				1
HT18		1				1
HT19		1				1
HT20			1			1
HT21			1			1
HT22				1		1
HT23				2		2
HT24				1		1
HT25				4		4
HT26				1		1
HT27				1		1
Σ	16	23	16	26	23	104

The respective values for the other populations were as follows: NF: five (55.6%) private out of nine haplotypes, SL: two out of five (40%), FM: six out of eight (75%), and RA: two out of four (50%). It has to be stated, however, that most of the private haplotypes occurred in low frequencies (16 of the 23 were only found once), which makes haplotype 2 even more re-

markable. This haplotype, exclusive to RA, was found in 11 of the 23 sampled individuals of that population.

Overall F_{ST} was 41.38%, the pairwise F_{ST} -values are shown in Table 4. 3. All but one pairwise value were significantly different from zero, the exception being the one between SL and NF, which was not significant at the 5%-level after Bonferroni-correction.

Table 4. 6. Results of the correlation analyses between distance measures. Correlation coefficients (normalized Z-values of the Mantel tests) are given below the diagonal, the respective p-values (Bonferroni-adjusted significance level $p = 0.005$) above the diagonal. Nei78: Nei's (1978) genetic distance corrected for small sample sizes, Chord: chord distances, $F_{ST}(ms)$: pairwise F_{ST} -values based on microsatellite allele frequencies, NND: net nucleotide diversities, $F_{ST}(mt)$: pairwise F_{ST} -values based on mitochondrial haplotypes.

	Nei78	Chord	$F_{ST}(ms)$	NND	$F_{ST}(mt)$
Nei78		0.011	0.009	0.062	0.042
Chord	0.85		0.01	0.071	0.052
$F_{ST}(ms)$	0.95	0.9		0.051	0.027
NND	0.63	0.69	0.79		0.011
$F_{ST}(mt)$	0.68	0.73	0.82	0.93	

4. 3. 4. Correlation analyses

There were no significant Spearman correlations between any two variability indices – neither between indices based on the same marker system nor between indices based on microsatellites and mitochondrial haplotypes. The only p-value below 0.05 was the one for the correlation between expected heterozygosity and allelic richness ($r_s = 0.821$, $p = 0.044$). This value, however, was not significant after Bonferroni-correction (the adjusted significance level with 10 pairwise comparisons was 0.005).

The results of the Mantel tests for matrix comparison are shown in Table 4. 6. None of the correlations was significant after Bonferroni adjustment of the p-values for multiple tests but there was a clear tendency towards low p-values: they ranged from 0.009 to 0.071 with the corrected significance level of the 5%-criterion being 0.005 (10 pairwise comparisons). Expectedly, the correlation coefficients (normalized Z-values) were higher for comparisons of parameters based on the same marker system (microsatellites or mtDNA sequences, respec-

tively), ranging from 0.85 to 0.95, than for comparisons between different markers ($0.63 < Z < 0.82$).

4. 4. DISCUSSION

The complete lack of polymorphisms at the reportedly polymorphic enzyme loci used in this study is astonishing. Herzog *et al.* (1993) found a lack of genetic variation in the transferrin system, a protein system highly polymorphic in other cervid species, in German roe deer populations but this monomorphism may be characteristic of the whole species whereas in the present case, the loci studied have been selected due to their known polymorphisms in roe deer. Unfortunately, Wang and Schreiber (2001) did not analyse the two roe deer populations from Schleswig-Holstein (which they genotyped for microsatellites) with regard to the allozymes they studied in other populations. Since these enzyme loci included three loci also examined in the present study (*Ak*, *Mpi*, *Pgm*) it would have been interesting to see if the allozymic homogeneity found by us is confined to our populations or if it is a more common phenomenon in northern Germany. The most probable explanation for this lack of variability is the demographic history of the roe deer in northern Germany: in the middle of the 19th century, the roe deer population was nearly driven to extinction through relentless persecution (Jessen 1988). This bottleneck may still be visible in the allozyme patterns of the species although introductions from other parts of Europe (e. g., Denmark, Poland and Hungary) have been carried out since then (Jessen 1988, Niethammer 1963). This hypothesis, however, stands in clear contrast to the very high variability of the microsatellites and the mitochondrial control region (see below). Alternatively, it cannot be ruled out that our results are an artefact of the eight loci studied and that allozymic variability would increase significantly if further loci were screened as was the case in red foxes and mustelids, which showed no polymorphisms at the loci studied by Simonsen (1982) but were highly polymorphic at the loci studied by Frati *et al.* (1998) and Hartl *et al.* (1988). Yet, given that exactly the loci which we analysed proved to be polymorphic in roe deer from very different parts of Europe, we consider this alternative to be rather unlikely.

Microsatellite variabilities in terms of expected heterozygosity were much higher in our five populations (0.74 – 0.79) than in the 12 Italian populations analysed by Lorenzini *et al.* (2002: 0.17 – 0.58) and in the 27 populations from Germany, the Netherlands and France studied by Wang and Schreiber (2001: 0.33 – 0.66; the two populations from Schleswig-

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Holstein exhibited values of 0.54 and 0.50, respectively). In the latter two studies, the microsatellite loci yielded distinctly lower numbers of alleles than in our study, which is probably the reason for the discrepancies in heterozygosity as another comprehensive study with an average of 14.1 alleles per locus showed values of expected heterozygosity between 0.53 and 0.79 (Randi *et al.* 2004). Taken together, the roe deer from northern Germany show a variability not unmatched by other populations but certainly at the upper end of the species' range.

Even more astonishing than the high variability at the microsatellite loci as compared to the allozymic homogeneity is the very high number of mitochondrial haplotypes in our roe deer which showed, on average, one distinct haplotype over 3.85 individuals ($104 / 27 = 3.85$). This is in line with previous results on control region variability: Randi *et al.* 2004 arrived at a respective ratio of 4.52 and Nies *et al.* 2005 even at a ratio of 1.71. If one further takes into consideration that the sequences analysed by the two other studies were longer (704 and 453 bp, respectively, as compared to 404 bp in our study) and that the geographic scale was much larger, the variability of the Schleswig-Holstein roe deer is even more remarkable and by far higher than comparable values for red deer as shown by Feulner *et al.* (2004: 582 bp of the control region analysed in 120 individuals from southeastern Europe yielded merely 14 haplotypes) and an unpublished analysis of red deer from the very area that the roe deer of the present study come from (Schleswig-Holstein, northern Germany; here, 437 bp of the control region sequenced in 52 specimens resulted in only 4 haplotypes). Also, similarly to the private microsatellite alleles, the number of private haplotypes is large but the very high proportion of 85.2% is due mainly to the high values of the two island populations. For the Föhr (FO) population, the repeated introductions may account for this, and the same almost certainly holds for Fehmarn (FM) in spite of the hunters' claim that after the initial introduction in 1935, there have been no further releases. This claim is definitely wrong as the five founder females could have possessed only five different haplotypes but we have found eight showing pairwise differences of 1 – 8 mutations! Thus, there must have been additional introductions of female roe deer to the island of Fehmarn in later times, a conclusion supported by the bottleneck analyses which found no detectable reduction of effective population size for either of the populations and also backed by the fact that the island populations – although showing on average lower values of genetic variability (Table 4. 1) - did not differ significantly from the mainland in terms of variability as measured by expected heterozygosity, inbreeding coefficient and allelic richness. The negative results of the bottleneck analyses in spite of the population breakdown due to overhunting in the 19th century may cautiously be interpreted as evidence that reintroductions were more common than mentioned in the literature.

With regard to genetic variability within populations, microsatellite and control region data are somewhat incongruent, in accordance with the lack of any significant correlation between the variability parameters. FO, e. g., has the lowest heterozygosity and allelic richness values and the second highest inbreeding coefficient but, at the same time, shows the highest haplotype and by far the highest nucleotide diversity. Similarly, RA yields the highest heterozygosity and allelic richness and the second lowest inbreeding coefficient but also shows the lowest haplotype and the second lowest nucleotide diversity, which is far lower than the values of the remaining three populations. In contrast to that, the different microsatellite-based variability indices yield more consistent results in that the populations with the highest heterozygosity (NF and RA) also have the highest allelic richness and the lowest inbreeding coefficients. Yet, this is not mirrored by the results of the Spearman correlations. Differences between the sequence-based indices are larger but this is due to the way they are calculated: low numbers of significantly different haplotypes will result in low haplotype but rather high nucleotide diversities while high numbers of only slightly different haplotypes will yield high haplotype and low nucleotide diversities. A possible reason for the striking differences in the FO population is the repeated introduction of individuals: at least eight females were released after the initial foundation of the population (Zachos *et al.* in press a.). Due to the generally much higher number of microsatellite alleles than haplotypes in a population, the introduction of small numbers of females is more influential in the mitochondrial gene pool than in the nuclear one. Moreover, the effective population size (N_e) for the mitochondrial genome is, due to its being haploid and inherited by one sex only, a priori only one fourth of the nuclear N_e . Thus, the addition of a new haplotype to the gene pool is a relatively larger increase in variability than the addition of two more microsatellite alleles.

As to the pairwise distance values, it is obvious that the population most differentiated from the others is FO, which might be accounted for by its hybrid origin from Danish and west-German animals. The mainland populations exhibit only little differentiation in the mitochondrial control region among each other (see pairwise F_{ST} -values and net nucleotide diversities) whereas the microsatellite-based indices do not show marked differences between islands and mainland. This may be either a result of random drift or of a male-biased introduction policy in the process of restocking the north-German populations in the second half of the 19th century. These males would have left their traces in the nuclear but not the mitochondrial genome. Interestingly, the only pairwise F_{ST} -value not significantly different from zero after Bonferroni-correction was the mtDNA-based one between SL and NF. This value was much lower than all the other mitochondrial ones and even lower than some of the microsatel-

lite-based F_{ST} -values. SL and NF are the only two populations without geographic barrier in between them since RA, the third mainland stock, is separated from SL and NF by the Kiel Canal which is known to be crossed by roe deer sometimes but which nonetheless considerably reduces the gene flow between the northern and the southern part of Schleswig-Holstein. In our opinion, the pairwise differentiation tests nonetheless suggest that all populations may be regarded as separate stocks since the microsatellite test showed a significant difference even for SL and NF. For the island populations, the detailedly known history of their origin can be viewed as further evidence of their distinct status.

Although the Mantel tests did not yield significant correlations after Bonferroni-corrections, most of the p-values were smaller than 0.05 with the highest one being 0.071. Nies *et al.* (2005) conducted Mantel tests for detecting correlations between allozyme-based Nei (1978) and modified Rogers distances on the one hand and net nucleotide diversities calculated from control region sequences in roe deer from Switzerland, Austria, Slovenia and Slovakia on the other. They found much weaker consistencies between nuclear and mitochondrial distances ($Z = 0.15$, $p = 0.172$ for Nei distances and net nucleotide diversities; $Z = 0.20$, $p = 0.128$ for Rogers distances and net nucleotide diversities; these values are not given in the original publication), which are, however, not directly comparable to our results because they are based on allozymes and sequences while the values given in Table 4. 6 refer to microsatellites and sequences.

There are a few other studies on roe deer using microsatellites and mitochondrial DNA data. Randi *et al.* (2004) conclude that both maternal and biparental markers describe concordant patterns of population structure with microsatellites being more capable of uncovering recent demographic events. Nevertheless, there are considerable differences in several of their populations between mitochondrial (haplotype diversity and average pairwise sequence divergence) and microsatellite variability (expected heterozygosity). Lorenzini *et al.* (2003), analysing mitochondrial RFLPs and microsatellites in Spanish roe deer populations, also found “highly concordant” results in their phylogenetic trees. Moreover, F_{ST} -values derived from mitochondrial RFLP data were significantly correlated with both Rho, an unbiased estimator of R_{ST} , and F_{ST} -values based on microsatellites ($p < 0.05$). The exact p-values are not given and Bonferroni-correction was probably less strict due to the smaller number of pairwise tests than in the present analysis. Similarly, Lorenzini *et al.* (2002), in an analysis of Italian roe deer, arrived at concordant results drawn from mitochondrial RFLPs and microsatellites, both with regard to their phylogenetic analysis and variability indices derived from the two marker systems.

4. 4. 1. Conclusions

The roe deer populations from northern Germany of this study exhibit a remarkable discrepancy between allozymic monomorphism on the one hand and high or even very high variability at microsatellite loci and the mitochondrial control region on the other. As in the case of the population from the island of Föhr, these differences are at least partly explicable when the demographic history is taken into account. In terms of microsatellite diversity, the Schleswig-Holstein roe deer are within the range of values reported in the literature for this species although variability is very high. The presented level of D-loop variability is one of the highest ever found in roe deer and very probably a result of extensive introductions and translocations. In particular, the island population of Fehmarn must have been repeatedly restocked with females, which shows that information from local authorities should be viewed critically when it comes to popular game species. Clearly, the north-German roe deer are genetically much more diverse than their sympatric relatives, the red deer, which have been listed as “near threatened” by human-induced habitat fragmentation and inbreeding in the latest regional Red List.

Concordance of microsatellite-based and control-region-based variability indices was low and not significant but genetic distances derived from the two marker systems showed a clear tendency to yield concordant results as judged from the low p-values most of which were below the usual significance level of 5% before Bonferroni-correction. These results suggest that the analysis of differentiation among populations may be less marker-dependent than studies of variability within populations. Especially when dealing with small populations, the different effective population sizes for diploid, biparental nuclear and haploid maternally-inherited mitochondrial markers may lead to distinctly different or even contradictory assessments concerning genetic variability. Analysis of both nuclear and mitochondrial marker systems is therefore strongly suggested when taxa relevant for conservation or management are examined with regard to genetic diversity within populations.

SUMMARY

The red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) are common free-ranging mammals and popular game species in Europe. Although they are not endangered in terms of population numbers both species' gene pools are affected by a variety of anthropogenic influences such as (re)introductions, translocations, habitat fragmentation, keeping of populations in enclosures and trophy hunting. Consequently, populations locally become increasingly isolated and mixed with animals of foreign populations. Many autochthonous stocks have been hybridized with introduced animals which blurs the historical boundaries between formerly natural populations. Genetic variability within and among populations is an important factor for the survival and adaptability of the stocks and is usually assessed by molecular approaches. Different molecular marker systems provide highly appropriate tools, e. g. allozymes, microsatellites and sequences of the mitochondrial control region. These marker systems have already been shown to be of great use in genetic studies of deer species dealing with general population genetics, conservation and phylogeography. The present studies constitute further applications of these markers to uncover genetic structures and phylogeographical patterns, to evaluate the conservation genetic status of populations and to analyse differences between the marker systems. These differences have an important bearing on the interpretation and comparability of studies using different markers.

The present dissertation is composed of four different studies.

Study 1:

Red deer of the endangered populations from Sardinia and Mesola Wood, northern Italy, were analysed with reference to genetic variation at 531 bp of the mitochondrial control region and twelve polymorphic nuclear microsatellite loci. A phylogenetic analysis was conducted including additional data from the literature to gain insight into the phylogeographic origin of the Sardinian subspecies *Cervus elaphus corsicanus*. Microsatellite variation was low in both populations but Sardinia showed comparatively high variability at the control region. Management recommendations are discussed. In particular, the Mesola red deer, the only remaining indigenous Italian population, ought to be managed to increase effective population size and subdivided into two or more populations. As to the phylogeography of the Sardinian population, microsatellite data favour mainland Italy as the place of origin in that Sardinia and Mesola show the smallest distance values and are paired together in respective trees with high bootstrap support. However, the mitochondrial data only partially confirm this

Summary

conclusion but show great similarity between Sardinian and Spanish red deer. Possible explanations for this discrepancy and general limits of mitochondrial sequences in resolving demographic and biogeographic processes of the recent past are discussed.

Study 2:

A total of 69 red deer from seven populations in Scotland and England were analysed regarding variability within and differentiation among stocks using eleven polymorphic microsatellite loci and 439 bp of the mitochondrial control region. The island population of Islay exhibited no mitochondrial variation at all but average microsatellite variability. Like in a population from northern Germany, the locus NVHRT16 was monomorphic in three populations (Islay, Dunachton and Achnacarry). Overall and pairwise F_{ST} -values indicate considerable differentiation among the populations studied. However, the two adjacent populations free from introductions, Dunachton and Achnacarry, only showed little differentiation and were paired in trees based on genetic distances. No statistically significant differences in terms of variability were observed between island and mainland populations and the overall test of isolation by distance was negative. Possible reasons (e. g., differences in human impact on the populations) for the genetic patterns observed as well as the discrepancy between analyses of haplotype phylogeny and haplotype distribution are discussed.

Study 3:

An analysis of three isolated red deer populations from Schleswig-Holstein was carried out. The total sample size was 105 individuals and the study was part of a more comprehensive project dealing with the effects of habitat fragmentation, low effective population size and inbreeding on red deer in northern Germany. Genetic variability as measured by expected heterozygosity was lower than in 12 other European red deer populations but higher than in the endangered indigenous red deer populations from Sardinian and Mesola. Together with data from other populations from Schleswig-Holstein and data from mitochondrial control region sequences, the results clearly show the genetic depletion of these north-German red deer and have implications for regional management plans, particularly in light of recent evidence of severe inbreeding depression (morphological malformations found in several individuals). Plans for future studies and the synthesis of the different subanalyses are outlined.

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Study 4:

Three mainland and two island roe deer (*Capreolus capreolus*) populations with a total sample size of 105 individuals from Schleswig-Holstein, northern Germany, were analysed with regard to genetic variability within and differentiation among populations as revealed by eight allozyme loci known to be polymorphic in roe deer, eight microsatellite loci and 404 bp of the mitochondrial control region. Surprisingly, the allozymes were completely monomorphic, but microsatellite and control region variability were high. Hypotheses as to demographic reasons for the variability patterns found, including bottlenecks, founder effects and translocations, are put forward. There were no statistically significant differences between the island and the mainland populations in terms of genetic variability as measured by expected heterozygosity, inbreeding coefficient and allelic richness. The correlations of the various variability indices were not statistically significant after Bonferroni-correction. Nevertheless, there was a clear tendency for differentiation indices to yield concordant results for microsatellite and mitochondrial markers.

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Declaration

DECLARATION

I hereby declare that I have carried out the analyses and written the thesis myself and that I did not use any devices or received relevant help from any persons other than those mentioned in the text. This dissertation has not been submitted before.

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