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Population genetic history of the British roe deer (*Capreolus capreolus*) and its implications for diversity and fitness

By

Karis Baker

School of Biological and Biomedical Sciences

Durham University

2011

Submitted for the degree of Doctor of Philosophy

Abstract

The first part of this study examined post glacial recolonisation by UK roe. Previous studies established three main roe deer lineages exist across Europe: a western (Iberian Peninsula), an eastern (Balkan region) and a central lineage (which spans across central Europe). It was unknown which group British roe deer populations belonged. Using a 419 bp region of the mt-DNA d-loop (HVR1) amplified from ancient and modern UK samples a direct comparison was made with previously published European data. Results showed that UK populations belong to the central lineage, indicating a post glacial re-colonisation that is likely to have occurred via an eastern route. The estimation of a substitution rate, which was applied to coalescent based methods, detected a signal for divergence of UK roe from continental roe at 5,600 YBP (HPD 3,500 - 11, 200 YBP), not long after the proposed date for the land bridge split (7,500 YBP).

Since post glacial re-colonisation, roe were known to have undergone severe fluctuations in population size. Perhaps the most significant fluctuation began during the medieval period, when roe suffered severe declines (bottlenecking) due to over hunting and deforestation. These declines were so severe that, by the 16th century, roe were believed to have been extirpated (locally extinct) from all southern areas of UK and considered scarce in northern areas. However, by the 19th century roe began to recover. Recovery in the south may have resulted solely from re-introductions (involving both native and non-native stocks) whilst, in the north, recovery resulted from natural re-colonisation from remnant native stocks. The second part of this study investigated the impacts of this more recent history.

This was first investigated using a 750 bp of the mt-DNA d loop region (HVR), 16 microsatellite loci and 18 skull traits from modern roe from across the UK to examine structure and diversity. Results based on both DNA and morphology revealed strong differentiation. Northern roe appeared least impacted by recent events; maintaining patterns of isolation by distance (IBD) and high genetic diversity (compared to southern populations). In contrast, southern roe appeared more strongly impacted by recent events; in particular, IBD was non-significant (although this may have been due to a sample size effect) and genetic diversity was lower (compared to northern populations). The roe re-introduction records indicated that the south western population was native in origin (Perthshire). Genetic

data showed that this population was, however, highly differentiated from its proposed source; which could reflect the powerful impact of genetic drift resulting from small founder populations. Alternatively, it may be that the ancestry of the south western population is more complex than previously assumed. For the other southern population (Norfolk), re-introduction records indicate a non-native (German) origin. In line with this, both genetic and morphological data implied that these roe were highly distinct.

The impacts of bottlenecks (including medieval declines and founder events) on roe populations were also examined. Bottleneck analyses examined ‘signatures’ in modern populations based on 16 microsatellites. The strongest evidence of bottlenecking was detected in the Norfolk population, consistent with the small founder group size introduced into this location relatively recently. For the other populations bottleneck signatures tended to be weak and non-significant. Direct comparisons of ancient (pre-bottleneck) and modern (post –bottleneck) populations were made based on 419 bp of mt-DNA d –loop (HVR1). Results showed considerable losses in genetic diversity between time frames consistent with medieval declines. Northern populations were also found to harbour the highest number of ‘native’ haplotypes and southern populations the lowest. The southern population of Norfolk exhibited only one ‘novel’ haplotype confirming its non-native origin.

The impacts of bottlenecks on populations are of concern because they have been shown to reduce population fitness and increase the risk of extinction. Therefore, fitness of roe was examined using fluctuating asymmetry (FA) of 10 skull traits as an indicator of developmental stability. Correlations of FA and genetic diversity indices were examined at the level of individuals within populations, across all populations and among populations. All correlations existed in expected directions; however, correlations tended to be weak and non-significant. Furthermore, among population level FA did not vary significantly across populations providing no indication as to whether fitness has been impacted by past population history.

Declaration

The material contained within this thesis has not previously been submitted for a degree at Durham University or any other university. The research reported here has been conducted by the author unless stated otherwise.

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Chapter 1 : General Introduction

1.1 Evolutionary forces that shape population genetic diversity and structure

Genetic structure and diversity of populations results from the joint action of four main evolutionary forces; mutation, natural selection, genetic drift and gene flow. Mutation, which results from the imperfect replication of DNA, generates all genetic variation for evolution (Barton and Partridge, 2000). However, mutations that directly impact evolution occur at very low frequencies (Papadopoulos et al., 1999) and take time, in the order of thousands of years, to occur. Natural selection is the only evolutionary process that directly results in populations becoming adapted to their environments (Brandon, 1990). For natural selection to occur there must be differences in fitness among individuals and a genetic basis for these differences (Endler, 1986). Over time, there will be a differential success of individuals contributing to the next generation where individuals most suited to the environment will contribute more. Gene flow acts to homogenise variation among populations by exchanging individuals among populations. There are many models that describe gene flow among populations including; the island model (Wright, 1931), the isolation by distance model (Wright, 1943) and the stepping stone model (Kimura and Weiss, 1964). In the absence of other evolutionary forces it is estimated that the exchange of one individual per generation can prevent significant genetic differentiation among populations (Wright, 1943; Mills and Allendorf, 1996). Genetic drift can simply be defined as a random sampling process of alleles between generations (Endler, 1986). The impact of genetic drift only becomes significant to a population when it becomes small. When population sizes are small, rare alleles are lost faster than they can be replaced by mutation (Slatkin, 1985). This has the effect of eroding genetic diversity and causing random changes in allele frequencies.

The relative effects of these evolutionary forces shape the genetic population structure of a species. For populations that have been in stable environments and connected by dispersal over long periods of time, an equilibrium will be reached where losses of alleles, as a result of drift, will be balanced by introduction of new alleles through migration (Wright, 1951). Most natural populations, are however, not in equilibrium (McCauley, 1993) because of disruption from at least the historical past and sometimes more recent ongoing processes

(Templeton et al., 1995). For such populations insufficient time has passed for the population to attain equilibrium.

1.1.1 Historical processes: impacts of the Pleistocene

During the last 65 million years (Myr) the Earth's climate became cooler with frequent oscillations (interglacial – glacial cycles) that increased in amplitude and led to a series of major ice ages of the Quaternary (2.4 Myr to present) (Hewitt, 2000). These fluctuations were significant for worldwide biota (Hewitt, 1996). During the height of each glacial cycle, ice sheets would have covered most of the northern hemisphere forcing temperate animal populations to survive in fragmented southern unglaciated refugia. During the warmer interglacials species would have been able to expand northwards to recolonise previously glaciated regions (Hewitt, 1996). This pattern of movement which coincided with the glacial cycles describes the basic expansion–contraction (EC) model; a simple paradigm for the demography of species (Provan and Bennett, 2008). This process of expansion and contraction served to cause differentiation between populations. During contraction phases, the refuges would have been isolated, breaking continuous distributions and causing significant genetic divergence (Hewitt, 2000). Although during the expansion phase isolated populations may have formed secondary contact zones, the length of time that populations were isolated would usually have meant that the signature of isolation would be retained. As a result, the genetic legacy of the Quaternary has been proposed to explain current intraspecific variation in a number of species (Hewitt, 2000). This intraspecific variation has been studied through phylogeography.

Phylogeography is 'the field concerned with the principle and processes governing the geographical distribution of genealogical lineages especially those at the intraspecific level' (Avise et al., 1987). This approach has become particularly useful in identifying congruent patterns of genetic variation among co-distributed taxa, enabling the determination of common refugia and post glacial recolonisation routes. In Europe in particular, three main recolonisation routes, repeated in many species, are exemplified by the patterns shown by the grasshopper, *Chorthippus parallelus*, the bear, *Ursus arctos* and the hedgehog, *Erinaceus europeus* (see Hewitt and Godfrey, 2004). Phylogeography has not only enabled insight to be gained on the spatial patterns of refugia and post glacial recolonisation, but also on estimating divergence times, made possible through the application of the molecular clock.

The molecular clock has become one of the most important aspects of evolutionary biology. Zuckerkandl and Pauling (1965) were the first to propose that evolutionary divergence could be estimated using calibrated sequence differences. The authors postulated that the amount of difference between DNA molecules was a function of time since evolutionary separation. In this way, translation of genetic variation into geological time could allow correlation of the origin or diversification of evolutionary lineages to the nearest major perturbation events approximating this geological time (Van Tuinen et al., 2004). Therefore, by applying the molecular clock to a set of sequences it can be seen whether past environmental change coincides with major evolutionary transitions. Despite the potentially valuable contributions the molecular clock can bring to studies date estimates have not been without controversy.

Molecular clocks can be difficult to implement. This is because in order for a clock rate to be estimated, reliable calibration points are needed to translate genetic measurements onto an absolute time scale. Traditionally, fossil or biogeographic sources have been used to calibrate the molecular clock (Villablanca, 1994). Using such points as calibration, universal clock rate estimates referred to as 'phylogenetic rates' have been established. For example, the phylogenetic rates of the mitochondrial protein-coding genes of birds and mammals have traditionally been recognized to evolve at approximately 1 % per million years (Myr) (Brown et al., 1979; Shields and Wilson, 1987), with the non-coding D-loop evolving several times more quickly. However, the recent development of ancient DNA technology which enables direct calibration of rate estimates within populations or species has recently challenged our views on the reliability of 'phylogenetic' rates.

Ancient DNA has enabled the molecular clock to be directly calibrated by using individuals from different time fames (Lambert et al., 2002). A number of recent studies that have obtained substitution rates in this way (e.g. Shapiro et al., 2004; Saarma et al., 2007; Ho et al., 2008; de Bruyn et al., 2009). Interestingly, rates estimated from these studies appear much more elevated than those obtained as 'phylogenetic rates'. As a result Ho et al., (2005) argued that when using recent (< 1–2 Ma) events to calibrate the molecular clock, an inverse relationship can be observed between the age of calibration and the estimated substitution rate. These authors referred to this relationship as a 'time dependency' of mutation rates. The exact biological causes of this effect are still partly unexplained; several factors could be responsible such as sequencing, calibration errors, saturation of mutations at fast evolving

sites and finally purifying selection (Ho et al., 2005). Ho et al., (2005) propose this latter factor, purifying selection, to be the most likely cause. Purifying selection removes sequence polymorphisms over a time scale correlated with deleteriousness. As Penny (2005) describes, when observation times diminish we should observe a greater proportion of slightly deleterious mutations that have yet to be lost, with the most deleterious observed only in the short term pedigree studies. Whatever the exact cause, if time dependency exists there may have been a systematic overestimation of molecular dating of Pleistocene events calculated using ‘phylogenetic rates’ (Saarma et al., 2007; Gratton et al., 2008).

Indeed, a number of recent studies have applied new substitution rates to understand past events specifically during the Pleistocene to infer impacts on population dynamics of species. For example, ancient genetic data assembled from both North American and Asian bison from the past 150,000 years were interpreted to show evidence of population growth until approximately 37,000 years ago, when the population suffered losses in genetic diversity consistent with population size decline (Shapiro et al., 2004). Shapiro et al. (2004) concluded that the decline was likely due to climatic change since the timing of the decline was coincident with initiation of the last glacial maximum in Beringia and preceded evidence of human migration into the Americas.

1.1.2 Recent processes: impacts of bottlenecks and founder events

Recent processes that impact population genetic structure and diversity generally involve those that are induced by humans within the past few hundred years. Typically they include bottlenecks.

A bottleneck will occur when a significant percentage of a population or species is killed or otherwise prevented from reproducing. Common causes are often due to human activities e.g. habitat loss, fragmentation and over exploitation which can result in near or complete extirpation of populations. Populations can also be established by another kind of bottleneck, a founder event. Founder events occur via either natural or non-natural (human mediated) dispersal where small numbers of individuals are transferred into a new location.

Under theoretical expectation bottlenecks should result in losses in genetic diversity as a result of strong genetic drift (Wright, 1969; Nei et al., 1975; Lacy, 1987). Small bottlenecked

populations may also lose genetic diversity (through accumulation of homozygotes) as a result of increased chance of inbreeding. Inbred populations are genetically more homozygous because the probability that individuals carry alleles that are 'identical by descent' increases (Ralls et al., 1986; Lacy, 1993).

Indeed, many empirical studies have shown that bottlenecks are typically accompanied by lowered levels of genetic diversity. Some studies have shown the effects of size reduction by direct comparison against 'control' populations to which levels of genetic variability can be compared. These controls are usually conspecific populations (e.g. Wayne et al., 1991) or closely related taxa (e.g. Wildt et al., 1987; Hoelzel et al., 1993; Beheregaray et al., 2000) that have no known history of population reduction. When controls exhibit substantially more genetic diversity than the population or species being studied a hypothesis of bottlenecking or founding is accepted. One example comes from a population of gray wolves (*Canis lupus*) on Isle Royale in Lake Superior which was likely founded by a single pair. Consequently, the population exhibited a reduced genetic variability in comparison to mainland populations. Specifically, the island wolves possess only 50 % of the allozyme heterozygosity relative to mainland populations (Wayne et al., 1991).

The use of control populations can only imply a population size reduction has occurred. Bouzat et al., (1998 b) consider that the use of controls may not represent historical levels accurately because these populations will have their own set of environmental conditions and demographic histories which predetermines genetic variability.

Alternative approaches for detecting bottlenecks in natural populations have, therefore, included looking for 'bottleneck signatures' including: heterozygosity excesses, mode shift distortions and reduced allele size in modern populations (Corunet and Luikart, 1996; Luikart and Cornuet, 1996; Luikart *et al.,* 1998; Garza and Williamson, 2001). These approaches have been devised on the basis of the theoretical effects of population declines. However, one problem with bottleneck signatures is that their relative sensitivity declines as the number of generations between the bottleneck and point of sampling increases (Spencer *et al.,* 2000). This limits the capacity of using such signatures to detecting only relatively recent bottlenecks. One way to get around this problem may be to incorporate ancient DNA as a more direct methodology, to show diversity declines by direct comparison before and after a presumed perturbation (Wandeler et al., 2007).

The first use of this methodology was a study of the Illinois Greater prairie chicken population (*Tympanuchus cupido*) (Bouzat et al., 1998 b). This population was known to have gone through a severe bottleneck; population numbers fell from millions of individuals in the 1860s down to less than 50 by 1993 (Westemeier et al., 1991). An initial study indicated that the extant individuals of the population harboured only very low levels of genetic diversity (Bouzat et al., 1998 a). In order to determine whether the low genetic diversity was due to the demographic contraction Bouzat et al., (1998 b) directly compared levels of past genetic diversity with the levels of extant population diversity. The results confirmed expectations; losses in genetic diversity were accompanied by losses in specific alleles referred to as 'ghost alleles'. Since this initial study many other studies have followed suit and used similar approaches to prove definitively that bottlenecking has caused losses in genetic diversity (for review see Leonard, 2008).

Other theoretical impacts of genetic drift should be that strong shifts in allele frequencies should occur. Indeed, this has once again been supported by empirical ancient studies. Harper *et al.,* (2006) studied both historical and contemporary populations of the Adonis butterfly. Results showed that along with losses in genetic diversity over time there had also been dramatic shifts in allele frequencies. The most notable shift occurred with the predominant LbG2 (microsatellite) ancestral allele that could not be found in any of the extant populations studied. In a converse situation, a study on the whooping crane showed that the most common modern mt-DNA haplotype could only be found at very low frequencies in pre-bottleneck populations (Glenn et al., 1999).

Both these examples demonstrate shifts in allele frequencies. The shifts are unlikely to have occurred in a population at equilibrium and therefore rapid genetic drift following one or more severe demographic changes can only be responsible. Furthermore, in the case of the adonis butterfly populations (Harper et al., 2006) the complete elimination of the most common ancestral allele could only have occurred during a very severe bottleneck as theoretical predictions suggest declines will generally only remove rare alleles (Nei et al., 1975; Leberg, 1992).

This section shows that populations that undergo size reductions will show decreases in diversity, increases in homozygosity and changes in allele frequencies. Ernst Mayr (1954)

was the first to describe these as ‘founder events’ that can generate considerable differentiation from ancestral populations and set the stage for ‘founder speciation’.

1.1.2.1 Founder speciation models

Founder events are considered to provide the conditions under which speciation may be facilitated, providing the population is isolated (Wright, 1942; Mayr, 1954). Mayr (1954) described the first models for how this may occur. Mayr (1954) considered that the accumulation of homozygotes may be one of the most important consequences of a founder event. He proposed homozygotes would be much more exposed to selection (founder selection). This selection would supposedly favour alleles that have a selective advantage as homozygotes. In turn this may lead to the generation of new epistatic gene complexes; which may play a major role in restructuring of fitness. In a strongly epistatic genetic system fixation at one critical locus could have cascading fitness effects (Mayr, 1954; Templeton, 1980). These ideas form the basis of Mayr’s ‘genetic revolution’ model. This model has been used to explain both the morphological and genetic divergence of many island species following founder events and isolation. The model put forward by Mayr (1954) has led to some controversy and this has mainly involved the role of ‘founder selection’ (Berry, 1998). As a result others have put forward modifications to the model (e.g. Carson, 1968; Templeton, 1980). Founder effect models tend to receive such critical attention because of their relevance to two important research areas; speciation and conservation of potentially endangered populations (see next section) (Grant, 2002). Nevertheless, as a general theory, founder effects have been a well received as a mode of speciation.

1.2 The importance of population genetic studies

The aim of population genetic studies is to describe the genetic composition of populations from which information on the evolutionary past, both in terms of historical and recent processes, can be extracted. This information is not only useful to evolutionary biology but also to conservation biology.

Conservation biology is a multi-disciplinary field of science that aims to provide the principle tools and knowledge for preserving the earth’s biodiversity. One way in which population genetic studies can contribute to this is by using knowledge on how past events have

impacted populations (Hoelzel, 2010). This is of paramount importance given that we are facing a period that is seeing unprecedented rates of climate change, human-population growth and habitat fragmentation (Hadly and Barnosky, 2009). In addition, genetic studies enable populations to be defined into management units (MUs) for conservation (Palsboll et al., 2007). The aim of defining MUs is to preserve as much of the legacy of the evolutionary past and its resulting diversity as possible. Indeed, the preservation of genetic diversity is a fundamental goal of conservation biology.

1.2.1 The preservation of genetic diversity

There are three fundamental levels of biodiversity; ecosystem, species diversity and genetic diversity that the world conservation union (IUCN) has recognised as being important to preserve. The third fundamental level, genetic diversity, is extremely important to conserve because genetic factors increase extinction risks of a species. Therefore, examining management regimes that minimise this risk has been the central tenet of the field of conservation genetics (Reed and Frankham, 2003). Heritable genetic variation is a prerequisite for evolution. Genetic variation enables a population to tolerate a wide range of environmental extremes (Hoffman and Parsons, 1997). Low levels of genetic diversity can therefore reduce the future adaptability and evolutionary potential of a population (Frankel and Soulé, 1981), thus compromising future fitness. Furthermore, populations with low genetic diversity may show signs of inbreeding which is also of major concern.

1.2.2 Inbreeding depression

Following demographic size reduction inbreeding can rapidly ensue. The amount of inbreeding will depend upon the severity of the demographic event. Inbreeding poses a serious threat to populations or species.

It is well established that inbreeding causes reductions in a population's fitness (under either over-dominance or partial-dominance hypotheses) a phenomenon known as inbreeding depression (Charlesworth and Charlesworth, 1987). The endangered African cheetah (*Acinonyx jubatus*) has been frequently cited as an example of a species' whose survival may be compromised by inbreeding depression. Early studies indicated that this species had significantly less genomic variation than did other felid or mammal species (Obrien et al.,

1986; Menotti-Raymond and Obrien, 1993; 1995). Apparently, correlated with the species genomic uniformity are a number of reproductive (including sperm abnormalities and low litter sizes) and immunological problems (increased susceptibility to disease). These correlations seemed to exemplify the perils that can threaten a species survival due to inbreeding. However, some researchers consider the relative role of inbreeding as being only minor in this species. For example, Merola (1994) considers the 'genetic constitution of the cheetah does not appear to compromise the survival of the species'. Moreover, Merola (1994) argued that conservation should be more effectively aimed at real, immediate threats such as loss of natural habitat. Indeed, it can be very difficult to provide convincing evidence for inbreeding depression in wild populations.

There are many reasons for why demonstrating inbreeding in wild populations is not trivial. Firstly, because to measure individual inbreeding coefficients the relatedness of both parents must be known which necessitates pedigree information beyond that available for most wild populations (Marshall *et al.,* 2002). Secondly, there must be knowledge of complete life history data, which is often very difficult to collect. Finally, evolutionary mechanisms (such as self incompatibility in plants and sex biased dispersal) act to reduce the amount of inbreeding in populations (Charlesworth and Charlesworth, 1987). Thus, inbreeding may only occur at very low frequencies; therefore the sample sizes involved in most studies may not afford sufficient statistical power to detect any effects (Kruuk et al., 2002).

An increased number of studies have circumvented these problems by exploiting the fact that inbreeding reduces genetic diversity (Hartl and Clark, 1997) therefore by recognising associations between genetic diversity and a trait presumed to be associated with fitness, inbreeding can be inferred (Allendorf and Leary, 1986).

1.2.3 Genetic diversity and fitness

Three measurements have been commonly applied in recent years in studies that deal with the interaction between genomic diversity and fitness. These measures are based on microsatellite markers and include: mean d^2, heterozygosity and internal relatedness. Mean d^2 is the squared difference in repeat units between two alleles at a locus averaged over all typed loci (Coulson et al., 1998); heterozygosity is the proportion of heterozygous loci within

an individual; and, finally, internal relatedness is a measure based on allele sharing where the frequency of every allele counts towards the final score (Amos et al., 2001).

Traits most commonly used as fitness measures include: life history traits (e.g. survival, growth, fecundity and reproductive success) or morphometric traits indirectly associated with fitness (e.g. plant height, fluctuating asymmetry).

Evolutionary theory predicts that life history traits should exhibit high levels of dominance variance and should, therefore, be more strongly affected by genomic diversity than weakly selected morphometric traits (Roff, 1997). Indeed, empirical evidence has supported such a theory (Coltman and Slate, 2003). A range of studies have shown correlations with genetic diversity and life history traits. For example, populations of the Natterjack toad (*Bufo calamita*) both larval growth and survival rates were positively correlated with heterozygosity (Rowe et al., 1999). In other studies reduced heterozygosity has been shown to directly result in increased risk of extinction. Saccheri et al., (1998) showed that, for a meta-population of Glanville fritillary butterfly (*Melitea cinxia*) located on an island off south-west Finland, inbreeding negatively impacted larval survival, adult longevity and egg-hatching rate. The main problem with the use of life history traits in these studies is that collection from natural populations is difficult; it usually involves careful monitoring over long periods of time.

Morphometric traits, on the other hand, can be collected more easily and quickly. Identifying suitable morphometric traits that can consistently index genetic stress holds enormous potential for the field of conservation science where it is often a priority to get data quickly from endangered species before it is too late for remedial action (Clarke, 1995). One specific morphometric measurement used to detect inbreeding is fluctuating asymmetry.

Fluctuating asymmetry (FA) refers to a pattern of bilateral variation in a sample of individuals where the mean of the right minus left values is zero and the variation is normally distributed about the mean (Palmer, 1994). FA is often taken as a measure of developmental stability 'the ability to attain equal development under given circumstances' (Zakharov et al., 1997). In principle developmental stability reflects an organism's ability to buffer development against disturbance (Moller and Swaddle, 1997). The efficiency of this stability mechanism is considered to be an integral component of an individual's fitness (Lacy, 1993; Clarke, 1995). More developmentally stable individuals are considered more fit then those

that are less developmentally stable. FA should reflect this stability as the development of each side of a bilaterally symmetrical organism is influenced by precisely the same genetic and environmental conditions; any disruption, i.e. asymmetries, presumably results from a developmental accident (Waddington, 1942).

1.2.4 Recovering from inbreeding effects

The most obvious way to overcome inbreeding depression in small populations is to introduce immigrants from elsewhere (Frankham, 1995). Immigration of individuals into a population can enable matings to occur between individuals of divergent origin. The result can be that offspring exhibit elevated levels of fitness (heterosis). Indeed, even low levels of immigration have been shown to increase population fitness. In an isolated population of Scandinavian wolves (*Canis lupus*) showing signs of inbreeding the immigration of a single male resulted in spurred population growth and rapid restoration of genetic diversity (Vila et al., 2002). In another isolated population of Swedish adders (*Vipera berus*) the deliberate introduction of individuals from a separate, unrelated population resulted in increased molecular genetic variation, recruitment of males and elevated population growth rates (Madsen, 1999). The results of these studies show how low levels of migration between populations (either natural or directed through relocation programmes) can be extremely effective in restoring genetic diversity and reduce extinction risks from inbreeding. However, the introduction of new individuals does not always result in the desired effect. Occasionally, populations can be at risk of outbreeding depression which can further increase risk of species extinction. Outbreeding depression occurs where offspring of crosses between animals adapted to different conditions have reduced survival rates and lower fecundity in both parental environments because they are not adapted to either of them (Ingvarsson, 2002; Edmands, 2007).

1.3 The European roe deer (*Capreolus capreolus*)

The spatial and temporal distributions of one species, the European roe deer (*Capreolus capreolus*), is well known to have been influenced by both ancient and contemporary evolutionary processes. These processes may have had important consequences for the genetic structure, diversity and fitness of populations.

1.3.1 The European roe deer: distribution and genetic differentiation

The roe deer *(Capreolus capreolus*) is a medium sized ungulate (weight >30kg) belonging to the deer family (Cervidae). It is a species that is widely spread across Europe, absent only from Ireland, some Mediterranean islands, northern Russia and the tundra regions. In Russia, its distribution is bordered by the western range of the Siberian roe deer (*Capreolus pygargus)* whose species status has been supported recently by both morphological and genetic evidence (Danilkin et al., 1995; Xiao et al., 2007).

The large distribution range of the European roe may be accounted for by its high adaptability to a wide variety of habitats with a preference for a mixture of woodlands and agricultural landscapes (Danilkin, 1996). In Northern Europe, the European roe's distribution is ultimately limited by cold winters, short growing seasons and high snow accumulation (Holand et al., 1998). In Southern Europe its range is limited by heat and dryness (Wallach et al., 2007). Fossil evidence suggests the roe deer has been present in Europe for at least 600, 000 years (Middle Pleistocene, Lister et al., 1998) and it has been known from both glacial and interglacial phases since then (Sommer et al., 2009). Specifically, during the late Quaternary the impacts of such phases have been fairly well established from patterns of genetic data (Randi et al., 2004; Lorenzini and Lovari, 2006). Three divergent mitochondrial lineages have been found; an eastern, a western and a central. The eastern and western lineage originated from Balkanic and Iberian refugia respectively. Their distributions are therefore correspondingly found mainly in Southern Europe (Greece, Serbia) and Northern Spain/Portugal. On the other hand, the location of the central lineage refuge is still unknown; however, it shows the most widespread distribution of the lineages being found across Central, Northern, Western and Eastern Europe. Although much of the roe deer's genetic structure and diversity may have been impacted by glacial –interglacial cycles more recent events are also likely to have been important.

Roe deer populations across Europe are known to have undergone recent and strong population fluctuations. Since pre-historic times the roe deer has been important for hunters across Europe. However, during the last few centuries the intensity of this hunting has caused many roe deer populations to become reduced in size and distribution. In some areas roe have even become extirpated. Following these declines, translocation has been an important management tool to aid the re-establishment of populations. Nearly all European roe

populations have been impacted by some level of restocking via translocation. Today, roe populations have greatly increased in density and are expanding geographically (Holand et al., 1998; Ward, 2005; Ward et al., 2008).

1.3.2 The British roe deer

The European roe deer, *Capreolus capreolus*, is one of two extant cervid species native to Britain, the other being the red deer, *Cervus elaphus*. The species has a very long history in the British Isles, first appearing in the fossil record during the Cromerian interglacial, 780,000 - 450,000 years before present (YBP) (Yalden, 1999). Since this time, successive glacial and interglacial periods have respectively seen the roe's disappearance and reappearance. For example, during the period encompassing the height of the Last Glacial Maximum (LGM, 23,000-18,000 YBP) (Kukla et al., 2002), the spread of permafrost and arctic tundra ecosystems across much of Britain forced roe to reside in southern European refugia (Sommer and Zachos, 2009). Following this ice age, climatic warming and de-glaciation enabled forests of birch and pine to re-colonise much of northern Europe, in turn attracting suitably adapted animal species such as the roe. The return of roe into Britain would have occurred via an expanse of land, named 'Doggerland', which once provided a direct connection to continental Europe (Figure 1). It is believed that 'Doggerland' existed until at least 9,450 YBP, if not until as late as 7,450 YBP (Coles, 1998), when its eventual loss resulted from warming which unlocked large quantities of water from ice caps and caused sea levels to rise. This period provided sufficient time for roe to re-colonise Britain following the end of the LGM.

Indeed, the first postglacial evidence of British roe populations originate from a Mesolithic site located in southern England (Thatcham) where a directly radiocarbon dated bone was aged 9,430 YBP (Sommer and Zachos, 2009). Other Mesolithic sites, such as Starr Carr and the Vale of Pickering in Yorkshire, provide an indication of roe's existence over a similar time frame (Yalden, 1999). By this time, Britain was almost completely re-covered in woodland and roe would have been able quickly to exploit new available habitat; expanding their distribution across the whole of England, Scotland and Wales. A previous study estimated that Mesolithic roe may have numbered over 800,000 (Maroo and Yalden, 2000) based on estimates of vegetation cover and population density. It is likely that this thriving population may have been sustained well into the Neolithic period.

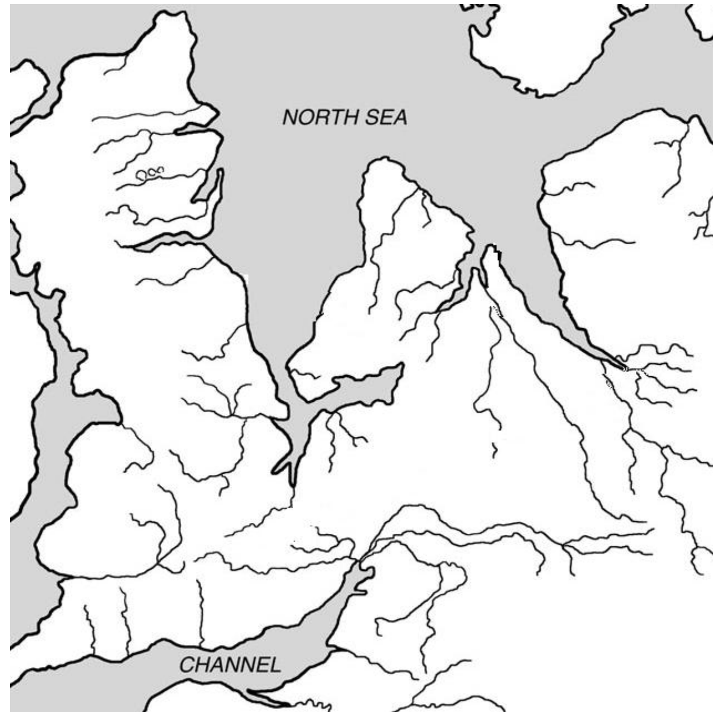


Figure 1.1 Hypothetical extension of Doggerland. From Coles (1999).

Although the Neolithic period witnessed an increased human population as well as the introduction of livestock (5,000 YBP), the impact for roe deer populations may have been minimal. The introduction of livestock could have released, to some extent, human hunting pressures on roe deer. At the same time, the requirement to control potential predators of livestock, such as brown bear, lynx and wolf may have also benefited the roe (Yalden, 1999). The impact of an increasing human population, therefore, may not have been felt until at least the Iron Age.

Evidence from the zooarchaeological record reveals that from the Iron Age onwards and up until the Roman period there was an increased interest in hunting (Sykes, 2007). However, with the withdrawal of the Roman Empire from Britain, around 410 AD, the enthusiasm for hunting did not last. In the early Anglo Saxon period (mid 5th to 9th centuries AD), hunting became less common and archaeological evidence shows that game contributed little to the human diet (Sykes, 2007). This was possibly due to fears over the supernatural creatures that the Anglo-Saxons believed inhabited the wild, as well as a preoccupation with farming. By the late Anglo Saxon period, around the mid 9th century AD, however, hunting increased substantially, especially amongst the social elite. By the end of the late Anglo Saxon period,

even the right to consume wild animals became restricted (Sykes, 2007). For example, 1016 saw the introduction into England of Forest Laws which exclusively restricted the hunting of 'beasts of the forest', including roe deer, to the King and the nobility in forests (Whitehead, 1964). Following the Norman conquest in 1066, archaeological data demonstrate an unparalleled increase in aristocratic hunting (Sykes, 2007). Figure 1.2 shows evidence of the overall increase in hunting from the early Anglo Saxon period onwards, as reflected by an increased number of wild mammal bones found in archaeological sites.

By the Norman period, roe becomes a less favoured quarry (Sykes, 2007). Furthermore, following this period from the mid 11th to mid 12th century AD, roe bones collected from archaeological sites show a rapid decline (Figure 1.3), which in spite of an overall increase in hunting (Figure 1.2) may reflect a reduced population size (Sykes, 2007). This possible decline would not have been helped by the amendment of Forest Laws in 1338 made by the court of the King's Bench when roe were relegated from a 'beast of the forest' to a 'beast of the warren' on the basis that it was a nuisance animal, responsible for driving away other more highly regarded game such as red and fallow deer. This change in law effectively enabled hunting of roe by commoners to take place. Furthermore, orders were given to the Royal Foresters to have roe eradicated from royal hunting preserves (Whitehead, 1964).

It was likely that the combination of habitat loss and increased hunting pressure over the centuries led to severe declines in roe populations. Indeed, both archaeological records (Figure 1.2) and historical documents imply that roe were increasingly scarce by the late medieval period. By the 16th century roe were reportedly absent from most of the midlands and the southern English counties (Ritson, 1933). By the 18th century some considered roe extinct throughout Britain with the exception of remnant populations in the Scottish highlands (Whitehead, 1964). This last claim should be viewed with caution, as although it is likely that *most* English roe populations were extinct, several pocket populations may have persisted in the northern border counties of England and lowlands of Scotland (Ritson, 1933; Whitehead, 1964).

Despite this period of severe decline, contemporary populations exhibit little evidence of a precarious past. With the turn of the 19th century, large scale re-planting of woodland provided suitable habitat for remnant populations over much of the north to re-colonise uninhabited areas (Taylor, 1948). In the south reforestation helped facilitate the successful re-

introduction of roe deer (using both native and non-native stocks) across much of Southern England (Whitehead, 1964; Prior, 1995). Reforestation and re-introductions have been so successful that roe deer populations are showing continual expansion and re-population over much of their historic range (Figure 1.4) (Whitehead, 1964; Ward, 2005; Ward et al., 2008). Recent population estimates suggest that roe deer have flourished well into the hundreds of thousands (500,000) (Harris et al., 1995). Increases in roe deer numbers have been so substantial that there are now conflicts with foresters and farmers, as well as problems with vehicle collisions (Cederlund et al., 1998).

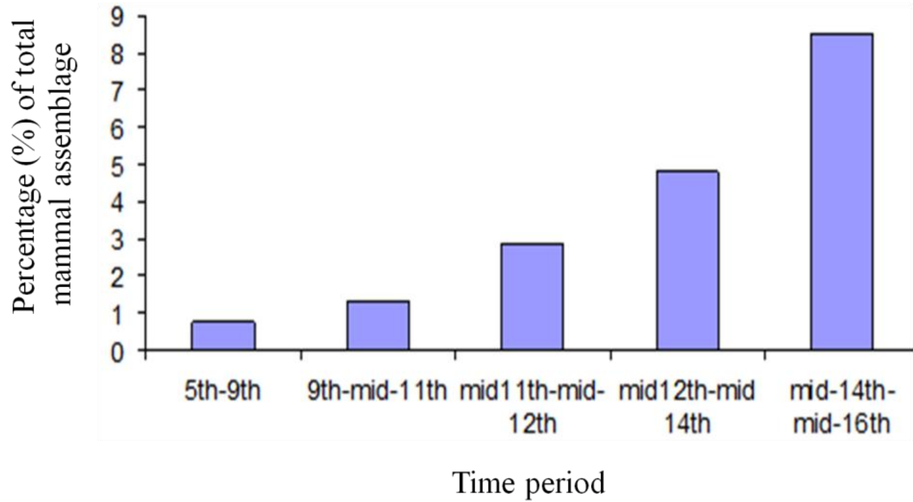


Figure 1.2. Representation of wild mammal bones found in archaeological sites (all site types combined) shown as a % of total mammal assemblage through time; 5th – 9th: Early Anglo Saxon, 9th – mid 11th: Late Anglo Saxon mid, 12th- mid 14th Norman mid 14th- mid 16th: Late Medieval. Data from Naomi Sykes.

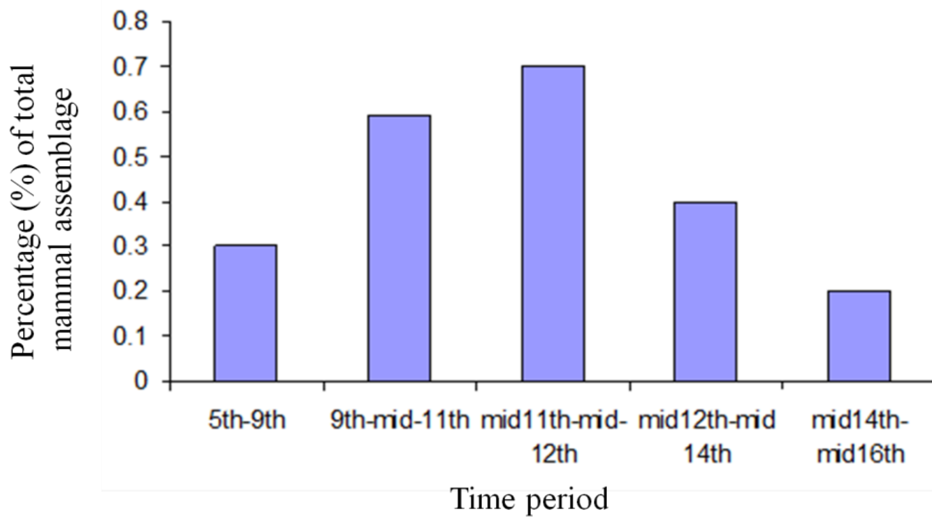


Figure 1.3. Representation of roe deer bones found in archaeological sites (all site types combined) shown as a % of total mammal assemblage through time. Data from Naomi Sykes.

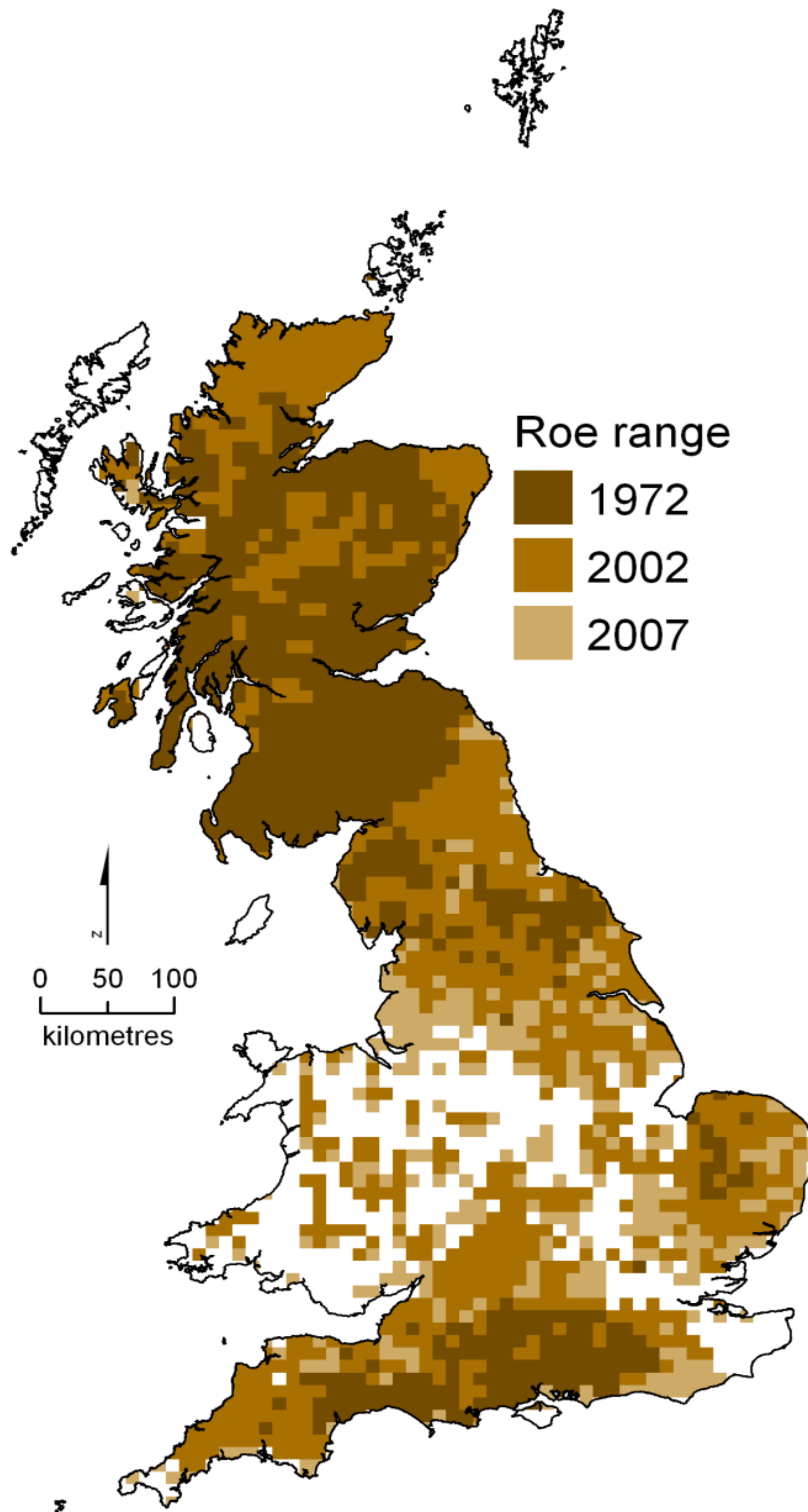


Figure 1.4. Observations of roe deer from 1972, (dark brown) 2002, (lighter brown) and 2007, (lightest brown) (after Ward et al., 2008).

With the well known history of the British roe deer the opportunity arises to use this species as a model to assess the genetic impacts of: Pleistocene climatic change, post-glacial recolonisation, human induced reductions in population size (bottlenecks), translocations and population expansions. In particular, there is the opportunity to test the consequences of re-introductions into regions where diversity has been lost, and assess the potential for genetic recovery. Using inference from ancient DNA, population dynamics and multiple introductions can be tracked over time, permitting some level of replication.

In support of that objective, this study aims to examine the population genetic history of the UK roe since early post glacial re-colonisation. As described in the previous section (1.3.1), the phylogeographic structure and thus the re-colonisation history of most European roe populations is now well established. However, the UK represents one of the few areas for which roe samples have yet to be obtained. Establishing the phylogeographic positioning of UK roe in relation to other European populations will provide new insight into re-colonisation history. Additionally, through the use of ancient DNA, substitution rates can be directly calibrated (see section 1.1.1) to examine the past demographic history of this species. Of course such work requires access to ancient DNA. Indeed, the roe is a species that is well represented in the fossil record. The use of modern and ancient DNA used in this initial study can then be used to address more recent processes impacting roe populations.

The use of modern DNA can enable a straightforward study on the genetic structure and diversity of the species to be carried out. This will enable the impacts of bottlenecking followed by founder events (through natural and non-natural dispersal) to be evaluated. Furthermore, such a study can help to define management units which can be important for guiding future roe management plans.

Given the importance of genetic diversity to a population (see section 1.2 above) it is important to identify populations that have undergone strong bottlenecking. Given that the roe deer is a species that is well known to have undergone bottlenecking, it is ideal for testing methods for detecting bottlenecks.

A further study will detect potential impacts of this species past on population fitness by examining relationships between genetic diversity and fluctuating asymmetry (see section 1.2.3). It may be that past bottlenecking has exposed populations to inbreeding depression (as

described in section 1.2.2). This assumption may, however, be complicated by the introduction of distantly related roe deer stock (from the continent) that could have induced heterosis (see section 1.2.4).

Collectively, the chapters in this thesis will provide new insight into the genetic history of the roe deer, of which there is currently very little understanding. Only one previous study based on British roe deer population genetics has been published (Hewison, 1995). This study used 15 allozyme markers on samples collected from a number of different locations across Britain. The results did not, however, show any significant genetic differentiation. Only one locus was shown to be polymorphic, which showed evidence of an east/west cline in southern populations. This scenario is consistent with the re-introduction records of roe deer. Hewison (1995) considered the low allozyme variability to be consistent with past bottlenecking. Allozyme markers are known to have low inferential power compared to more modern DNA markers such as microsatellites and mt-DNA (Zachos et al., 2006) which this study will employ.

1.4 Molecular markers

Mitochondrial DNA is distinctive and universally distributed in the animal kingdom with a remarkably uniform gene content and order (Avise, 1994). The control region (d-loop) is the most variable portion of mt-DNA (Moritz *et al.,* 1987). It therefore has great utility in providing resolution for populations that are expected to show little differentiation. Mitochondrial DNA is especially useful for studies based on ancient DNA. For such studies mt-DNA has tended to be the genome of choice (Hoelzel, 2005) because it is present in vast numbers of copies per cell (500-10^3) compared to other molecular markers e.g. nuclear markers (~2 copies) (Avise, 1994; Hillis *et al.,* 1996). Therefore, the chance of finding intact mt-DNA is greater (Hoelzel, 2005). One property of mitochondrial DNA that can limit inferences made is that it is maternally transmitted. Although this can make information easily interpretable because all parts of the molecules share the same historical pattern of common descent (Avise 1998) this marker will only be able to track female movement.

Unlike mt-DNA, microsatellites are markers that are biparentally inherited. These markers are short (< 10 bp) tandemly repeated DNA units with high degrees of size polymorphism. Furthermore, they are highly abundant and have broad distributions across the eukaryotic

genome. Owing to these characteristics microsatellites are currently considered the marker of choice for population genetic studies (Bruford and Wayne 1993: Jarne and Lagoda, 1996). Information from this marker can be particularly useful in addressing a range of biological questions from fine scale determination of individual identity and relatedness to broader scale questions, including determination of population genetic structure, history and evolutionary relationships (Zhang and Hewitt, 2007). One limitation of this marker is that amplification and analysis from low quality and quantity DNA samples (e.g. ancient) can be difficult (Pompanon et al., 2005).

1.5 Thesis outline and aims

The thesis will begin, in Chapter 2, with a broad-scale phylogeographic study to establish the relationship of British roe deer populations to other European populations.

Chapter 2:

1. Examine the phylogeographic position of UK in relation to European roe to:
   a) Provide information on the post-glacial re-colonisation route of roe into the UK.
   b) Determine a population splitting time of UK roe European populations and determine whether this is consistent with the land bridge split.

Chapter 3 will investigate the differentiation and genetic diversity across modern day populations to address how recent processes may have impacted populations.

Chapter 3:

2. Examine genetic and morphological differentiation of British roe deer to:
   a) Determine relative impact of past events such as bottlenecks and re-introduction.

Chapter 4 will examine evidence for bottlenecking by examining both bottleneck signatures (from modern genetic data) and temporal changes in genetic diversity (from modern and ancient DNA).

Chapter 4:

3. Examine evidence for past bottlenecks on populations to:

a) Determine which roe populations have been most impacted by bottlenecks.

b) Assess the value of different methods used to detect bottlenecks.

Finally, Chapter 5 will expand upon chapter four and address whether variation in levels of current genetic diversity in roe has influenced population fitness as measured by fluctuating asymmetry (FA).

Chapter 5:

Examine roe population fitness using fluctuating asymmetry to:

a) Determine whether this species past history of bottlenecking and founder events has impacted population fitness. b) Provide further support to FA- genetic diversity relationships.

Chapter 2 : Phylogeography and re-colonisation history of British roe deer

2.1 Introduction

The advance and retreat of ice sheets through multiple glacial cycles in the Quaternary has had a major impact on the present day distribution of species (Hewitt, 2004). During each glacial cycle, temperate species were confined to separate southern refugia. During warmer inter-glacial periods, species were able to expand northwards and recolonise previously glaciated regions (Hewitt, 1996).

The field of phylogeography which examines the geographic distributions of contemporary genetic lineages helps to elucidate both refugial areas and postglacial re-colonisation pathways (Avise, 1994). Such studies have provided useful insight into the impact of the Quaternary on a number of different species (Hewitt, 2000). In Europe in particular, a number of concordant phylogeographic patterns between species now indicate possible re-colonisation routes from at least three main European refugia which exist in the Iberian, Italian and Balkan peninsulas (Hewitt, 1996; Taberlet et al., 1998). More recent studies have also argued the case for refugia further north in central and western Europe (Kotlik et al., 2006). The existence of these refugia is consistent with the distribution of the ice caps, which apparently covered Scandinavia and the northern belt of central Europe (Lorenzini and Lovari, 2006). However, the exact delimitation of refugia as well as the process of re-colonisation can differ between species, depending on factors such as population density in the refuge area, food resources and dispersal capability (Taberlet et al., 1998; Saarma et al., 2007).

For one European species, the roe deer (*Capreolus capreolus*), the impact of the Quaternary has been described in some detail from studies based on both phylogeographic and fossil evidence (Randi et al., 2004; Lorenzini and Lovari, 2006; Sommer et al., 2009; Sommer and Zachos, 2009). The geographical distribution of genetic variation in the roe deer divides sequences of the mitochondrial DNA control region into three major lineages (or clades); central, eastern and western. The central lineage is widespread throughout Europe, while the eastern lineage is found mainly in Greece and Serbia and the western lineage is mainly in Spain and Portugal. In addition to these divisions, significant internal structuring has been detected in roe sampled from the Iberian and Italian peninsulas. This supports the existence

of subspecies in both Italy (*C. c. italicus*) (Lorenzini et al., 2002; Randi et al., 2004) and Spain (*C. c. garganta*) (Royo et al., 2007). The European structuring of the roe deer suggests at least two refugia in Iberia. One of these refuges, located in the north western part of Iberia, gave rise to the western lineage whilst the other, located in the central- southern part of Iberia, gave rise to the sub species *C. c. garganta* (Royo et al., 2007). In other parts of Europe, at least one refuge existed in the southern Italian Apennines and this gave rise to the sub species *C. c. italicus* (Randi et al., 2004). In addition, at least one refuge was located in the Balkans giving rise to the eastern lineage of roe. Finally, the refuge that gave rise to the central lineage remains unknown. Both molecular and fossil evidence point to an eastern refuge origin rather than a western (Iberian) one (see Randi et al., 2004; Lorenzini and Lovari, 2006; Sommer and Zachos, 2009). Furthermore, it is thought that ice sheets of the glacial periods which probably covered northern and central mountainous chains in the Iberian Peninsula may have acted as a sufficient barrier to expansion from low altitude Iberian refugia (Lorenzini and Lovari, 2006).

Although for most parts of Europe there is a good representation of roe samples from phylogeographic studies, there is a lack of relevant published data from the UK, and it is therefore unknown which lineage these roe belong to. Membership to the western lineage would suggest re-colonisation from Iberia, while membership of the central lineage may suggest an eastern colonisation route. Given that the central lineage is most widespread across Northern Europe, it is likely that UK populations belong to this group; this study will provide the first evidence of whether this is the case. One problem with phylogeographic interpretation is that recent, re-introduction events into the UK from the continent may have blurred the true phylogeographic patterning of this species (Randi et al., 2004). One way to minimise this problem is to incorporate ancient samples (that pre- date introduction events) in combination with modern samples.

Examining the phylogeography of roe deer not only provides a better understanding of re-colonisation histories but also enables population divergences and expansion events to be dated. This can be achieved by examining parameters of the demographic history of a population through tracking genealogies backwards in time on the basis of their coalescent (Kingman, 1982). The coalescent is a mathematical description of the genealogy of a small sample of individuals taken from a large background population. More specifically, it is a statistical description of the amount of time individual genealogical lineages take to coalesce

to a common ancestor. Coalescent methods are powerful for detecting important environmental or geological changes that occurred in the past, especially when ancient DNA is incorporated (Willerslev and Cooper, 2005). This is because ancient DNA provides a more inclusive representation of coalescent events and also enables an accurate interpretation of the substitution rate to be directly calibrated (Hoelzel, 2005).

Accurate substitution rates are reliant on suitable calibration points to translate onto an absolute time scale (Saarma et al., 2007). Traditionally, most estimates have been derived from comparative approaches among living taxa, where sequence divergence is calibrated against geological estimates of divergence time (Cann et al., 1987). Such rates are referred to as ‘phylogenetic rates’. Based on these ‘phylogenetic rates’, estimates of 1 % divergence per million years (Myr) have been commonly applied to mitochondrial protein-coding genes of both birds and mammals (Brown et al., 1979; Ho, 2007). However, a growing number of studies have recently challenged the validity of these phylogenetic rates.

Recent advances have now made it possible to directly calibrate substitution rates for species or populations by using dated ancient DNA sequences (Drummond et al., 2002). One of the notable characteristics of rates obtained from ancient DNA datasets, is that they generally yield much higher substitution rate estimates (Ho et al., 2007). In fact, it seems substitution rates can change by an order of a magnitude when these internal calibration points are used (Lambert et al., 2002). This amount of rate change brings all estimated dates of divergence or expansion events forward, radically altering our understanding of how the past has influenced populations (Ho et al., 2005; Ho et al., 2008).

Previous roe deer studies have examined expansion events in European populations. For example, Randi et al. (2004) estimated two roe expansions to have occurred around 200 000 and 130 000 years ago which (roughly) coincided with the penultimate and last interglacial respectively. However, this estimation was based on a ‘phylogenetic rate’ and therefore may be a gross over-estimation.

The calculation of a new substitution rate for roe deer, directly calibrated using ancient DNA, provides the opportunity to re-examine the species’ evolutionary history. Furthermore, the new rate can be validated by examining whether estimated points of divergence coincide with known points of divergence between certain populations.

The objective of this study is to examine the phylogeographic position of the UK population in relation to roe deer found elsewhere across Europe. This will allow better inference on the process of post-glacial re-colonisation, the direct calibration of a substitution rate specific to roe deer, and the validation of this rate through application to independently supported division times. Specifically, the following predictions will be tested:

1. UK roe will be most closely related to the central clade, given that this clade is already widespread across Northern Europe. This would support an eastern re-colonisation route into the UK.
2. The splitting time of UK and European populations, using a substitution rate calibrated using ancient DNA will occur after the land bridge split.

2.2 Materials and methods

For this chapter, most data were based on mitochondrial DNA (mt-DNA) sequences. However, for one part of the analyses (IM – see below), 16 microsatellites (as described in Chapter 3) were incorporated from modern populations of Dorset and Perth.

2.2.1 Collection of modern and ancient samples

A total of 314 modern samples were obtained from 14 UK wide locations for mt-DNA analysis. Details of the samples amplified for mt-DNA for each location are provided in Figure 2.1 a.

A total of 168 ancient samples were collected from 16 sites from across the UK. Table 2.1 lists the locations from which samples were obtained, and whether they were successfully amplified for mt- DNA. For samples that were successfully amplified their locations are represented on a map (Figure 2.1b).

2.2.2 Modern DNA extraction, amplifications and sequencing

A small part of the tissue sample (approximately 5mm^3) was finely cut using a scalpel. Samples were then incubated overnight at 37 ˚ C in digestion buffer (50 Mm Tris Ph 7.5, 1Mm EDTA, 100 Mm NaCL, 1 % w/v SDS) with 30 µl proteinase K (10mgµl^{-1}). The DNA was then extracted using a standard phenol: chloroform extraction (Sambrook et al., 1989). The presence of genomic DNA was then confirmed by viewing results on 1.2 % agarose gels which were run for 20 minutes alongside a 1 Kb DNA ladder.

Amplification and sequencing

In order to gain sequence data comparable to previous studies (e.g. Randi et al., 2004) the entire mt-DNA control region, spanning 750 base pairs (bp), was successfully amplified using a previously published primer set; Lcap Pro and Hcap Phe (Table 2.2) (Randi et al., 1998). However, for this chapter only the first part of the sequences, hyper variable region 1 (HVR1) was used (spanning 399 bp).

Table 2.1. Detailed description of samples including the location, site and site code origin of each sample, the number of samples extracted (N extracted) and successfully amplified for DNA (N successful) and, finally, the approximate date range of samples (provided from stratigraphic information).

Location	**Site**	**Site code**	**N extracted**	**N successful**	**Approximate date range (AD/BC)**	**Period**
London	Moor House	MH	40	40	1180-1300 AD	Medieval
	Moorgate	MRG	2	2	145-170 AD	Roman
	Wood Street	WOO	1	1	190-400 AD	Roman
	Wood Street	WOO	1	1	1050-1100 AD	Norman
	Baltic Exchange	BAX	2	2	100-250 AD	Roman
	Baltic Exchange	BAX	1	1	250-400 AD	Roman
	Fenchurch Street	FEH	2	2	1050-1150 AD	Norman
	Fenchurch Street	FEH	1		50-80 AD	Roman
	London Bridge	LBI	4	4	70-160 AD	Roman
	Regis House and Ridgeway House	KWS	1	1	no date	Unknown
	Upper Thames Street	UP	1	1	970-1050 AD	Late Anglo Saxon
	Borough High Street	BGH	2	2	100-160 AD	Roman
	Walthamstow	WAL	3			Pleistocene
Kent	Bishopstone	BIS	8	8	900-1000 AD	Late Anglo Saxon
Oxfordshire	Banbury Castle	BAN	7	4	1095-1292 AD	Late Anglo Saxon/early Medieval
Gloucestershire	Salmonsbury Camp	SAL	1	1	700 BC - 43 AD	Iron age
Hampshire	Faccombe Netherton	FAC	7	4	850-1070 AD	Anglo Saxon
	Faccombe Netherton	FAC	9	4	1066-1154 AD	Norman
	Faccombe Netherton	FAC	5	2	1154-1485 AD	Medieval

Table 2.1 continued; Detailed description of samples including the location, site and site code origin of each sample, the number of samples extracted (N extracted) and successfully amplified for DNA (N successful) and, finally, the approximate date range of samples (provided from stratigraphic information).

Location	**Site**	**Site code**	**N extracted**	**N successful**	**Approximate date range (AD/BC)**	**Period**
Wiltshire	Durrington Wells	DW	5	3	2600 BC	Late Neolithic
	Boscombe Down	BD	1		2300 - 700 BC	Bronze Age
Sussex	Fishbourne Roman Palace	FB	12	12	45-180 AD	Roman
	Whitehawk Camp	WC	4		4000 -2500 BC	Neolithic
Somerset	Glastonbury Lake Village	GLV	3		700 - 400 BC	Early Iron Age
Hereford	Cathedral House	CH	3	3	1100-1200 AD	Medieval
	Gaol Street	GAO	3	2	1066-1485 AD	Medieval
Chester	Unknown	CHE	1	1	1200-1350 AD	Medieval
Lincolnshire	Welland Bank Quarry	WBQ	4	1	1300 - 700 BC	Bronze Age
Derbyshire	Carsington cave	CPC	14	14	5678 - 3447 BC	Neolithic
	Carsington cave	CPC	2	2	1248- 630 BC	Late Bronze age/early Iron Age
Yorkshire	Staple Howe	STP	9	1	700 BC - 43 AD	Iron Age
Durham	Barnard Castle	BAR	1	1	1095-1292 AD	Medieval
	Arbeia Roman Fort	ARB	2	2	200-350 AD	Roman
Perthshire	Horse Cross	HC	3	3	1400 AD	Medieval
	Horse Cross	HC	2		1100-1250 AD	Norman
	Holyrod, Edinburgh	HLY	1	1	1500 AD	Medieval
	Total		**168**	**126**		

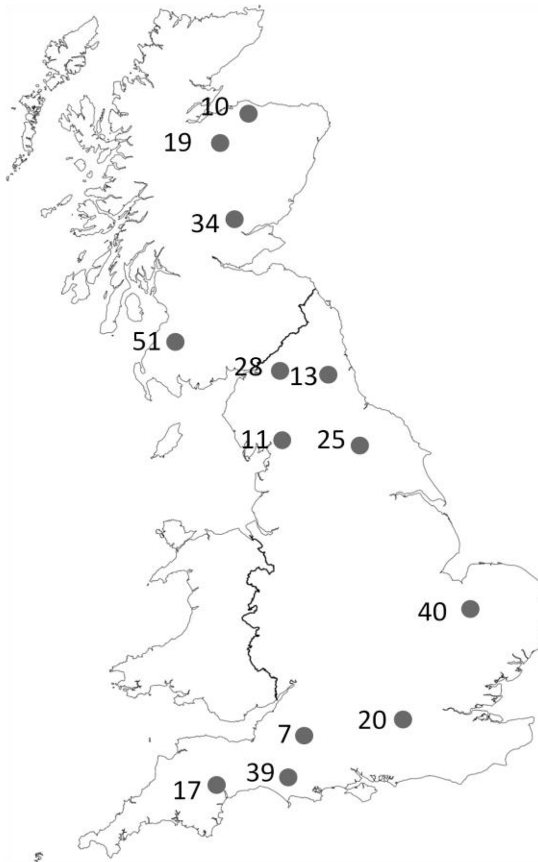


Figure a

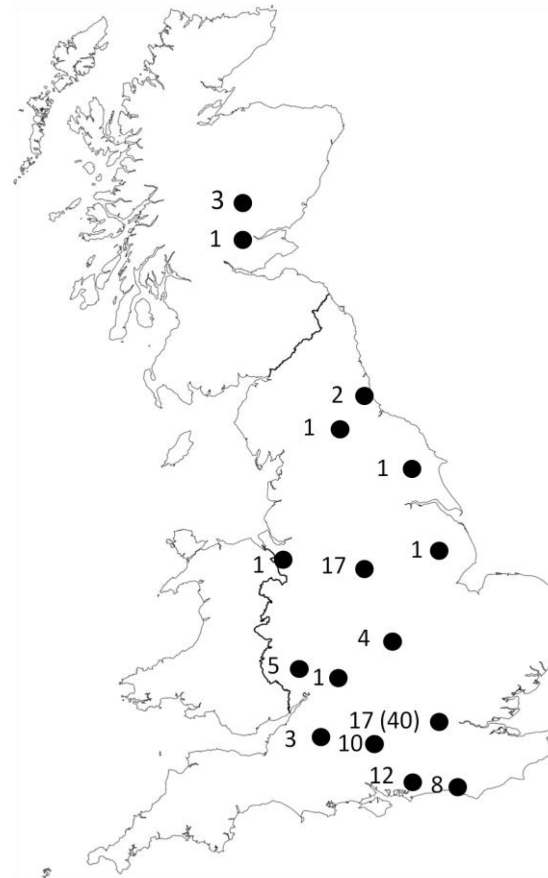


Figure b

Figure 2.1.Map showing locations from which modern (Figure a) and ancient (Figure b) samples were obtained along with sample sizes successfully amplified for mt-DNA analysis. In figure b, bracketed samples represent Moor House samples that were excluded.

All amplifications were carried out using the polymerase chain reaction (PCR) (Saiki et al., 1988). Reactions (20 µl) were prepared on ice and carried out in 0.2ml reaction tubes. Each 20 µl contained 0.2 pM/µl each primer, 0.2 mM each dNTP, 10Mm Tris-HCL Ph 9.0, 1.5 Mm MgCl2 and 0.4 units of *Taq* polymerase (New England Biolabs). 1µl of DNA template was added.

Amplifications were then performed in a G-storm (Biotechniques) with the following cycles: 95ºC for 5 minutes; 35 cycles at 94ºC for 45s, 51ºC for 45s and 72ºC for 45 s; 72ºC for 5 min.

Table 2.2. Forward and reverse primers used to amplify a 750 bp region of the mt-DNA d loop (after Randi et al., 1998).

Gene	Primer	Primer sequence
D-loop		
Forward	Lcap Pro	5'-CGT CAG TCT CAC CAT CAA CCC CCA AAG - 3'
Reverse	Hcap Phe	5'-GGG AGA CTC ATC TAG GCA TTT TCA GTG -3'

PCR products were purified with Qiagen PCR purification columns and directly sequenced (in the forward direction) using the ABI dye-terminator method on an ABI 377 automated sequencer (Applied Biosystems). Resulting chromatograms were viewed and edited in Chromas V 2.01 (Technelysium, Helensvale, Queensland, Australia).

PCR controls and interpretation of results

Negative controls, which were master mixes without DNA template, were used alongside all PCR amplifications to detect evidence of any reagent or sample cross contamination. After all PCR reactions, finished products were run on a 1.2 % gel to confirm amplification and lack of contamination.

2.2.3 Ancient DNA extraction

Prior to DNA extraction all samples were prepared. Bone, antler and teeth were prepared as outlined below.

(a) Bone and antler

A variable speed Dremmel™ drill was used to collect powder from bone/antler samples. To start, all samples were surface sanded extensively with disposable rotary tips of the Dremmel tool to ensure removal of surface contaminating DNA. The outer layer of powder was then discarded. The drill was then used to cut ~ 1cm³ internal segment of the cortical part of the bone where DNA was likely to be best preserved (MacHugh et al., 2000). Further drilling was carried out to yield up to ~ 1g of powdered bone. After each sample was collected all drill parts were soaked in 10% bleach, 70% ethanol and then treated with UV to reduce problems arising from cross contamination.

(b) Teeth

The surface of tooth samples were decontaminated by soaking in 10% bleach and rinsed in sterile water. Teeth were then wrapped in UV treated foil and broken into fragments using a mortar. The pestle and mortar were then used to generate dental powder from larger fragments. All samples were collected in a sterile 1.5 ml micro-centrifuge tube and stored at ambient temperature until digestion. Between samples, pestle and mortars were soaked in 10% bleach and autoclaved. Blank controls (1 in 5, containing no bone) were extracted in parallel to monitor contamination from laboratory reagents and equipment.

Enzymatic digestion was carried out by adding 0.5 ml of digestion buffer (0.425 M EDTA pH 8, 0.5% sodium dodecyl sulphate, 0.05 M tris, PH 8.5) along with 50 µl of proteinase K (20 mg/ml) to powdered samples. Samples were then incubated for a minimum of 24 hours at 50 º C with constant agitation. DNA was extracted using a QIAquick purification kit™ following the manufacturers guidelines.

Amplification and sequencing

Due to the degradation of ancient DNA, short primer sets spanning no more than 300 bp were specifically designed to amplify the HVR1 of the mitochondrial control region. Primer design followed the key principles outlined by previous workers (e.g. Innis and Gelfand, 1990). Primers were designed using the programme Primer 3 (Rozen and Skaletsky, 2000). The primer sets and sequences used to amplify this region are given in Table 2.3.

Table 2.3. Forward and reverse primers used to amplify ancient mt-DNA d-loop.

Gene	Primer	Primer sequence
D-loop		
Forward	Roe_1F	5'-ATT ATA TGC CCC ATG CTT AT- 3'
Reverse	Roe_1R	5'-CCT GAA GAA AGA ACC AGA TG-3'
Forward	Roe_2F	5'-AAC CAA GAA CTT TAC CAG- 3'
Reverse	Roe_2R	5'-GGG ACA TAA TGT ACT ATG-3'

The primer pairs Roe_1F with Roe_1R and Roe_2F with Roe_2R amplify fragments of 244 and 267 bp respectively (including primers). Together these were combined to give a total of 419 bp to be used for genetic analysis.

The HVR1 was amplified using PCR (Saiki et al., 1988). Reactions (25µl) were prepared on ice and carried out in 0.2 ml reaction tubes. Each 25-µl PCR contained 0.2 pM/µl each primer, 0.2 mM each dNTP, Platinum 1X High Fidelity Buffer [60 mM Tris-SO4, pH 8.9/18 mM (NH4)2SO4 (Invitrogen)], 1.5 mM MgCl2, and 1 unit of Taq High Fidelity DNA polymerase (Invitrogen). 2µl of DNA template was added.

Amplifications were performed in a G-storm with the following cycles: 95ºC for 5 minutes; 45 cycles at 94ºC for 45s, 51ºC (roe_1F, 1R) or 55ºC (roe_2F, 2R) for 45s and 68ºC for 45 s; 68ºC for 5 min.

Precautions and replication for ancient DNA

Contamination is frequently a problem with old samples (Paabo et al., 2004), so careful precautions were taken during every stage of aDNA extraction and PCR set up. DNA extraction and PCR setups took place in a separate laboratory dedicated to ancient DNA research, where no amplified or contemporary roe deer DNA had ever been present. No laboratory materials or clothing were transferred from the post amplification rooms to the ancient laboratory. All work surfaces and equipment were thoroughly rinsed and wiped down with 10% bleach (sodium hypochlorite) followed by 70% ethanol. Surfaces, equipment and solutions (e.g. extraction buffers, digestion buffer and PCR water) were also routinely exposed to UV light for at least 10 minutes. Gloves, tucked into the sleeves of a lab coat (to

prevent exposure of wrist skin), were worn at all times and frequently changed. All extractions and PCR work was carried out under a fume hood.

Negative extraction and PCR controls were both included to detect potential contamination in reagents and cross contamination between samples. Negative controls were reagents without DNA template. Negative controls were carried out for 1 sample in every 5. PCR reactions were carried out on all controls and finished products were run on a 1.2 % gel to confirm lack of contamination.

For mitochondrial sequences, 50% of samples were replicated by extracting twice from independent samples of the same bone. DNA sequences were considered authentic when independent extracts from the same individual yielded identical sequences.

2.2.4 Statistical analysis

2.2.4.1 Phylogeography, diversity and expansion signals

Three sequence data sets were obtained for analysis in this chapter. Two of these data sets (ancient UK and modern UK) were obtained using the methods described above. The third data set involving European sequences was obtained from a previous study (Randi et al., 2004). The European sequences were freely downloadable from Genbank (accession numbers: AY625732-AY625892). All data sets sequences were aligned against each other using the programme CLUSTAL X (Larkin et al., 2007). A 399 bp region of the mt-DNA control region was used for all subsequent analyses.

The European data set analysed in this chapter was divided into the clades identified by previous authors (Randi et al., 2004; Lorenzini and Lovari, 2006); Eastern, Western and Central as well as the sub species *C. c.italicus.* Summary statistics were calculated in DNA sp 10.4.9 (Rozas et al., 2003) for each of the clades individually and combined, as well as for the ancient and modern UK samples individually and combined.

The relationship between European and UK populations (using both ancient and modern data) was investigated using a median joining network (MJN) constructed using the programme NETWORK (Bandelt et al., 1999). Additionally, an analysis of molecular variance (AMOVA) was used to calculate both F_{ST} and Φ_{ST} values between groups using

Arlequin v 3.0 (Excoffier et al., 2005). Φ_{ST} differs from F_{ST} in that it incorporates both haplotype frequencies and the number of nucleotide differences between each pair of haplotypes (Excoffier et al., 1992). Two tests of selective neutrality were performed in DNA sp 10.4.9 (Rozas et al., 2003): Tajima's D (Tajima, 1989) and Fu's *Fs* test (Fu, 1997). These tests determined whether sequences are evolving randomly, as expected under neutral theory, or if they are affected by alternative mechanisms such as selection, gene flow, demographic expansion or decline. A population that has experienced any of these alternate mechanisms will result in a rejection of the null hypothesis of neutrality. These tests can, therefore, identify the effects of demographic changes. For both statistics, a demographic expansion produces large negative values.

Mismatch distributions, examined using ARLEQUIN 3.0 (Excoffier et al., 2005), were also used to evaluate possible events of expansion and decline (Rogers and Harpending, 1992). Mismatch distributions were analysed using the sudden expansion model (Rogers and Harpending, 1992) and goodness of fit tests (sum of squared deviations, SSD; Harpendings raggedness index R; Schneider and Excoffier 1999) of the observed to the estimated mismatch distributions were computed. Tau (τ; calculated using ARLEQUIN 3.0) was used to estimate expansion time (T) using the equation: $T = \tau/2\mu$ where μ is the mutation rate in units of substitutions per locus per generation (Rogers and Harpending, 1992). The substitution rate calculated from BEAST (see below) is in units of substitutions per site per year. Therefore this value was converted by multiplying by the length of the sequence (399bp) and by the generation time (3 years; after Randi et al. 2004).

Neutrality tests and mismatch distributions were carried out on both UK (ancient and modern separately and combined) and European data (each clade separately and combined).

2.2.5 Demographic analysis

2.2.5.1 BEAST

BEAST is a powerful and flexible Bayesain method which employs MCMC simulation analyses for the joint estimation of genealogy, demographic patterns and substitution parameters (Drummond and Rambaut, 2007). This program was used to obtain direct substitution rate estimates and to explore past demographic changes in roe deer, using the Bayesian Skyline Plot (BSP).

The dataset used for BEAST analysis was ancient samples only (excluding Moor House samples, see Chapter 4). Substitution rates were estimated from temporally spaced sequence data (Drummond et al. 2002), obtained by stratigraphic dating. Although such dates will have associated errors, they are expected to provide sufficient calibration information for the estimation of rate and divergence dates (Drummond et al 2002), and are more appropriate than using an external calibration point (Ho and Larson, 2006). All dates were provided as years before present (YBP).

Input files were first generated with BEAUTi version 1.4.2 (Rambaut & Drummond 2007a). Estimates of substitution rates have been shown to be relatively robust to the assumed demographic model (Ho et al., 2007). Nevertheless, 2 demographic coalescent models (constant and Bayesian skyline plot; BSP) were assessed.

For each model applied, three independent MCMC runs of four chains each were run for 20,000,000 iterations, of which the first 10% were discarded as burn-in. Samples from two runs (which yielded similar results) were combined to estimate model parameters. Genealogies and model parameters were sampled every 2,000 iterations. An explicit post mortem damage (PMD) model was incorporated into each run (Rambaut et al., 2009). This model allows each site in an alignment probabilistically to be the result of a PMD event. As DNA damage will accumulate through time, it assumes that it is more likely for sequences derived from older specimens to have miscoding lesions. To model this, the probability that any given nucleotide remained undamaged is assumed to decay exponentially with sample age (Rambaut et al., 2009). A strict molecular clock model was applied. To determine the model of sequence evolution to use in this program a hierarchical likelihood test in Mr. MODELTEST 2.2 (Posada and Crandall, 1998) was performed. The substitution model chosen was Hasegawa, Kishino and Yano (HKY) (Hasegawa et al., 1985). Independent runs were combined using Tracer 1.4 (Rambaut and Drummond, 2007) to generate credibility intervals that represent the coalescent model and phylogenetic uncertainty and to produce final estimates. For combined runs, effective sample sizes (ESSs) for each parameter exceeded 100, which indicated efficient mixing (i.e. low autocorrelation in the Markov chain) and sufficient sampling of model parameters. Model selection (constant versus BSP) was performed by comparison of average marginal posterior likelihoods and their differences in harmonic means using approximate Bayes factors. Rate estimates for further analyses were

derived under the BSP model. A BSP coalescent model is preferable as it 'averages out' the demographic history of the sample.

2.2.5.2 Isolation with migration (IM)

For the final part of this chapter's analysis, the program isolation with migration (IM) was used. The IM analysis model assumes an ancestral population splits into two descendant populations with gene flow possibly continuing between the divergent populations. To fit the IM model, a Bayesian coalescent method is used to integrate all possible genealogies using MCMC simulations.

This program simultaneously estimates six basic demographic parameters. These include: ɵ (theta) of the ancestral and two descendant populations scaled by mutation rate (μ) ($\theta = 4N_e\mu$, where N_e = effective population size of either two descendant populations or the ancestral population); directional gene flow rates per gene copy per generation (m1 from population one to two and m2 from population two to one; $m = \mathrm{m}/\mu$, where m = genes moved from one population to the other); and time (t) since population divergence from an ancestral panmictic population ($t = \mathrm{t}\mu$, where t = generations since population splitting).

IM makes several assumptions including: selective neutrality; that the two populations being tested are each other's closest relatives; and random sampling from a panmictic population (Hey and Nielsen, 2004). Taking these assumptions into account, splitting between the UK population and its most closely related European population (clade central) was examined. All samples, from clade central as stated both by Randi et al., (2004) and from my MJ network (excluding those from UK) were used to define this clade. For the UK populations both ancient and modern samples were incorporated but haplotypes that were identified as introductions from the continent by man (e.g. the Norfolk haplotype, see Chapter 3 & 4) were eliminated to minimise negative impacts on parameter estimates.

One further population split was also estimated. This was carried out to validate the mitochondrial substitution rate. As will be discussed in Chapter 3, a series of known translocations took place in the 19th century to facilitate the recovery of the southern roe after extinction. One well documented re-introduction occurred from Perthshire into Dorset, Milton Abbas in 1800 (i.e. 210 YBP). It was therefore tested whether IM was able to estimate

accurately the known date of introduction. The complete, 750 bp, mitochondrial DNA control region as well as 16 microsatellite markers (as described in Chapter 3) were used for this population split.

For both population divergences the program was set up as follows. For mtDNA the program was run under an HKY model of evolution. The IM program scales parameters to the neutral substitution rate/generation and, therefore, a good estimate of the substitution rate was critical to convert these parameters to biologically informative values (Hey and Nielsen, 2004). The substitution rate estimated from BEAST (for mtDNA) was therefore incorporated into the model. For the additional data set, which included microsatellites, simulations were conducted using a stepwise mutation model (SMM). A neutral mutation rate of $\mu = 5 \times 10^{-4}$ per generation for microsatellite loci was assumed. This microsatellite mutation rate was chosen because it is considered the average rate over many species (Ellegren, 2000; Estoup et al., 2002; Sun et al., 2009). In total, three runs were conducted in IM using a two-step heating increment. Each Markov chain was run for 100,000,000 generations after discarding 10% burn in. The first run was conducted to determine appropriate priors for subsequent runs; unrealistic upper bounds for priors were used in this preliminary run. Uninformative priors (i.e. ranges that encompassed the entire posterior distributions) were then set for the final two runs. The final runs were conducted using identical conditions but different random number seeds to test whether multiple runs gave similar results. To ensure convergence, simulations were run until the smallest effective sample size (ESS) estimates were at least 100 (Hey, 2005). For the dataset, both runs gave similar results; the data from the longer of the two runs are therefore presented. The mode is reported along with the 95% HPDs (highest posterior densities; Hey and Nielsen 2004).

2.3 Results

2.3.1 Phylogeography, genetic diversity and expansion signals

The data taken from Randi et al. (2004) divide into three separate clades and the sub species *C. c. italicus*. With the addition of UK samples this structure was maintained (Figure 2.2). The inclusion of UK data increased, to 104, the number of haplotypes found. Some of the haplotypes found in both ancient and modern UK populations were shared with the central European clade, and the network showed close association between ancient and modern UK samples and the central European clade (Figure 2.2). This was consistent with the results of the AMOVA (Table 2.4), which showed both F_{ST} and Φ_{ST} values were lowest between the central clade and UK populations.

Table 2.4. Pairwise F_{ST}s (above diagonal) and Φ_{ST} (below diagonal) for roe deer between European clades and ancient and modern UK populations for 399bp of the mt-DNA control region. Values in bold indicate significance after Bonferroni correction.

	1	2	3	4	5	6
1. Central clade	0	**0.13**	**0.35**	**0.1**	**0.07**	**0.14**
2. Western clade	**0.56**	0	**0.51**	**0.15**	**0.16**	**0.22**
3. C. *c. italicus*	**0.58**	**0.84**	0	**0.39**	**0.44**	**0.44**
4. Eastern clade	**0.57**	**0.69**	**0.67**	0	**0.12**	**0.18**
5. Ancient UK	**0.09**	**0.61**	**0.75**	**0.6**	0	**0.14**
6. Modern UK	**0.16**	**0.66**	**0.74**	**0.64**	**0.17**	0

Overall, levels of mitochondrial diversity showed a wide range within Europe (see Table 2.5). The central European clade had the highest levels of diversity and the sub species *C. c.italicus* the lowest (Table 2.4). When all European data were combined, mitochondrial diversity was high (Table 2.5) with an average of one distinct haplotype over 8.9 individuals (81/721 individuals). Levels of diversity were examined (for the same 399 bp region) in both ancient and modern UK populations to compare with the European data (Table 2.5), and levels of diversity in ancient UK populations were relatively high. In fact, diversity in ancient UK populations was similar that of the main European clades, while modern UK populations were less diverse (Table 2.5).

Neutrality tests yielded no evidence of expansion (see Table 2.5) within any of the individual clades or the subspecies *C. c. italicus*. However, mismatch distributions showed evidence of expansion in the eastern clade and the sub- species *C. c. italicus* but not in other individual clades [for significance of difference from model (SSD) and raggedness (R) data see appendix one]. The Eastern clade and *C. c. italicus* showed evidence of expansion at $\tau = 3.84$ and 3.36 (appendix one). Using this value of τ, estimated expansion times of 8,098 and 7,086 YBP were calculated respectively using the substitution rate calculated from the BEAST analysis described in the next section (2.3.2). Strongest evidence of expansions was observed for all European data combined, ancient UK populations and ancient and modern UK populations combined, as discussed below.

For all European data combined, there was evidence of expansion from some methods (Fu's $F_s = -51.362$; $P<0.02$; see also mismatch distribution, Figure 2.3 a) but not all (Tajima's D = -0.748 P>0.10). Goodness of fit tests for the mismatch distribution were not significant (SSD = 0.006 P>0.05, R= 0.018, P >0.05) (Figure 2.3 a). The main expansion event occurred at τ = 6.41, which corresponded to an estimated expansion time of 13,523 YBP. A signal of expansion was also detected for ancient UK populations from Fu's *Fs* (Table 2.5) and the mismatch analysis (SSD = p>0.05, R = P>0.05) (Figure 2.2 b). The main expansion event occurred at $\tau = 2.66$. This corresponded to an expansion event at 5,612 YBP. The modern UK data showed strong evidence for expansion only when combined with ancient UK data (see mismatch distribution, Figure 2.3 c, and Fu's *Fs*, Table 2.5).

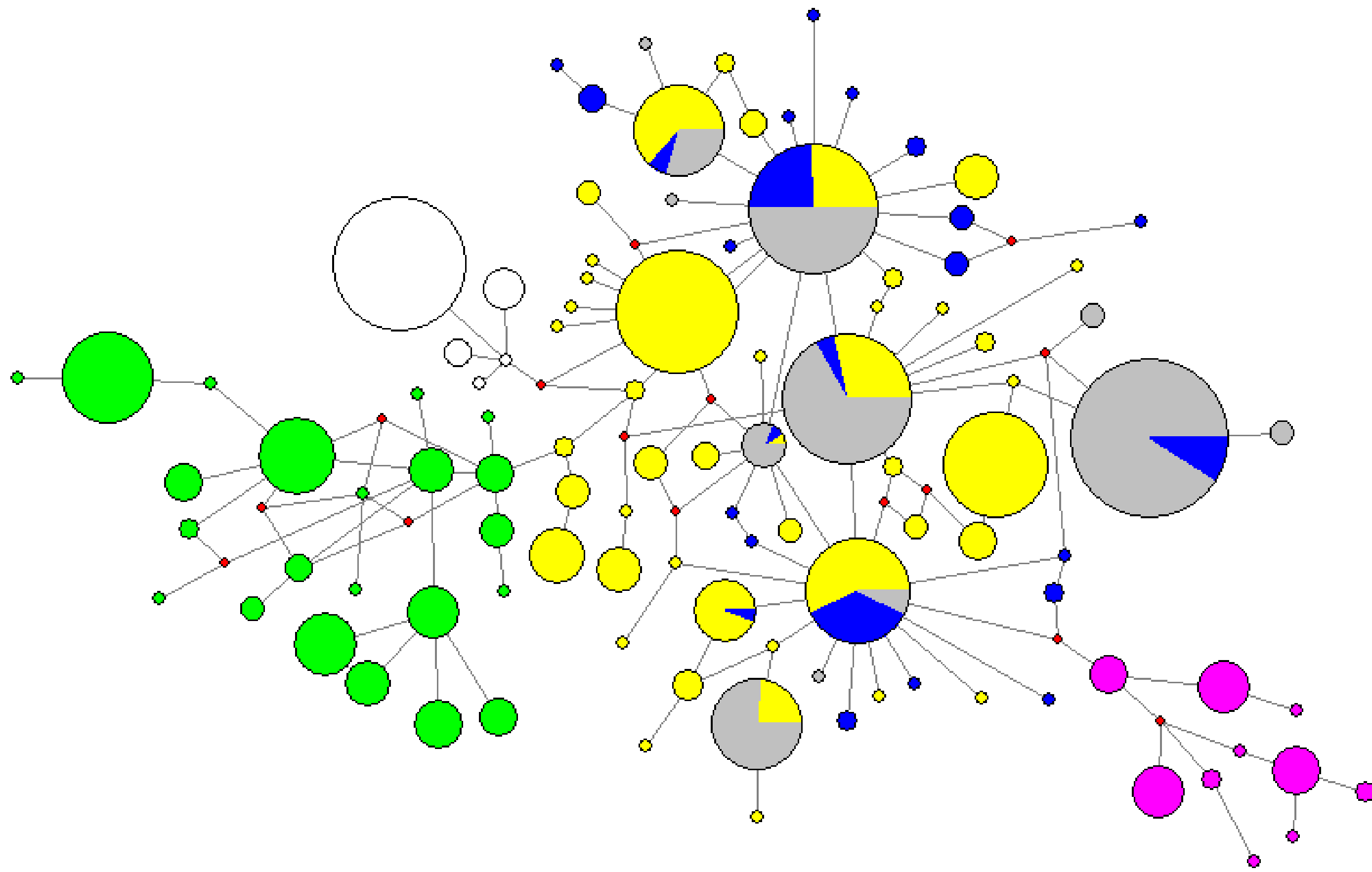


Figure 2.2. Median joining network (MJN) computed using 104 haplotypes from European clades along with ancient and modern UK populations. Circle size is proportional to haplotype frequencies. The coloured circles represent the clades formerly defined by Randi *et al.,* (2004) ● central, ● eastern, ● western, ○ the sub species *C. c.italicus,* ● ancient UK populations, ● modern UK populations and ● inferred intermediate haplotypes not represented by any sampled roe.

Table 2.5. Population genetic summary and demographic statistics for European and UK populations; n: number of individuals; nh: number of haplotypes; hd: haplotype diversity (s.d.); π: nucleotide diversity; k: average number of nucleotide differences; *Fs*: Fu's *Fs*; D: Tajima's D.

	n	nh	hd ± sd	π	k	F's	D
Clade central	376	44	0.91 ± 0.0006	0.0075	2.98	-26.94	-1.12
Clade east	184	22	0.89 ± 0.0001	0.0080	3.20	-4.75	-0.32
Clade west	55	10	0.82 ± 0.0005	0.0061	2.41	-0.97	0.3
C.C. Italicus	105	5	0.26 ± 0.0540	0.0013	0.50	-1.44	-0.69
European clades and *C.c. Italicus* combined	721	81	0.952 ±0.0001	0.0130	5.16	**-51.362**	-0.748
Ancient UK	87	24	0.88 ± 0.0004	0.0061	2.41	**-14.4**	-1.5
Modern UK	279	12	0.76 ± 0.0003	0.0056	2.21	-0.21	0.33
Ancient and Modern UK combined	366	30	0.82 ± 0.0130	0.0059	2.36	**-13.75**	-1.35

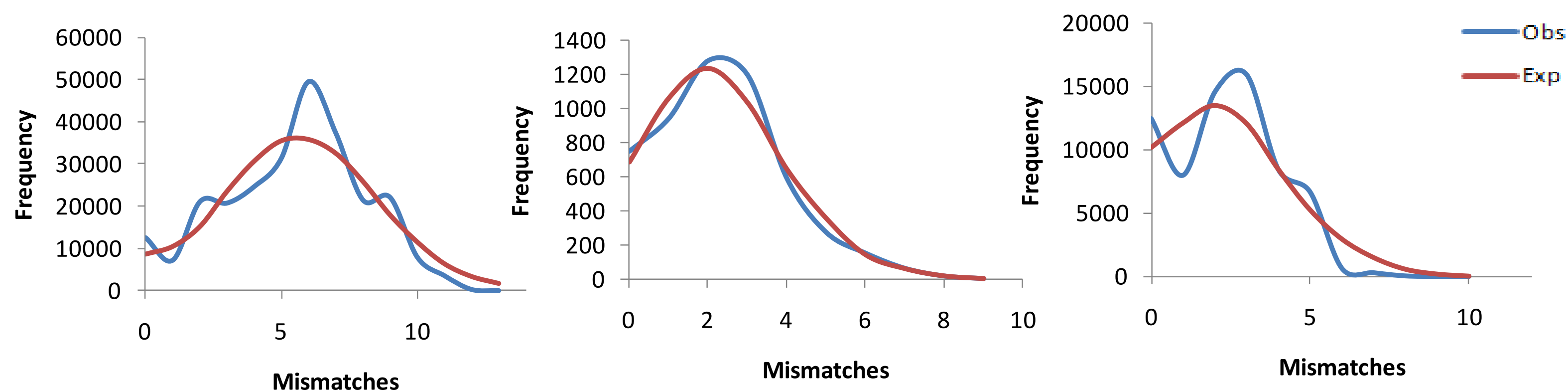


Figure a

Figure b

Figure c

Figure 2.3. Mismatch distributions for mitochondrial DNA haplotypes sampled from; all European clades and *C. c. italicus* combined (fig. a), ancient UK (fig. b) and ancient and modern UK populations combined (fig. c).

2.3.2 BEAST

The substitution rate was calculated under two models of population size (BSP and constant). In each case, ESSs for mean evolutionary rate, population size and posterior likelihood were all found to exceed 100.

Substitution rate estimates were very similar under the different population growth models (see Table 2.6). This shows that the estimated rate was not particularly sensitive to model assumptions about demographic history. The models were compared using the average marginal posterior probabilities of the data given the model (Table 2.6). This indicated that a BSP growth model (ln L=-837) fitted the data better than the constant growth model (ln L= 840). This was further confirmed by examining the differences in harmonic means of the sampled marginal likelihoods using approximate Bayes factors (Table 2.6).

Therefore, assuming a BSP model for the UK ancient DNA using data calibrated against the estimated calendar age of the samples (from stratigraphic data; Table 2.1 in methods) the substitution rate estimate was 3.69×10^{-7}(95% highest posterior density interval; HPDI; 1.82×10^{-7} - 5.82×10^{-7}) substitutions per site per year (s.s.yr $^{-1}$) (Table 2.6). The posterior estimates of the substitution rate were found to be distinctly unimodal (Figure 2.4). In terms of magnitude, the roe deer rate estimate is shown to be in agreement with estimates that have been obtained from other, intraspecific, ancient DNA datasets (Figure 2.5). A coalescent reconstruction of past population dynamics of British roe deer was also carried in BEAST using the Bayesian Skyline Plot (BSP) (Figure 2.6). The plot shows a rapid expansion in the effective number of roe deer between 5,000 and 6,000 YBP. After that time frame, roe deer numbers appear to remain relatively stable.

Table 2.6. Evolutionary rate estimations using different population growth models (BSP v Constant) with a HKY model of evolution and a strict molecular clock.

	Ancient UK samples			
Parameters	Lower 95% HPD limit	Median	Mean	Upper 95% HPD limit
	Bayesian Skyline plot (BSP)			
10^{-7} x substitution rate (subs/site/year)	1.82	3.55	3.69	5.80
Mean ln (Posterior)			-837	
Bayes factor			26.93	
	Constant population size			
10^{-7} x substitution rate (subs/site/year)	1.57	3.71	3.88	6.49
Mean ln (Posterior)			-840	
Bayes factor			0.004	

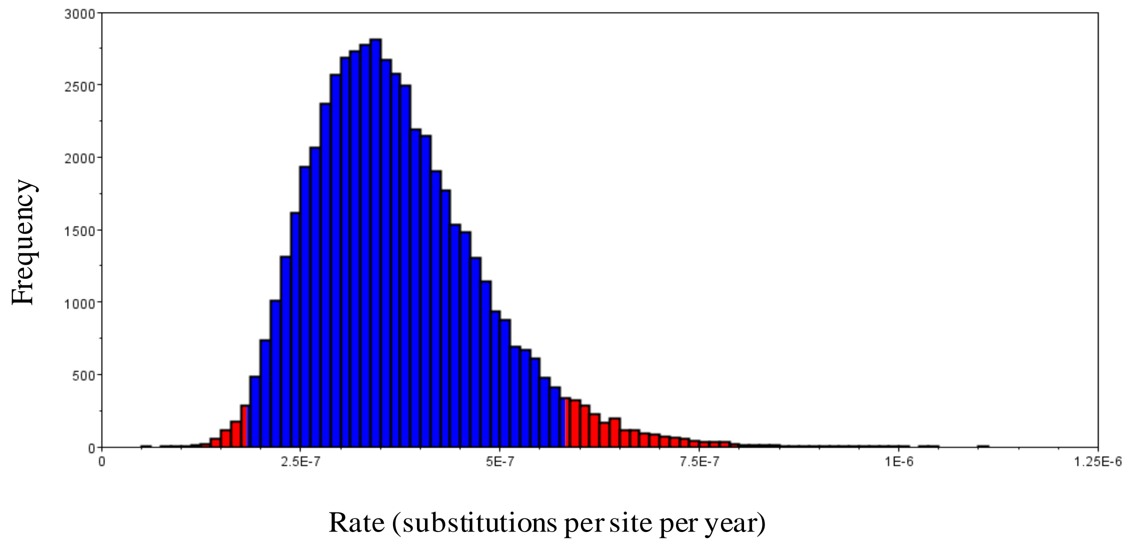


Figure 2.4. BEAST output of roe deer substitution rate estimated under a BSP population size model from 86 roe deer stratigraphic date samples from the UK.

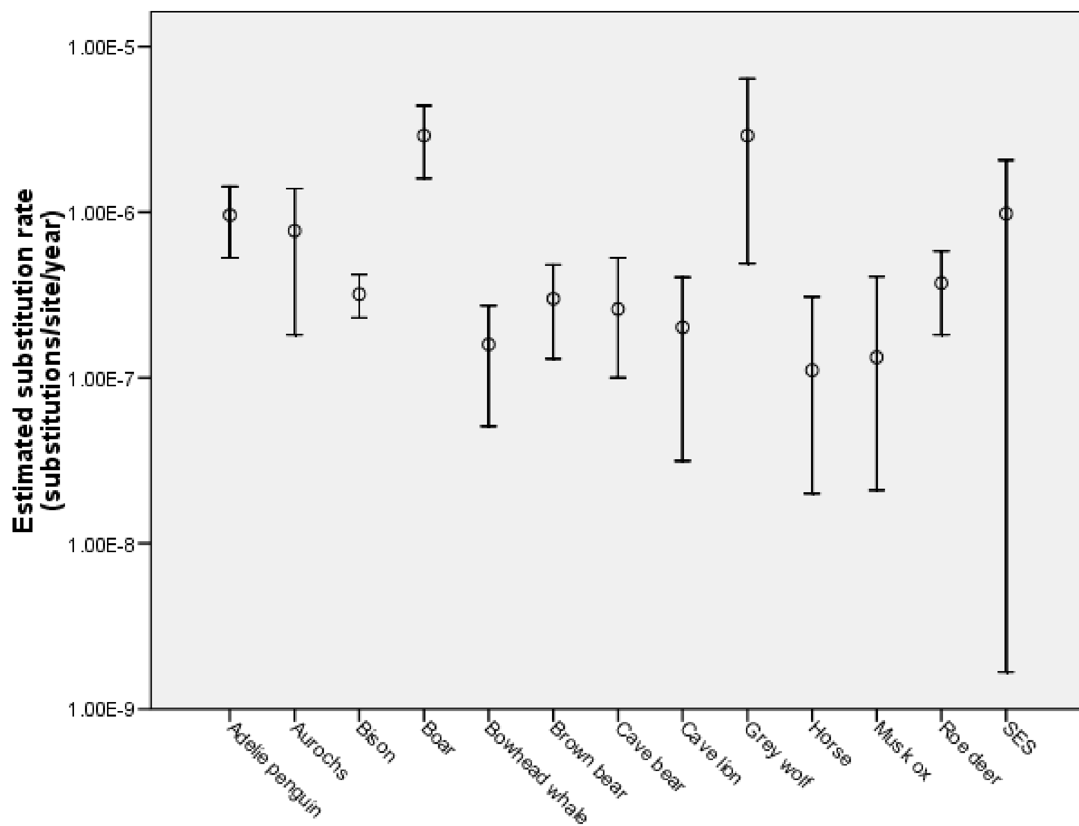


Figure 2.5. Rate estimates from a range of ancient DNA data sets, for rates and sources see appendix two. The roe deer represents the rate calculated from this study. Bars denote 95 % HPD, SES = Southern elephant seal.

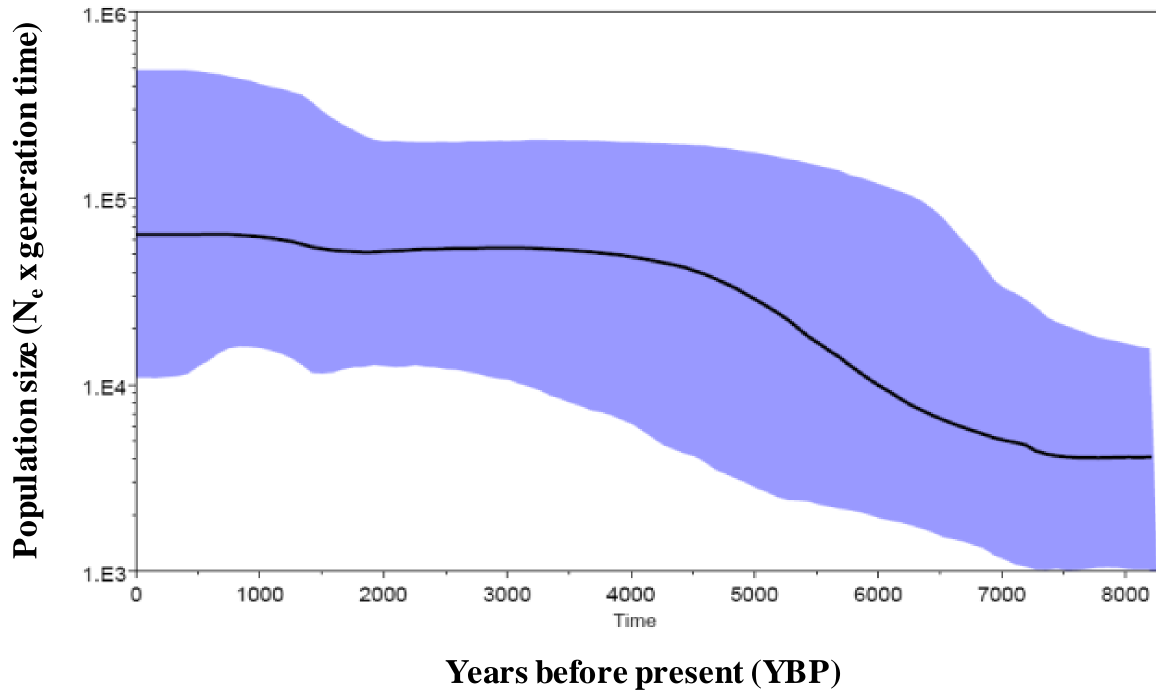


Figure 2.6 A Bayesian skyline plot derived from an alignment of ancient UK roe deer mtDNA d-loop sequences (399 bp). The x axis is in units of years before present, and the y axis is equal to population size (the product of the effective population size and the generation length in years = 3). The thick black line is the median estimate, and the thin blue lines show the 95% highest posterior density intervals.

2.3.3 Isolation with migration (IM)

The divergence time for ancient UK and clade central is well resolved (Figure 2.7), with a posterior distribution that has a distinct peak and bounds that fall within the prior distribution. For ancient UK and clade central, the position of the peak indicates a population split at t = 0.88 (Table 2.7) which corresponds to 5,600 YBP with 95% equally tailed Highest Posterior Density (HPD) spanning from 3,500 to 11, 200 YBP. The effective population sizes for both the ancient UK and the central clade are shown to be similar. The ancestral population is, however, shown to be approximately ten times smaller than the two recent populations. The estimated rate of mt-DNA gene flow into the UK and into Europe is very small suggesting that, after separation, the populations remained isolated until later anthropogenic introductions.

The divergence time for Dorset and Perth is also well resolved (Figure 2.8), the position of the peak indicates a population split at t = 0.10 (Table 2.8) which corresponds to 209 YBP with 95% equally tailed Highest Posterior Density (HPD) spanning from 106 to 622 YBP. The effective population sizes for Dorset and Perth are shown to be similar. The ancestral population is, however, shown to be approximately 20 times larger than the two recent populations. The estimated rate of mt-DNA and microsatellite gene flow into Dorset is larger than the gene flow into Perth, consistent with an introduction from Perth to Dorset.

Table 2.7. Maximum likelihood estimates of isolation and migration model parameters and their respective demographic conversions for the UK populations and the European clade central. The model parameters given in italics (*t*, *m* and *ɵ*) are scaled by µ. The demographic parameters (not italicised) are based on an estimate of µ (see text) where: Ne = effective population size; t = divergence time in years; and m = average number of migrants per 1000 generations per gene copy.

Parameter	Ancient UK	Clade central
t	0.88 (0.6- 1.8)	
t	5625 (3541-11201)	
ɵ	58.1 (31.8-69.3)	57.8 (39.2-69.2)
ɵ (ancestral)	5.65 (1.4 - 29.6)	
Ne	31042 (16991-37037)	30817 (20963-37000)
Ne (ancestral)	3016 (730-15830)	
m	0.01 (0.01-1.2)	0.19 (0.03-1.4)
m	0.004 (0.004-0.60)	0.0889 (0.01-0.65)

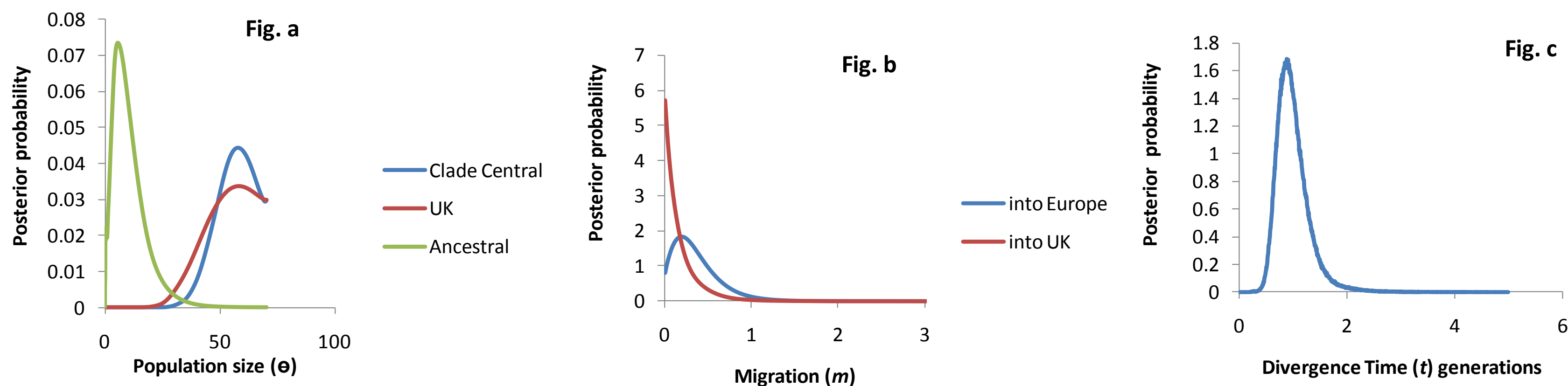


Figure 2.7. The posterior probabilities of model parameter estimates: theta, population size of ancient UK, clade central and the ancestral (Fig. a); *m,* migration rates into ancient UK populations and into the European clade central (Fig. b); and *t*, divergence time of ancient UK and clade central (Fig. c).

Table 2.8. Maximum likelihood estimates of isolation and migration model parameters and their respective demographic conversions for contemporary populations of Dorset and Perth. The model parameters given in italics (*t*, *m* and *θ*) are scaled by μ. The demographic parameters (not italicised) are based on an estimate of μ (see text) where: Ne = effective population size; t = divergence time in years; and m = average number of migrants per 1000 generations per gene copy.

Parameter	**Dorset**	**Perth**
t	0.10 (0.05 - 0.3)	
t	209 (106-622)	
θ	0.21 (0.14-0.56)	0.77 (0.46-1.59)
θ (ancestral)	24.64 (13.82-49.95)	
Ne	36.9 (26.3-100.3)	137.6 (82.6-284.5)
Ne (ancestral)	4417 (2477-8953)	
m	4.15 (1.1 -17.7)	1.24 (0.14 -7.9)
m	5.78 (1.5-24.8)	1.74 (0.20-11.1)

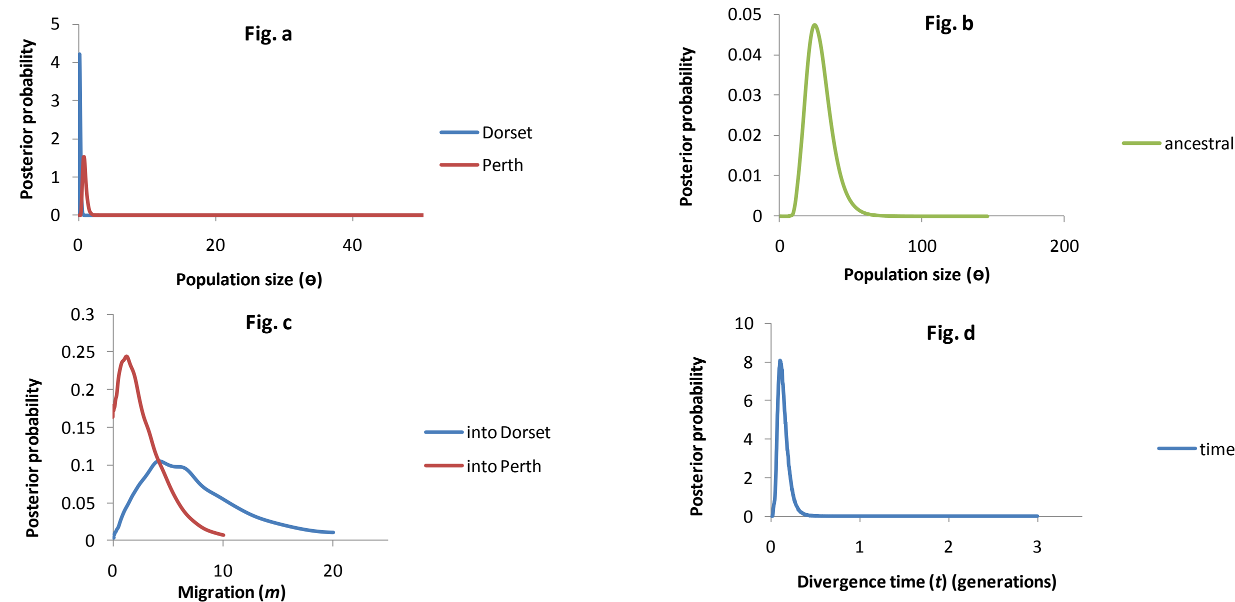


Figure 2.8. The posterior probabilities of model parameter estimates: theta, population size of Dorset, Perth (Fig. a) and the ancestral population (Fig. b); *m*, migration rates into Dorset and into Perth (Fig. c); *t*, divergence time of Dorset and Perth (Fig. d).

2.4 Discussion

2.4.1 Phylogeographic patterns

Randi et al. (2004) identified the existence of three main roe deer clades in Europe (central, eastern and western) and the sub species *C. c. italicus* based on a 750 bp region of the mitochondrial DNA. The same structure was evident for the 399bp fragment analysed in this study. The central clade harboured the greatest diversity, consistent with its widespread distribution (Randi et al., 2004), while the sub species *C. c. italicus* harboured little genetic diversity, consistent with its confinement to the central and southern Apennines (Lorenzini et al., 2002; Randi et al., 2004). Modern UK roe deer populations show lower diversity than any of the European clades or the ancient UK sample, consistent with bottlenecks and founder effects (see further discussion and analysis in Chapter 4). The level of diversity in the ancient sample is comparable to that seen in modern European lineages. However, this comparison is limited by the fact that all recent populations have been impacted by hunting, and therefore contemporary comparisons are more meaningful.

The combined dataset (incorporating the UK samples from this study) added 17 now extinct haplotypes from the ancient UK sample as well as 5 extant haplotypes from modern UK populations. The phylogeography revealed in the MJN had defined lineages in different geographic regions (likely representing glacial refugia; Randi et al. 2004), and the incorporation of UK data strongly supported the origin of the UK populations from just one of those lineages: the central clade (see Figure 2.3).

Previous phylogeographic studies have found a wide array of species colonised northern Europe from the Iberian Peninsula after the ice ages, including: brown bear, *Ursos arctos;* chaffinch, *Fringilla coelebs*; natterjack toad, *Bufo;* and ivy, *Hedera spp*. (Taberlet et al., 1998; Hewitt, 1999). Here, the results suggest that a parallel recolonisation route for roe deer is unlikely. A colonisation route from Iberia would be more supported by the coincidence of UK samples with the western clade.

The fact that UK roe are a subset of samples belonging to the central clade instead supports an eastern colonisation route, as proposed for various other species including: grasshopper, *Chorthippus parallelus;* alder, *Alnus glutinosa*; beech, *Fagus sylvatica;* and newt, *Triturus*

cristatus (Hewitt, 2000). Both recent fossil and molecular data suggest that the central clade originated from the east, although the exact location is unknown (see Randi et al., 2004; Lorenzini and Lovari, 2006; Sommer and Zachos, 2009). An eastern rather than a western colonisation route is further supported by the lower F_{ST} values shared between the UK and the eastern clade. This also correlates with patterns of broad-leaf forest expansion (Petit *et al.* 2003) which is the preferred habitat of the roe deer (Putman and Langbein, 2003).

2.4.2 Rate of molecular evolution and a time frame for demographic and evolutionary history of *Capreolus capreolus*

Both neutrality tests and mismatch distributions suggested strong expansion events for all European clades combined, the ancient UK sample alone, and the ancient and modern UK samples combined. Calculating tau (τ) from the mismatch distributions and using the substitution rate calculated in BEAST, there was evidence of expansion at, 13,500 YBP (HPD; 29,500-9,200) for the central European clade, and 5,600 YBP (HPD; 12,200-6,000) for the UK population. The expansion date for the UK population was consistent with the BSP graph, which showed that roe populations expanded over a similar timescale (Figure 2.6).

The analyses show that roe populations expanded after following the last glacial maximum (LGM). The LGM, which occurred between 23 000–18 000 YBP (Kukla et al., 2002), confined roe and other temperate species to separate southern glacial refugia. This was due to the permafrost and Arctic tundra ecosystems which were widespread in central Europe down to a latitude of 45° (Andersen and Borns, 1997). Following the LGM, species were able to recolonise by expansion into formerly glaciated regions. For the roe deer, evidence from the fossil record infers that during initial de-glaciations (18,000–14,700 YBP) no distributional change is evident (Sommer and Zachos, 2009). However, following the period of de-glaciation a period of warming (14,700 – 11,600 YBP) enabled a rapid expansion of roe into Europe (Sommer et al., 2009; Sommer and Zachos, 2009). The first calculated expansion date (13,500 YBP) is consistent with this fossil evidence. The second expansion date for historical populations of UK roe occurred much later (5,600 YBP). According to fossil evidence, it seems that roe did not re-colonise areas of the northern European lowlands (such as the UK) until the early Holocene (Sommer and Zachos, 2009). Indeed, the first evidence of roe in the UK comes from radiocarbon dated bones with a result of 9,439 ± 100 YBP from a

site in Southern England (Thatcham; (Sommer et al., 2009). During this time, the central European lowlands were apparently being slowly recolonised by birch and pine woods (Usinger, 2004) which would have in turn improved the environmental conditions for roe. However, it was not until 6,000 YBP that the vegetation pattern broadly resembled that of today (Hewitt, 1999). Therefore, the later expansion signal of ancient UK roe suggests a response to the improved environmental conditions of the Holocene; taken together, results indicate that, following the end of the last glaciation, new habitat was quickly exploited by expanding roe populations.

Randi et al. (2004) proposed two expansion events (based on 750bp segments of the mt-DNA control region), scaling values of τ from their mismatch analyses with a 'phylogenetic rate' of 4-6% per million years (Myr). The resulting expansion times were estimated to have coincided with the penultimate (*c*. 200 000 years ago), and the last (*c*. 130 000 years ago) inter-glacials. Using the same data but with the new substitution rate calculated in this study (37% per myr) it is estimated that expansions instead occurred at 13, 300 (HPD; 8,400-22,900) YBP and 8,400 YBP (HPD; 5,300-17,100) respectively. Both of these expansion dates are consistent with the European expansion signal proposed above, based on the same data, but with a reduced sequence length (399bp).

The results of this study are consistent with a number of recent studies that have replaced 'phylogenetic rates' with substitution rates directly calibrated using ancient DNA. Specifically, close matches of the substitution rate from this study (3.69×10^{-7} s.s.yr $^{-1}$) were detected over a similar time frame for brown bear (Saarma et al., 2007) and bison (Shapiro et al., 2004) (3.2×10^{-7} and 3.0×10^{-7} s.s.yr $^{-1}$ respectively). The consistency between ancient DNA studies provides strong evidence that substitution rates are higher than conventional 'phylogenetic rates'. Unless all ancient DNA data sets are confounded by error, which is unlikely (as these would need to have been made systematically and substantially; [113]Ho et al., 2007) then rates can be accepted with some confidence. The use of this substitution rate suggested that divergence and population expansions occur over much shorter timescales than previously proposed. As for previous studies, the more recent dates (based on higher substitution rates) are consistent with expectations based on historical environmental events (e.g. Drummond et al. 2005, de Bruyn et al. 2009). For the roe deer in Europe, the expansion

date is consistent with post-glacial expansions suggested for various other species. For the UK sample, it is consistent with the separation of the UK from the European landmass.

When the Scandinavian and British ice sheets reached their maximum extent, and the North Sea as a consequence receded to its lowest level (Fairbanks, 1989), Britain was connected to the continent by a land bridge. This dry land, referred to as Doggerland, would have allowed roe deer to migrate from central Europe. Coles (1998) suggests that Doggerland existed until at least ca. 9,450 YBP and possibly until as late as 7,450 YBP. Consistent with this scenario, calibrated results (Figure 2.7c; Table 2.7) indicate that the divergence of the two populations occurred not long after the UK split from the continent.

Finally, the substitution rate is further supported by the estimated population splitting time between the contemporary populations of Dorset and Perthshire, which was accurately estimated by IM analyses to within 1 year of the actual known date (Table 2.8).

2.5 Conclusion

This is the first study to determine that UK populations group within the central lineage of European roe. This information provides insight into the possible re-colonisation route of this species into the UK. Re-colonisation of the roe across Europe seems to have occurred very rapidly as environmental conditions improved (evident from expansions). Shortly after roe re-colonised the UK, populations became isolated as the land bridge was cut from mainland Europe. The genetic data put forward provide a plausible time frame for when UK and European populations diverged. Overall, this study has provided information on evolutionary events that have shaped European roe during its recent post-glacial history. The information gained from this study could be considered of particular importance as it indicates the response of roe to climatic changes. Today, rapid climate change is beginning to impact the biology of many species (Bradshaw and Holzapfel, 2006) and unravelling specific responses to past climatic change may be a powerful way to help to predict the future (Hadly and Barnosky, 2009), which may be fundamental to long term conservation and species management planning (Leonard, 2008).

Chapter 3 : Genetic and morphological differentiation in British roe deer populations (*Capreolus capreolus*)

3.1 Introduction

Over the last few centuries anthropogenic activities have had profound impacts on mammalian populations, causing severe demographic fluctuations. Populations have gone from being reduced in size, fragmented and even locally extinct (extirpated) to showing near to complete recovery. Recovery of a population has generally occurred very rapidly once the initial factor causing decline has been alleviated or removed.

Typically, recovery can occur as a species naturally disperses and re-colonises areas formerly occupied from remnant populations. This process of natural re-colonisation usually occurs when degraded habitats are restored and corridors are created between remaining former colonised areas and other populations (Hochkirch et al., 2007). With time passed, the naturally re-colonising populations may be well connected with neighbouring populations and, genetic relationships may follow a model of isolation by distance (IBD). IBD describes the tendency that individuals will find mates from nearby populations rather than distant populations (Sokal and Wartenberg, 1983). As a result, populations that live near each other will be genetically more similar than populations living further apart (Wright, 1942).

In some situations, however, habitat restoration may be poor or natural populations too fragmented to enable re-colonisation of distant areas holding suitable habitat (Cheyne, 2006). In these situations, populations may be restored through human intervention using translocations. Translocation is defined as the movement of living organisms from one area with free release in another (IUCN, 1987). One type of translocation that has become increasingly popular is re-introduction (Lipsey and Child, 2007). Re-introduction is the intentional movement of an organism into a part of its native range from which it previously disappeared or became extirpated in historic times (Griffith et al., 1989; Armstrong and Seddon, 2008). Re-introductions can have important consequences for the genetic structure of populations, whereby expected patterns of IBD may be obscured (Mock et al., 2004; Latch and Rhodes, 2005).

Re-introductions frequently result in population bottlenecks (Griffith et al., 1989). The impact of bottlenecks on a population is well documented (this has been covered in more detail in Chapter 1: introduction). However, in brief, populations that have experienced bottlenecks through re-introduction (founding) face a host of interrelated demographic and genetic problems as a result of their small effective population sizes (O'Brien and Evermann, 1988). These problems include genetic drift.

Genetic drift is a stochastic process that causes major reductions in genetic diversity and rapid differentiation between source and founder populations (Mayr, 1954; Nei et al., 1975). The extent to which genetic drift acts on a population will depend on the numbers of founder individuals, as genetic drift occurs at a rate inversely proportional to the effective population size (Crow and Kimura, 1970). Therefore, losses of genetic variation and genetic divergence will be greater in populations established with fewer individuals (Baker and Moeed, 1987; Merilä et al., 1996; Mock et al., 2004). Even when a high number of individuals are translocated, genetic diversity harboured in subsequent generations may still be low. This is because only a few of the introduced individuals may contribute to future generations (see Schwartz and May, 2008). Clearly, the number of individuals can significantly alter the amount of genetic variation and divergence occurring among re-introduced populations. Other factors that can exacerbate these impacts are the characteristics of the population used as a source.

Geographically close source populations are usually remnants that survived the pressures acting to cause populations elsewhere to become extirpated. As such, these populations are likely to have been influenced by demographic contraction. Impacts on the genetic diversity of translocated populations can, therefore, be compounded. One way to get around this problem can be to use geographically distant populations. However, such populations are also likely to be genetically distant. Therefore, although impacts on genetic diversity may be lowered (depending on effective population sizes, as above), genetic distances from native populations may be substantially altered. The use of such populations also raises issues regarding the spread of unsuitable genotypes (Akeroyd, 1994), which is a concern, as spread and hybridisation with native taxa can cause loss of local adaptation. This can occur through introgession of non-indigenous alleles and loss of local alleles, otherwise known as outbreeding depression (Rhymer and Simberloff, 1996).

Irrespective of the exact parameters of a re-introduction, there are clear genetic implications for future populations. These include reductions in genetic diversity within (sub) populations and increased genetic differentiation among them.

These ‘genetic signatures’ have been documented in a number of re-introduced populations. Good examples have been provided by studies based on re-introduced Alpine ibex, *Capra ibex ibex* (Biebach and Keller, 2009), wild turkey, *Melegris gallapavo silvestris* (Latch and Rhodes, 2005), white tailed deer, *Odocoileus virginianus* (Deyoung et al 2003) and fisher, *Martes pennanti* (Williams et al., 2000). A few studies have even shown populations with morphological alongside genetic divergence. Morphological change following re-introduction seems to be a relatively under-studied subject. However, where it has been found, this has been suggested to be due to strong genetic drift (Reznick and Ghalambor, 2001; Rasner et al., 2004; Pruett and Winker, 2005), although the exact cause is not well understood.

In spite of the foregoing, not all re-introduced populations necessarily exhibit strong ‘signatures’ of re-introduction. For example, when introduced populations rapidly expand, relatively high levels of genetic diversity can be retained (e.g. Zenger et al., 2003). Such expanding populations may even overcome isolation, enabling genetic exchange between neighbouring populations. Genetic exchange has the potential to introduce new genetic variation (Hicks et al., 2007; Le Gouar et al., 2008) and limit genetic divergence (Hicks et al., 2007).

It is important to study populations that have been impacted by re-introduction as these can have implications for the design of optimal re-introduction strategies for conservation and management.In this study the effects of population declines followed by re-colonisation via natural and non-natural dispersal on population structure and diversity of the British roe deer (*Capreolus capreolus*) were examined. The British roe deer is one of two cervid species indigenous to the UK, the other being the red deer (*Cervus elaphus*). First post-glacial records of this species date back to between 10,050 and 9,600 YBP from a site found at Thatcham in Berkshire (Yalden, 1999). Since these prehistoric times, roe have been exposed to various anthropogenic influences that have had major impacts on populations. One of the most significant impacts occurred during the late medieval period when roe deer populations were severely reduced as a result of overhunting and deforestation. Historical documents indicate that these declines were so severe that roe were confined to parts of Scotland and possibly

some of the northern border counties (Whitehead, 1964). In most of the midlands and southern English counties roe were reportedly absent by the 16th century (Ritson, 1933). During the 1800s roe deer populations began to recover and, since then, this recovery has been remarkable (see Ward, 2005). Recovery in northern parts of the UK can generally be attributed to natural expansion of remnant populations into formerly occupied areas following afforestation (Taylor, 1948). Contrastingly, in southern parts of the UK all populations are believed to have descended from re-introduction events (see table 3.1) (Whitehead, 1964).

Table 3.1. Summary of all known roe deer introductions into southern UK (after Whitehead, 1964).

Site of introduction	Date	Site of origin	Number released
Milton Abbas, Dorset	1800	Perth, Scotland	4
Petworth, Sussex	1800	Unknown	
Abbotsbury, Dorset	1820	Unknown	
Windsor Park, Berks	1825	Dorset	4
Windsor Park, Berks	1850	Petworth	
Epping Forest, Essex	1883	Dorset	6
Epping Forest, Essex	1884	Unknown	8
Thetford, Norfolk	1884	Wurttemberg, Germany	12
Petworth, Sussex	1800s	Unknown	
Petworth, Sussex	1890	Scotland	
Brentwood, Essex	1892	Unknwown	2
Horsham, Sussex	1931	Unknown	

The first recorded re-introduction occurred into Milton Abbas, Dorset in 1800 when 4 to 5 individuals were translocated from Perthshire. This introduction must have been a success because, by 1825, individuals from the Dorset population were translocated into Windsor Great Park, Berkshire.

Another important southern re-introduction occurred into Santon Downham, Norfolk in 1884. This involved the introduction of 12 geographically distant roe from Wuttermburg, Germany. It is believed that this introduction has been responsible for re-establishing all populations within Norfolk (Whitehead, 1964; Hugh Rose Pers. comm.). Further introductions into southern Britain have also taken place; however, both the origins (likely to

be Scotland; Whitehead 1964) and the numbers of individuals involved are not very well documented (see table 3.1).

Although most introduction events are recorded for southern parts of the UK, one significant introduction event is recorded further north. This involved the introduction of 12 Austrian roe into Windermere, Cumbria in 1915. At the time of this introduction native roe already existed within Cumbria; however, the Austrian roe were apparently introduced to 'improve the local breed'. It is thought that these introduced roe bred and multiplied considerably (see Prior, 1995). Excluding the one continental introduction that took place in Windermere, Cumbria there are few other *known* introductions that could have influenced the re-colonisation of roe in Scotland and Northern England. The origin of roe deer from these parts is thus linked directly to the dispersal of individuals that survived in remnant populations since bottlenecking. It is for this reason that both Northern English and Scottish roe deer populations are believed to represent ancient indigenous stock of the British roe deer.

It is likely that these events have resulted in important consequences for genetic structure and diversity of roe. Historic bottlenecks may have lowered levels of genetic diversity in all UK roe populations. However, subsequent founder events, which may have been strongest in the southern populations, as a result of re-introduction, may have led to further losses in genetic diversity. In addition, such strong founder events may have disrupted patterns of IBD that might otherwise have been found. Currently, whether this is the case is very poorly understood. Here, a population genetic and morphological analysis of roe deer from different localities from across England and Scotland is presented to evaluate population diversity and structure of this species. Specifically the study will address the following predictions:

1. Levels of genetic diversity will vary across populations with the lowest levels occurring in southern UK where populations are likely to have undergone strong founder events as a result of introduction.
2. Patterns of IBD explain genetic structure in northern populations where natural re-colonisation has taken place following declines. Contrastingly, IBD may be less important in southern populations.
3. Overall, population differentiation will be high across the UK, in line with this species' history.

3.2 Methods

3.2.1 Sample sites and collection of samples

Tissue samples were taken from 367 male and female culled roe deer from 15 main sampling areas across the UK during 2007-2009 (See Figure 3.1 and Table 3.2). In addition, complete heads were taken from 114 of the sampled females across 9 locations (see Chapter 5) across the UK for morphological measurements. Locations were chosen to ensure both re-introduced and remnant native populations were sampled.

All tissue samples were stored in 20% DMSO/saturated NaCl solution (Amos and Hoelzel, 1991) and placed in the freezer (-20˚C) to minimise DNA degradation.

Complete heads were stored at -20˚C until ready for preparation. For skull preparation all heads were defrosted thoroughly and then skinned using a sharp domestic knife. Skulls were then placed in a boiling 10% solution of a commercially available enzymatic detergent in water (with enough water to cover the entire skull). Skulls were then boiled for approximately 1 ½ hours. After this time they were cooled slightly and more of the flesh, now softened, was removed by hand. After this process some flesh still remained. Therefore cold water maceration was used to remove all remaining flesh. This involved filling Rubbermaid tubs with the 10% solution of detergent plus water and placing skulls in the water for up to 3 weeks. Putrid water was poured off periodically (every 3-7days) and replaced with fresh water. After this time, the skull was rinsed under running water and finally cleaned with a stiff wire brush. Once skulls were completely clean, they were placed in 100% ethanol for 10 minutes and then air dried overnight. During every stage of the above process all biological waste that was generated was disposed by double bagging and placing in clinical waste for incineration (in accordance with Durham University guidelines).

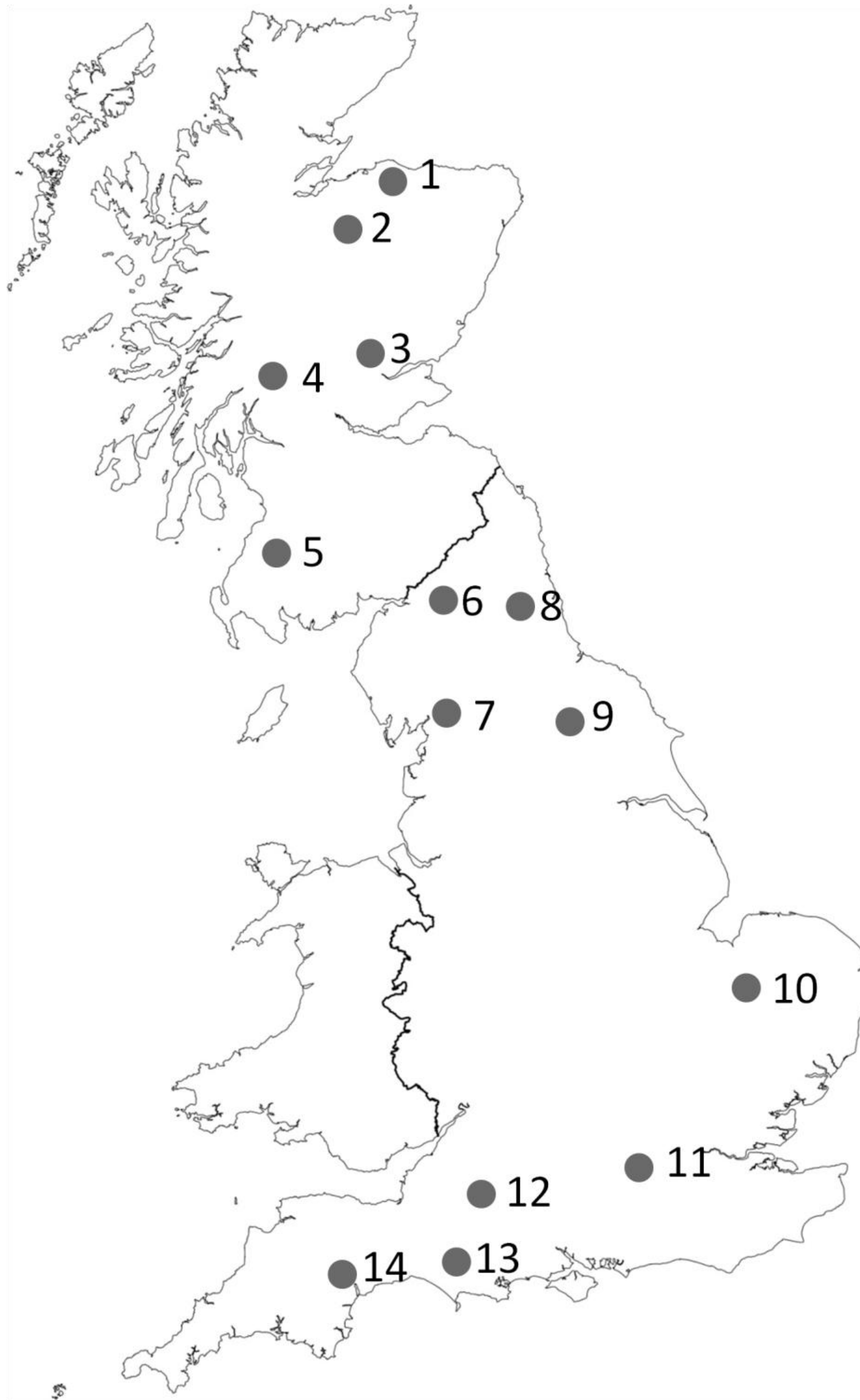


Figure 3.1. Map showing the 14 main areas where roe deer were sampled within the UK between 2007 and 2009. 1) Morayshire: Elgin 2) Morayshire: Aviemore 3) Perthshire 4) Dunbartonshire 5) Ayrshire 6) Cumbria 7) Lancashire 8) Durham 9) Yorkshire 10) Norfolk 11) Berkshire: 12) Wiltshire 13) Dorset 14) Somerset

Table 3.2. Regions, counties and locations with number (n) of roe deer samples collected from across the UK used for microsatellite and mitochondrial DNA analysis

Region	Map no.	County	Locations	Mitochondrial samples (n)	Microsatellite samples (n)
Scotland	1,2	Moray	Elgin, Aviemore	29	39
	3	Perthshire	Balmanno, Moncreiffe, Kirkton, Dupplin, Abernethy	34	39
	4	Dunbartonshire	Bardowie, Garlochhead, Loch Green		9
	5	Ayrshire	Girvan, Blairquhan, Kilkerran	51	59
North West	6	Cumbria	RAF Spadeadam, MOD Longtown	28	29
	7	Lancashire	Silverdale, Langdale, Witherslack, Ashstead,Longshedds	11	17
North East	8	Durham	Hexham, Consett	13	18
	9	North Yorkshire	Pickering	25	29
South East	10	Norfolk	Santon Downham	40	44
South West	11	Berkshire	Windsor Great Park, Swinley Park	20	18
	12	Wiltshire	Tisbury	7	7
	13	Dorset	Muckleford, East Lulworth, Alton Common, Up Sydling, Okeford	39	39
	14	Somerset	Escot, Ugbrooke, Peamore	17	20
Total				314	367

3.2.2 DNA extraction

A small part of the tissue sample approximately ($5mm^3$) was finely cut using a scalpel. Samples were then incubated overnight at 37 ˚ C in digestion buffer (50 Mm Tris Ph 7.5, 1Mm EDTA, 100 Mm NaCL, 1 % w/v SDS) with 30 µl proteinase K ($10mg\mu l^{-1}$). The DNA was then extracted using a standard phenol: chloroform extraction (Sambrook et al. 1989). The presence of whole genomic DNA was then confirmed by viewing results on 1.2 % agarose gels which were run for 20 minutes alongside a 1 Kb DNA ladder.

3.2.3 Amplification and genotyping of microsatellites

Eighteen published microsatellite loci were used in this study. The loci chosen were known to be polymorphic in roe deer. The loci, their annealing temperatures, primer sequences, dye, size range, number of alleles and source references of the microsatellite are provided in Table 3.3 and 3.4.

In each primer set, one tenth of one of the primers in each reaction was labelled at the 5' end of the oligonucleotide with a fluorescent ABI prism dye to allow for sizing of the PCR product.

Microsatellites were amplified by polymerase chain reaction (PCR) using a Qiagen™ Multiplex kit. Multiplex enables the simultaneous amplification of many targets of interest in one reaction using several pairs of primers. Generally, multiplex conditions followed those presented by Galan et al., (2003). However, additional microsatellites were also used, for these several PCRs were carried out for optimisation and choice of suitable primer pair combinations.

Each of the multiplex mixtures contained HotstarTaq™ DNA Polymerase, Qiagen buffer (containing 6mM $MgCl_2$) and dNTP mix of dATP, dCTP, dGTP and dTTP. To this master mix equimolar amounts of each primer (0.2 pM/µl) were added along with 1 µl of DNA template. Amplifications were performed in a G-storm (Biotechniques) with the following cycles: 95ºC for 15 minutes; 35 cycles at 94ºC for 30s, annealing for 90s and 72ºC for 30 s; 72ºC for 5 min.

DBS Genomics (Durham University) ran results in a 3730 ABI DNA Analyser. As mentioned in the earlier section on PCR, each product had been labelled by use of a fluorescent labelled primer, allowing the product to be detected by the sequencer. ABI prism labels of FAM, HEX and NED were used. The PCR products were then added in specific amounts (0.2µl for FAM dyed products, 0.3 for HEX dyed products and 0.4 for NED dyed products) to a 1.625 µl mixture of ABI loading buffer containing ROX labelled DNA size ladder to allow the sizing of PCR products. Sets of loci and multiplex conditions were set taking care not to overlap allele size on the same given dye before running together on the sequencer. Visualisation of PCR sizes to a resolution of 1 bp was possible on a chromatogram produced by analysis of the output of the automated sequencer using Peak scanner ™ software v 1.0 (Applied Biosystems).

Table 3.3. Loci, primer sequences and additional characteristics of 18 microsatellites selected to genotype roe deer. * Ref gives the source of the microsatellite.

PCR no.	Ta	Locus	Primer Sequence	Dye	Size range	No. of alleles	Ref
PCR1	56.5	MCM505	ATC AGC ACC ATC TTA GGC CTA GA	HEX	114-134	12	1
			TGT AGA TTC CCT CAA TAT AAA AAT GGT				
		MCM131	ATT CAC AAA GCC GCG CTT G	HEX	260-284	10	1
			ATC AAG CTC CCC TCT TCG GT				
		ILST011	GCT TGC TAC ATG GAA AGT GC	FAM	82-112	6	2
			CTA AAA TGC AGA GCC CTA CC				
PCR2	54.7	CSSM39	AAT CGG AAC CTA GAA TAT TTT GAG	FAM	178-186	8	3
			AGA TAA AAT GTG AGT GTG GTC TCC				
		IDVGA 8	CTC TTG GGG GCG TGT TGT CT	HEX	210-228	9	3
			TAG CAG AAA GCA CAG GAG TC				
		BM1706	ACA GGA CGG TTT CTC CTT ATG	FAM	238-250	8	3
			CTT GCA GTT TCC CAT ACA AGG				
		IDVGA29	CCC ACA AGG TTA TCT ATC TCC AG	HEX	142-148	3	3
			CCA AGA AGG TCC AAA GCA TCC AC				
PCR3	62	OarFCB304	CCC TAG GAG CTT TCA ATA AAG AAT CGG	HEX	150-182	19	3
			CGC TGC TGT CAA CTG GGT CAG GG				
		MAF70	CAC GGA GTC ACA AAG AGT CAG ACC	NED	130-152	11	2
			GCA GGA CTC TAC GGG GCC TTT GC				
		BM848	TGG TTG GAA GGA AAA CTT GG	HEX	356-368	7	3
			CCC TCT GCT CCT CAA GAC AC				
PCR4	50	NVHRT24	CGT GAA TCT TAA CCA GGT CT	FAM	132-140	10	4
			GGT CAG CTT CAT TTA GAA AC				

Table 3.4. Loci, primer sequences and additional characteristics of 18 microsatellites selected to genotype roe deer. *Ref gives the source of the microsatellite.

PCR no.	Ta	Locus	Primer Sequence	Dye	Size range	No. of alleles	Ref
PCR5	51.9	HUJ117	TCC ATC AAG TAT TTG AGT GCA A	HEX	198-220	12	3
			ATA GCC CTA CCC ACT GTT TCT G				
		Bmc1009	GCA CCA GCA GAG AGG ACA TT	NED	280-292	10	3
			ACC GGC TAT TGT CCA TCT TG				
		RT1	TGC CTT CTT TCA TCC AAC AA	NED	222-240	16	5
			CAT CTT CCC ATC CTC TTT AC				
PCR6	55.1	CSSM43	AAA ACT CTG GGA ACT TGA AAA CTA	HEX	238-246	7	3
			GTT ACA AAT TTA AGA GAC AGA GTT				
		CSSM41	AAT TTC AAA GAA CCG TTA CAC AGC	HEX	120-124	3	3
			AAG GGA CTT GCA GGG ACT AAA ACA				
		Bm757	TGG AAA CAA TGT AAA CCT GGG	NED	172-204	19	3
			TTG AGC CAC CAA GGA ACC				
		NVHRT48	CGT GAA TCT TAA CCA GGT CT	FAM	86-90	4	3
			GGT CAG CTT CAT TTA GAA AC				

* Ref: 1. Hulme et al., (1995) 2. Crawford et al., (1995) 3. Galan et al., (2003) (and references therein) 4. Roed and Midthjell (1998) 5. Wilson and Strobeck (1997)

3.2.4 Amplification and sequencing of mitochondrial DNA

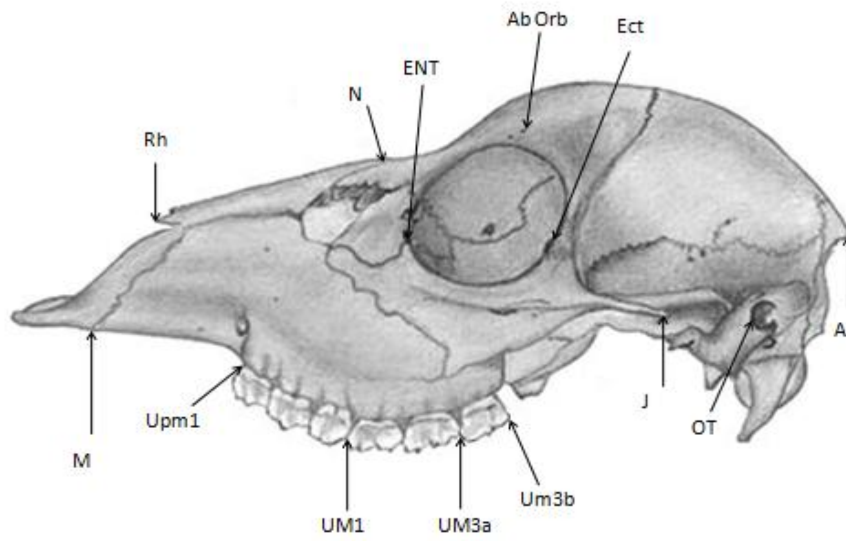
The entire mt-DNA control region, spanning 744 base pairs (bp), was amplified for use in this study using the primer set; Lcap Pro and Hcap Phe (Randi et al., 1998). The primer set, amplification and sequencing methods are provided in Chapter 2.

3.2.5 Morphology methods

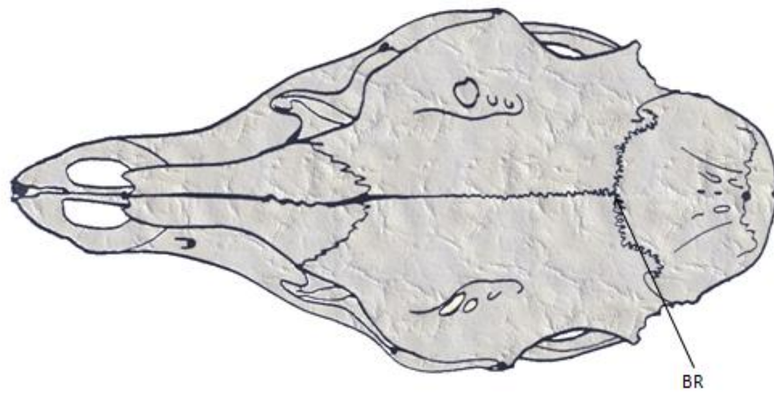
Seventeen sets of measurements were taken from each skull. Two measurements were taken sagitally along the midline of each skull (A-Upm1 and A-P) and one further measurement was taken as width of the cranium (Zyg- Zyg). All other traits were bilateral, taken from both the cranium (10) and mandible (4); however, for this morphological analysis, only measurements from the left side of the skull were used. Measurements for both sides were used to assess fluctuating asymmetry. All traits were derived after von den Driesch (1976) and are shown and described in Figure 3.2 and Tables 3.5 a and b.

All sets of measurements were taken using precision callipers and measured exclusively by one person (KHB) to avoid possible inter-observer variability (Lee, 1990). Each measurement was taken at least twice (and then averaged) to the nearest 0.01mm. Repeat measures were always taken 'blindly', that is with the measurer having no knowledge of the previous measurements. Several days elapsed between each complete set of measurements on each skull as this has been shown to give the most reliable estimate of measurement error (Palmer, 1994). No measurements were attempted on missing or worn structures; therefore there are missing data.

a)



b)



c)

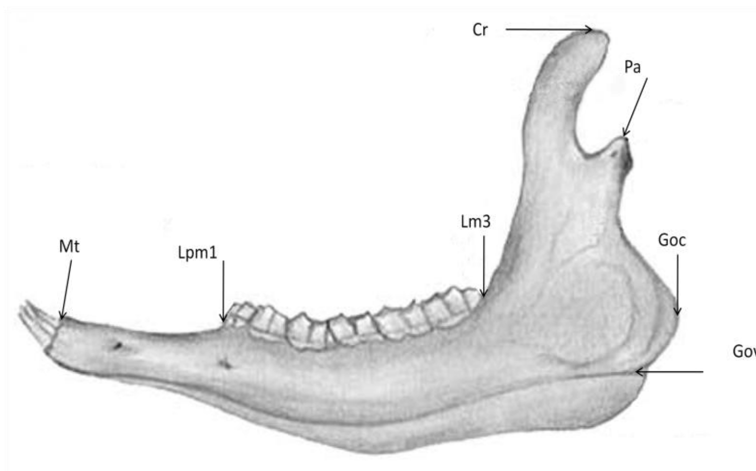


Figure 3.2. Traits measured in the skull of the roe deer a) Lateral view of skull b) Dorsal view of skull c) Lateral view of mandible.

Table 3.5 a) Codes and definitions of the measuring points taken on the cranium b) Measurements and their respective descriptions that were taken on both cranium and mandible.

a)

Definitions of measuring points of the cranium	
Code	**Point on cranium**
A	Akrokranion
Rh	Rhinion
N	Nasion
Ent	Entorbitale
Ot	Otion
Zyg	Zygion
Br	Bregma
Upm 1	Upper premolar 1
J	Jugal
M	Maxillary tip
Um 3a and 3b	Upper molar 3
Um 1	Upper molar 1
Rh	Rhinon
Ab Orb	Above orbitale
Gov	Gonion ventrale
Cr	Coronion
Goc	Gonion caudale
Pa	Processus articulus
Mt	Tip of mandible
Lpm 1	Lower premolar 1
Lm3	Lower molar 3

b)

Bilateral measurements of the cranium	Description
Um3b-M	Upper third molar to tip of maxillary
Um3b-Upm1	Upper tooth row length
Um3b-Um1	Upper molar length
Um3a-3b	Length of 3rd molar
J-Upm1	Jugal to 1st premolar
J-M	Jugal to tip of maxillary
N-Rh	Nasal length
Ot-Ab Orb	Otion to above orbitale
Ot-Ent	Otion to ectorbitale
Ot-Br	Otion to bregma
Bilateral measurements of the mandible	**Description**
Gov-Cr	Dental height
Goc-Mt	mandibular length
Lm3-Lpm1	Lower teeth row
Lpm1-Pa	Processus articulurus to lower 1st premolar
Sagital measurements of overall cranium size	**Description**
A-Upm1	Length of skull to upper 1st premolar
A-P	Overall length of skull
Zyg-Zyg	Zygomatic breadth of the skull

3.2.6 Statistical analyses

3.2.6.1 Microsatellite loci analyses

Incorrect assignment of microsatellite genotypes can occur due to DNA degradation, low DNA concentrations and primer-site mutations. Null alleles, stuttering and large allele dropout are the main genotyping errors that can occur. Null alleles result when an allele fails to amplify, stuttering can results from slippage during PCR-amplification and allele dropout can occur as large alleles may not amplify as efficiently as smaller alleles (Van Oosterhout et al., 2004). It is important that such genotyping errors are detected as they can potentially bias population genetic analyses. The presence of genotyping errors was therefore checked using the software Micro-checker (Van Oosterhout et al., 2004). This program uses a Monte Carlo simulation (bootstrap) method to calculate expected homozygote and heterozygote allele size difference frequencies and the Hardy-Weinberg theory of equilibrium to calculate expected allele frequencies. The program then highlights possible genotyping errors within individual microsatellites. Homozygote excess may indicate evidence of null alleles or when this excess is biased towards either extreme of the allele size distribution allele drop out may be occurring. Alternatively, a deficiency of heterozygotes with alleles differing in size by a single nucleotide repeat can indicate stutter.

The program ARLEQUIN 2.000 (Schneider, 2000) was used to calculate observed and expected heterozygosity. Deviation from Hardy-Weinberg equilibrium was tested using a method analogous to Fisher's exact test using a modified version of the Markov chain method (Guo and Thompson, 1992). Tests for linkage disequilibrium were carried out for each pair of loci using an exact test based on a Markov chain method as implemented in Genepop 3.4 (Raymond and Rousset, 1995).

Allelic richness for each locus and population was calculated using the program FSTAT 2.9.3 (Goudet 2001). This parameter yields a measure of allelic diversity independent of sample size by adjusting measures of alleles per locus in a sample. This programme was also used to assess the degree of non-random mating using Wright's F_{IS} (Wright, 1965). Non- random mating is a cause of reduced heterozygosity at any given locus therefore the degree of non random mating can be assessed comparing the observed and expected heterozygosity values.

For all of these analyses, the sequential Bonferroni technique was used to eliminate false assignment of significance by chance (Rice, 1989).

The level of genetic differentiation between populations was quantified using F_{ST} (Weir and Cockerham, 1984). F_{ST} estimates of genetic differentiation between populations assumed an infinite sites mutation model (IAM). For this measure significance was tested with 1000 permutations of individual genotypes between populations by performing a two level analysis of molecular variance (AMOVA) (among and within populations) with the software Arlequin v 2.0 (Schneider *et al*. 2000). As the AMOVA involves using multiple tests Bonferroni was applied.

Relationships between geographic distance and genetic distance first in Southern areas (including locations in the region of South West and South East) and then separately for Northern areas (including all locations in the regions of Scotland, North East and North West) were investigated using Genepop 3.1b.These two areas were separated on the basis that southern populations may have been influenced strongly by re-introduction events. Correlations between genetic distance (F_{ST}/1- F_{ST}) (Rousset 1997) and geographic distance were assessed with a Mantel test (10,000 permutations). Geographic distances were calculated, in kilometres, from the approximate centre of each of the sampling locations.

Patterns of differentiation were also visualised using a factorial correspondence analysis (FCA) of population multi-locus scores using GENETIX 4.0 (Belkhir et al., 2000). The use of FCA to analyse genetic data has been described by She et al. (1987) and is used in order to give a visual representation of individuals clustering on the basis of their relative allele frequencies.

The program STRUCTURE 2.0 was used to assign putative populations (K) (Pritchard et al., 2000). Structure divides sampled individuals into a number of clusters (K) independent of locality information (i.e. based only on multi locus genotypic data), so as to minimize deviations from Hardy–Weinberg and linkage equilibrium. The program uses a Markov chain Monte Carlo (MCMC) procedure to estimate P ($X|K$), the posterior probability that the data fit the hypothesis of K clusters.

Four independent runs for each value of (K = 2–9) were performed at 10^6 MCMC repetitions and 10^5 burn-in period using no prior information and assuming correlated allele frequencies and admixture. The posterior probability was then calculated for each value of K using the estimated log likelihood of K to choose the optimal K.

3.2.6.2 Mitochondrial DNA

All sequences were aligned using Clustal X (Larkin et al., 2007). The programme DNA sp 10.4.9 (Rozas et al., 2003) was used to identify haplotypes and calculate mitochondrial DNA polymorphism estimated as haplotypic diversity, (*hd*; Nei and Tajima, 1981), nucleotide diversity (π, Nei 1987), and average pairwise nucleotide divergence (k).

The relationship amongst haplotypes was examined by constructing a Network. Networks are better suited than phylogenetic methods to infer haplotype genealogies at the population level because they explicitly allow for extant ancestral sequences and alternative connections (Bandelt et al., 1999). Complete alignments were used with the median joining network procedure (Bandelt et al., 1999) implemented in NETWORK 3.1.1.1.

AMOVA was used to analyze how genetic variability was partitioned within and among populations using F_{ST} and its analogue Φ_{ST} using Arlequin v 2.0 (Schneider, 2000). Φ_{ST} differs from F_{ST} in that it incorporates both haplotype frequencies and the number of nucleotide differences between each pair of haplotypes (Excoffier et al., 1992).

Distributions of mt-DNA haplotypes were examined within subpopulations by plotting haplotypes (excluding singletons) onto a location map of the UK where samples were collected. In order to see whether differences in haplotype distributions across the UK could define population structure –a spatial analysis of molecular variance in the SAMOVA software (Dupanloup et al., 2002) was implemented.

The SAMOVA method defines groups of local populations that are geographically homogenous and maximally differentiated from each other. The method is based on a simulated annealing procedure that aims to maximize the proportion of total genetic variance due to differences between groups of populations, measured by the F_{CT} coefficient of the AMOVA F-statistics (Excoffier et al., 1992). The SAMOVA procedure finds a structure based solely on genetic data and geographical location of populations. However, this

approach requires *a priori* definition of the number of groups (K). Thus, SAMOVA was run successively with a different K, ranging from 2 to 10. Analyses were run twice for each value of K to check consistency between runs. For each run, 100 simulated annealing processes were performed. The composition of the K groups was identified by observing the maximum F_{CT} index, which is the proportion of total genetic variance due to differences between groups of populations (Dupanloup et al., 2002).

3.2.6.3 Morphology

The locations compared for morphological variation represented 7 of 8 populations that were defined based on microsatellite analysis in STRUCTURE: Durham, Carlisle, Dorset/Wiltshire, Perth, Moray, Norfolk and Lancashire. However, due to the low number of skulls collected from Durham (n= 5) and its close affiliation with Carlisle based on F_{ST} values, Carlisle and Durham samples were combined.

The level of variability of each of the cranial traits was compared between populations using mean and standard deviation. For the remainder of the analyses all measurements had to be standardised over the total length of the skull (A-P) to control for size. However, many of the skulls had lost nasal bones and therefore had missing values recorded for all measures that involved the prosthion (P). As an alternative the measure A- Upm1 which is also a measure of skull length (but to the upper first molar) was used as an alternative. This measure 'A-Upm1' was found to be highly correlated with A-P (R = 0.946 for 85 skulls). Therefore to correct for size, a regression analysis was carried out where each trait was regressed against this measure of skull length (A-Upm1).

Discriminant function analysis (DFA) was used to classify the roe deer into one of the six putative populations based on discriminant functions (see Tabachnick and Fiddell, 1996). The adequacy of the classification was determined by the percentage of correct classification, assuming there is an equal probability (16.6 %) of being classified into any of the groups by chance alone. Classification percentage greater than 16.6 % for any given population was therefore used to indicate whether the discriminant functions were satisfactory for predicting group membership. The Wilks' test was used to determine if classification by the DFA into the discriminant functions was significant (Field, 2005).

3.3 Results

3.3.1 Microsatellites

Genotyping errors, Hardy Weinberg equilibrium and linkage disequilibrium

Micro checker revealed null alleles existed in all populations for the loci IDVGA- 29 and MCM131, furthermore significant homozygote excess was also detected for all populations, these two loci were therefore excluded from further analyses.

No evidence of genotyping errors were found for any further loci. However, the estimation of exact P values by the Markov chain method for HWE revealed deviations after Bonferroni adjustment for 2 population- loci combinations: IDVGA – 8 in Norfolk and ILST011 in Moray (see tables in appendix 3). As deviations for these loci only occurred in one sub population each and omissions did not change patterns of differentiation these were retained for the remainder of the analysis.

The test of genotypic disequilibrium for each pair of the 16 microsatellite loci over all populations gave 11 significant values ($p = < 0.05$) for 224 comparisons (14 significant values are expected by chance at the 5 % level). After Bonferroni correction, six combinations were significant ($p<0.0031$) at the experimental level, 3 of these occurred in the Norfolk population. Despite these differences no clear patterns across samples were observed.

Microsatellite genetic diversity

Overall, genetic variation expressed as mean He (expected heterozygosity) was 0.65 (range 0.59-0.76) and mean Ho (observed heterozygosity) was 0.62 (range 0.49-0.74) (Table 3.6). In general, He values were higher than Ho values resulting in positive F_{IS} values (see Table 3.6). Negative F_{IS} values resulted when Ho was higher than He. Average F_{IS} was 0.034.

Microsatellites were highly polymorphic showing an average of 10.06 alleles per locus in the total sample (n=372) and from 3.75 alleles (in roe deer from Somerset and Wiltshire) to 6.38 alleles (in roe deer from Moray) in the local samples. The difference in the average number of alleles per locus in the total and local samples indicates that the distinct alleles are differentially distributed in the sampled populations.

All analysed loci were moderately variable, showing between 3 (CSSM41) and 19 (BM757/Oarfcb304) alleles per locus. Allelic richness, which provides a measure of allelic diversity corrected for differences in sample sizes, was lowest in the Somerset and N Yorkshire populations (3.21 and 3.33 respectively) and highest in the Glasgow population (5.03).

Table 3.6. Inter population indices of British roe deer; n, number of individuals; A, number of alleles; AR, allelic richness; F_{IS}; Ho observed heterozygosity; He expected heterozygosity; P values are indicated for multi-locus Hardy-Weinberg equilibrium tested against an alternative hypothesis of heterozygote deficit.

		Mean over all loci					
	N	A	AR	F_{IS}	Ho	He	P
Norfolk	45	4.19	3.34	0.145	0.49	0.59	<0.0001
Berks	20	4.19	3.61	-0.064	0.65	0.62	0.9530
Dorset	38	4.69	3.58	-0.008	0.60	0.60	0.1930
Wiltshire	9	3.75	3.65	0.051	0.57	0.64	0.2550
Somerset	20	3.75	3.21	0.047	0.52	0.57	0.0019
Durham	19	4.44	3.77	0.064	0.59	0.65	0.1090
N York	29	3.94	3.33	0.013	0.58	0.60	0.2520
Carlisle	30	4.75	3.77	-0.044	0.67	0.65	0.9200
Perth	30	6.25	4.68	0.034	0.69	0.72	0.0100
Moray	30	6.38	4.57	0.070	0.64	0.70	0.0050
Aviemore	21	5.88	4.67	0.081	0.65	0.72	0.0341
Glasgow	10	5.25	5.03	0.003	0.74	0.76	0.5230
Ayrshire	52	5.81	4.05	0.028	0.64	0.66	0.0040
Lancashire	18	5.00	4.39	0.053	0.63	0.68	0.1440
All	371	4.88	3.97	0.034	0.62	0.65	<0.0001

Microsatellite genetic structure

F_{ST} values were used to assess genetic differentiation among populations. The roe deer from the UK were assigned to each of their populations on the basis of the area from which they were sampled. The Wiltshire samples were combined with those from Dorset due to their low sample size ($n < 10$); this combination was in H-W equilibrium. Thirteen different locations in total were therefore examined. The overall F_{ST} value was 0.17 ($P < 0.001$). The AMOVA analyses indicated that 82% of genetic variation was contained within populations and 17% among populations.

The pairwise F_{ST} values between the inferred populations ranged from 0.02 to 0.32 (Table 3.7). All values were significantly different from zero. The greatest differentiation occurred between the Norfolk population and the Somerset population. Overall, levels of differentiation were highest against all other populations and the Norfolk population (range 0.19-0.32). As a general trend lower differentiation was found between Northern populations (Scotland and Northern England; Durham, Carlisle, Lancashire, N York) and higher differentiation in Southern populations (Norfolk, Berks, Dorset, Somerset). Between Northern roe populations microsatellite F_{ST} ranged between 0.02 and 0.16, (61% of values were <0.10). Between Southern roe populations microsatellite F_{ST} values ranged between 0.09-0.32 (66% of estimates yielded $F_{ST} > 0.16$).

A Mantel test was carried out to identify whether Northern and Southern populations (tested separately) followed an IBD pattern. Figures 3.3 and 3.4 represent correlations between geographic and genetic distances; southern populations did not show significant IBD ($P > 0.05$) whereas northern populations did ($P < 0.001$).

Table 3.7. Pairwise values of F_{ST} using 16 microsatellite loci, significant values following Bonferroni adjustment are in bold (p= <0.003).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1. Moray	0.00												
2. Aviemore	**0.03**	0.00											
3. Perth	**0.07**	**0.05**	0.00										
4. Glasgow	**0.05**	**0.03**	**0.03**	0.00									
5. Ayrshire	**0.09**	**0.08**	**0.09**	**0.05**	0.00								
6. Carlisle	**0.09**	**0.07**	**0.08**	**0.05**	**0.03**	0.00							
7. Lancashire	**0.12**	**0.12**	**0.10**	**0.09**	**0.03**	**0.02**	0.00						
8. Durham	**0.12**	**0.12**	**0.12**	**0.09**	**0.10**	**0.08**	**0.10**	0.00					
9. N York	**0.16**	**0.16**	**0.14**	**0.10**	**0.06**	**0.05**	**0.04**	**0.11**	0.00				
10. Norfolk	**0.19**	**0.20**	**0.19**	**0.20**	**0.20**	**0.20**	**0.22**	**0.19**	**0.24**	0.00			
11. Berks	**0.24**	**0.18**	**0.20**	**0.16**	**0.19**	**0.21**	**0.24**	**0.20**	**0.23**	**0.27**	0.00		
12. Dorset/Wilts	**0.24**	**0.22**	**0.20**	**0.18**	**0.21**	**0.22**	**0.25**	**0.24**	**0.23**	**0.27**	**0.13**	0.00	
13. Somerset	**0.26**	**0.25**	**0.23**	**0.25**	**0.27**	**0.28**	**0.31**	**0.28**	**0.31**	**0.32**	**0.19**	**0.09**	0.00

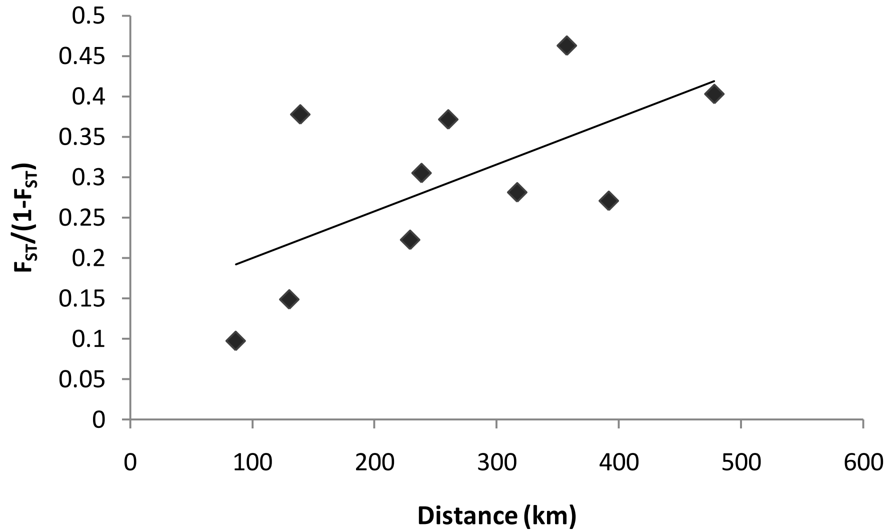


Figure 3.3. Isolation by distance tests for correlation between genetic differentiation and geographic distance between southern roe based on microsatellites (R^2 0.40, P > 0.05).

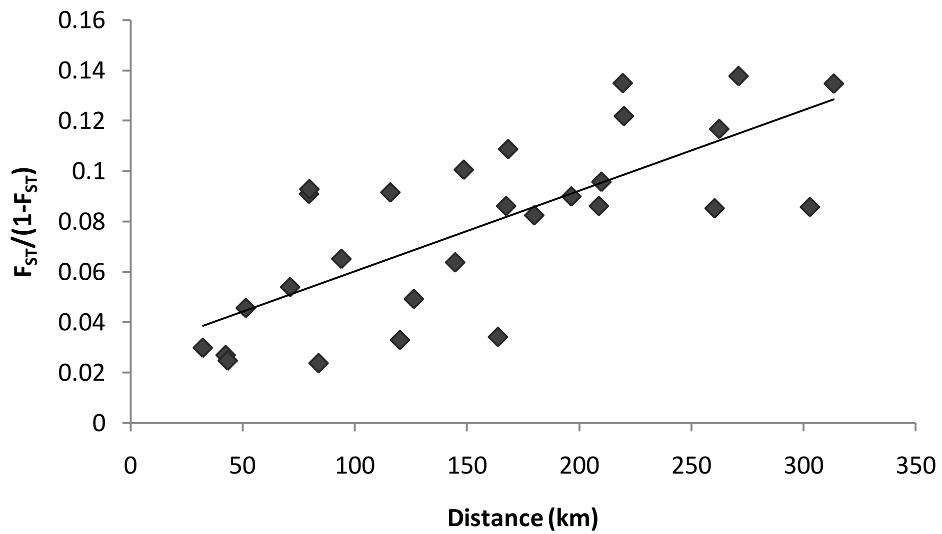


Figure 3.4. Isolation by distance tests for correlation between genetic differentiation and geographic distance between northern roe based on microsatellites (R^2 0.55, P < 0.001).

An FCA plot (Figure 3.5) provided some geographic resolution, with the putative populations from Berkshire, Dorset and Somerset (southern England) forming a distinct cluster, Norfolk samples forming another, and most of the rest together in a third cluster (northern England and Scotland). FC-1 explained 26.01% of the total genetic variation, while FC-2 explained 22.4%. The Lancashire population clustered with samples from both Norfolk and Northern UK.

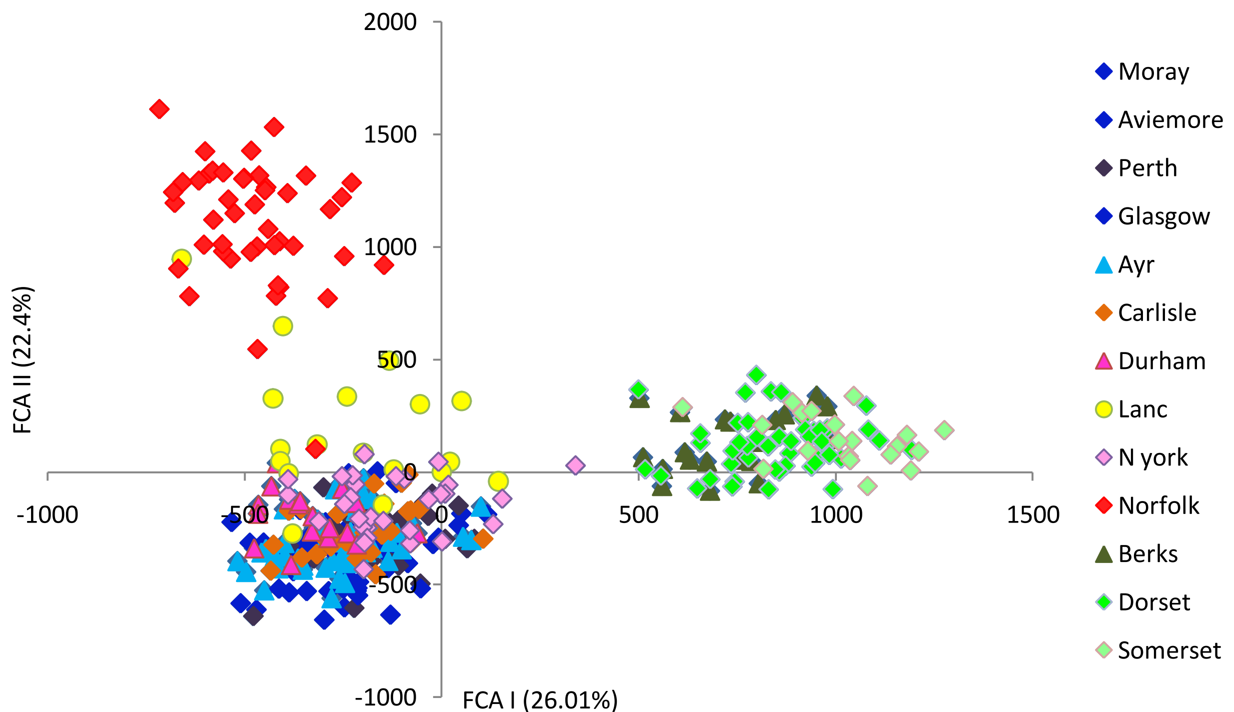


Figure 3.5. Factorial correspondence analysis (FCA) of population multi locus scores computed using GENETIX. Multi-locus scores are computed in the bivariate space defined by the first two factorial components.

Analyses using STRUCTURE indicated that the highest posterior probability was for eight sub-populations (*K*= 8; see Table 3.8). This analysis also permitted each individual to be assigned a likelihood of belonging to each population (Figure 3.6).

Table 3.8 Estimated posterior probabilities of *K*

K	LnPr (X/*K*)
3	-16240
4	-15728
5	-15588
6	-15545
7	-15423
8	**-15416**
9	-15500

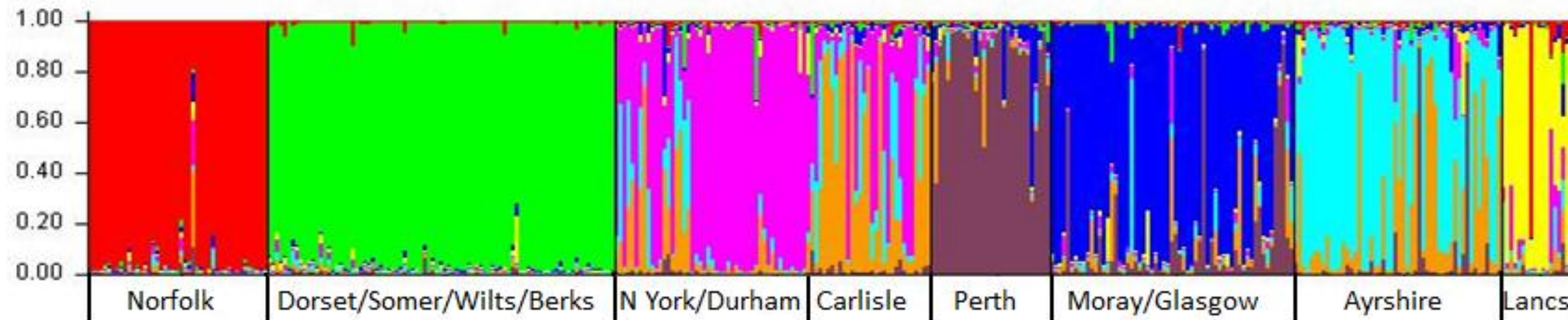


Figure 3.6. Plot obtained with the software STRUCTURE to determine the number of roe deer genetic groups across Britain. Each vertical line in the plot represents an individual and is broken into different coloured segments indicating the proportion of their genetic make-up assigned to each of the different genetic groups.

3.3.2 Mitochondrial DNA

Mitochondrial genetic diversity

The sequencing of 744 bp revealed 22 variable sites (18 transitions and 4 transversions) and resulted in the detection of 27 putative haplotypes. The haplotypes occurred between one and 115 times (distributions are represented in Table 3.10). Haplotypic diversity (*hd*) (Table 3.9) across all populations was 0.81. On average there was one distinct haplotype over 11.1 individuals (312/27). However, across populations haplotypic diversity was variable, with no diversity observed in either the Norfolk or Somerset populations even though sample sizes were relatively high (41 and 19 respectively). This clearly suggests that these populations are genetically very homogenous. In the other localities *hd* ranged between 0.21 in Carlisle and 0.82 in Lancashire. Nucleotide diversity ranged from 0.0008 (Carlisle) to 0.0044 (Perth and Moray).

Table 3.9. Mitochondrial control region diversity indices for roe deer from different sampling locations in the UK : n, number of individuals; n*h*, number of haplotypes; *hd*, haplotypic diversity; π nucleotide diversity; *k*, average pair wise sequence divergence.

Location	n	n*h*	*hd*	Π	*k*
Norfolk	41	1	0.00	-	-
Berks	19	3	0.61	0.0038	2.83
Dorset	43	3	0.53	0.0018	1.30
Somerset	19	1	0.00	-	-
Durham	13	3	0.60	0.0042	1.84
N York	25	5	0.29	0.0025	3.60
Lancashire	11	4	0.82	0.0041	0.56
Carlisle	28	3	0.21	0.0008	3.10
Perth	34	7	0.78	0.0044	3.30
Moray	29	9	0.81	0.0044	3.19
Ayr	50	7	0.36	0.0024	1.78
All locations	**312**	**27**	**0.81**	**0.0057**	**4.23**

Table 3.10. Distribution of mitochondrial haplotypes among the five roe deer populations studied, singletons marked in grey.

	Dorset/ Wilts	Somerset	Berks	Norfolk	Moray	Perth	Ayr	N York	Durham	Carlisle	Lancs	∑
Hap 1	7											7
Hap 2	28	19	2									49
Hap 3				41								41
Hap 4	8		7		2							17
Hap 5			10		2	7	40	20	7	25	4	115
Hap 6						4						4
Hap 7					12	13		3	5	1		34
Hap 8						7						7
Hap 9									1	1		2
Hap 10											4	4
Hap 11											2	2
Hap 12					2	1						3
Hap 13					3	2	4					9
Hap 14					3							3
Hap 15					2							2
Hap 16					2							2
Hap 17							2					2
Hap 18											1	1
Hap 19											1	1
Hap 20										1		1
Hap 21						1						1
Hap 22					1							1
Hap 23								1				1
Hap 24							1					1
Hap 25							1					1
Hap 26							1					1
Hap 27							1					1
∑	43	19	19	41	29	35	50	24	13	28	12	**313**

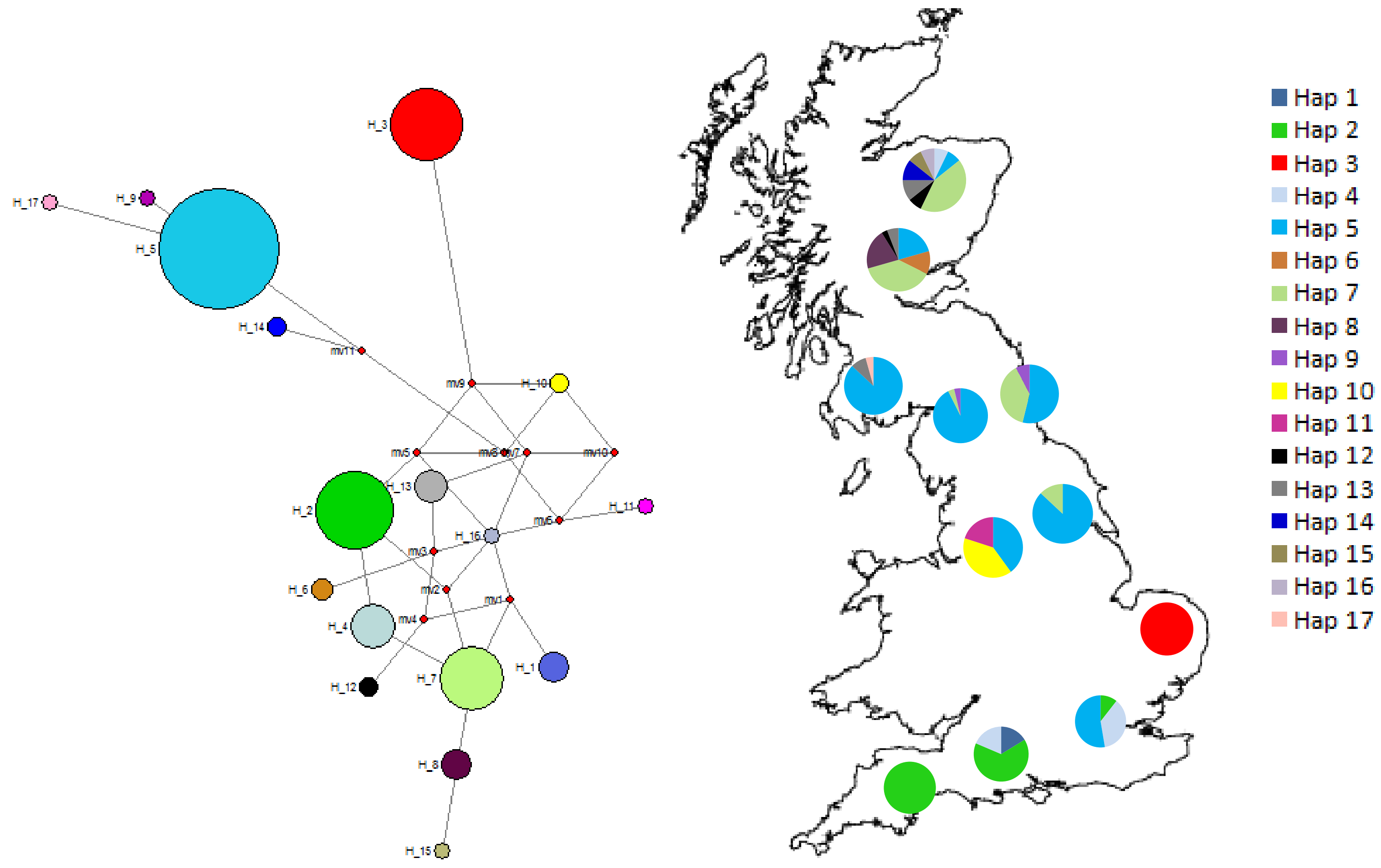


Figure 3.7. a) Median joining network of phylogenetic relationships among modern mitochondrial haplotypes where the size of the circle indicates relative frequency of the haplotype. Haplotypes represented are based on 744 base pairs of the mt-DNA d-loop and exclude singletons (Table 3.10; Hap 18-Hap 27). b) Modern roe haplotypes (excluding singletons) and their distributions across the UK.

The haplotype distribution (Table 3.9) and median joining network with distribution map (Figure 3.7 a & b) showed clear differences for some of the roe sampling locations. Norfolk was most notable with only one unique, divergent haplotype (hap 3; Figure 3.7).

The Southern populations were also fairly distinct from all other locations. The most frequently sampled haplotype (hap 2) was unique to the region (and the only haplotype found in Somerset). The Dorset populations contained two other haplotypes (hap 1 and 4). Haplotype 1 was unique to Dorset whereas haplotype 4 was also sampled in the Berkshire populations and interestingly the Moray population. The Berkshire population contained one additional haplotype (hap 5) which was the most broadly distributed across Northern parts of the UK (especially in Carlisle, N York) and Scotland (especially Ayr).

The Lancashire population also shows some distinction from all other populations. It contains many unique haplotypes (hap 9, 11, 18 and 19; Table 3.9) and shares only one common haplotype (hap 5) with other populations. All other Northern English populations (N York, Durham and Carlisle) showed similar haplotype distributions but in varying frequencies.

Mitochondrial population structure

Global F_{ST} was high and significantly greater than zero ($F_{ST} = 0.611$; $p < 0.001$). Sixty one percent of variation occurred among populations and 38 % within populations. The patterns of significance and relative values between locations were similar for both F_{ST} and Φ_{ST} (see Table 3.11). Most pair-wise comparisons were found to be significant ($p < 0.01$). Overall, the pair-wise comparisons between the 11 putative populations suggested high levels of genetic divergence.

Table 3.11. Pairwise F_{ST}s (below diagonal) and Φ_{ST} (above diagonal) for roe deer between locations in the UK for 744bp of the mt-DNA control region. Values in bold indicate significance ($P < 0.05$).

	1	2	3	4	5	6	7	8	9	10	11
1 Moray	*	0.01	**0.57**	**0.65**	**0.24**	**0.33**	**0.49**	**0.75**	**0.27**	**0.27**	**0.43**
2 Perth	0.03	*	**0.53**	**0.60**	**0.21**	**0.34**	**0.45**	**0.73**	**0.25**	**0.30**	**0.43**
3 Ayrshire	**0.41**	**0.33**	*	0.01	**0.20**	**0.40**	0.01	**0.86**	**0.25**	**0.73**	**0.79**
4 Carlisle	**0.45**	**0.37**	0.01	*	**0.34**	**0.57**	0.04	**0.97**	**0.37**	**0.82**	**0.94**
5 Durham	**0.11**	0.06	**0.19**	**0.25**	*	**0.24**	0.08	**0.87**	0.00	**0.55**	**0.70**
6 Lancs	**0.17**	**0.15**	**0.28**	**0.36**	**0.14**	*	**0.36**	**0.85**	**0.23**	**0.55**	**0.66**
7 N York	**0.36**	**0.29**	0.01	-0.01	0.12	**0.28**	*	**0.89**	**0.15**	**0.71**	**0.81**
8 Norfolk	**0.64**	**0.63**	**0.81**	**0.92**	**0.84**	**0.78**	**0.89**	*	**0.83**	**0.86**	**1.00**
9 Berks	**0.24**	**0.21**	**0.19**	**0.26**	**0.16**	**0.15**	**0.20**	**0.79**	*	**0.47**	**0.60**
10 Dorset/Wiltshire	**0.33**	**0.35**	**0.56**	**0.61**	**0.45**	**0.37**	**0.57**	**0.73**	**0.35**	*	**0.15**
11 Somerset	**0.54**	**0.54**	**0.76**	**0.88**	**0.75**	**0.67**	**0.83**	**1.00**	**0.66**	**0.17**	*

Table 3.12. Population structures inferred by spatial analysis of molecular variance (SAMOVA). Proportions of variation explained by groupings are indicated.

K	**Groupings**	**Among groups (F_{CT})**	**Among populations within groups (F_{SC})**	**Within populations (F_{ST})**
2	[Norfolk] [Moray,Perth, Ayr, Carlisle, Dur, N York, Berks, Lancs, Somerset, Dorset]	0.441	0.531	0.738
3	[Norfolk] [Moray,Perth, Somerset, Dorset] [Ayr, Carlisle, Dur, N York, Berks, Lancs]	0.565	0.267	0.681
4	[Norfolk] [Moray,Perth] [Ayr, Carlisle, Dur, N York, Berks, Lancs] [Somerset, Dorset]	0.592	0.176	0.664
5	[Norfolk] [Moray,Perth] [Ayr, Carlisle, Dur, N York, Berks] [Somerset, Dorset] [Lancs]	0.612	0.116	0.657
6	[Norfolk] [Moray,Perth] [Ayr, Carlisle, Dur, N York] [Berks] [Somerset, Dorset] [Lancs]	0.618	0.070	0.645
7	**[Norfolk] [Moray,Perth] [Ayr, Carlisle, N York] [Dur] [Berks] [Somerset, Dorset] [Lancs]**	**0.623**	**0.031**	**0.638**
8	[Norfolk] [Moray,Perth] [Ayr, Carlisle, N York] [Dur] [Berks] [Somerset] [Dorset] [Lancs]	0.613	0.036	0.627

The results of SAMOVA indicated significant population genetic structure for each assumed number of groups, from 2 to 10 (P <0.00001 in each case) (Table 3.12). The F_{CT} value reached its highest at K =7 when all parameters began to stabilise. Thus, K = 7 was identified as the most probable number of groups. These results indicate a pattern of population structure similar to the F_{ST} and Φ_{ST} values (Table 3.11).

3.3.3 Morphological results

The basic statistics for each trait among populations are shown in Table 3.13. This table shows that, on average, skulls from the Norfolk population were short and narrow. In contrast, skulls from the Perthshire and Dorset/Wiltshire populations were shown to be longest and widest, respectively.

Table 3.13. Mean and standard deviation of measurements for each population. All traits are reported in cm.

Measure	Description of measure	Norfolk (n =23)	Carlisle/Dur (n=23)	Dorset/Wilts (n = 29)	Moray (n= 15)	Perth (n=15)	Lancs (n=9)
Cranium							
A-Upm1	Length of skull to upper 1st premolar	12.74 ± 0.39	12.95 ± 0.39	13.38 ± 0.48	12.93 ± 0.44	13.48 ± 0.41	13.32 ± 0.38
Zyg-Zyg	Zygomatic breadth of the skull	7.79 ± 0.28	7.76 ± 0.20	8.12 ± 0.32	7.77 ± 0.21	8.08 ± 0.33	8.01 ± 0.20
Um3b-M	Upper third molar to tip of maxillary	8.17 ± 0.28	8.44 ± 0.38	8.66 ± 0.36	8.19 ± 0.37	8.53 ± 0.32	8.54 ± 0.27
Um3b-Upm1	Upper tooth row length	4.85 ± 0.23	4.86 ± 0.26	4.99 ± 0.30	4.88 ± 0.25	4.86 ± 0.29	5.02 ± 0.23
Um3b-Um1	Upper molar length	2.52 ± 0.16	2.31 ± 0.16	2.42 ± 0.13	2.37 ± 0.17	2.35 ± 0.20	2.41 ± 0.13
Um3a-3b	Length of 3rd molar	0.42 ± 0.08	0.34 ± 0.07	0.36 ± 0.07	0.33 ± 0.07	0.33 ± 0.08	0.37 ± 0.07
J-Upm1	Jugal to 1st premolar	8.17 ± 0.36	8.35 ± 0.30	8.61 ± 0.39	8.41 ± 0.40	8.62 ± 0.33	8.65 ± 0.39
J-M	Jugal to tip of maxillary	11.24 ± 0.46	11.80 ± 0.49	12.30 ± 0.52	11.73 ± 0.67	12.22 ± 0.38	12.06 ± 0.43
N-Rh	Nasal length	4.17 ± 0.40	4.52 ± 0.30	4.79 ± 0.54	4.72 ± 0.55	5.02 ± 0.49	4.81 ± 0.45
Ot-Ab Orb	Otion to above orbitale	5.96 ± 0.32	5.86 ± 0.25	6.12 ± 0.24	5.84 ± 0.29	5.96 ± 0.20	6.00 ± 0.29
Ot-Ent	Otion to ectorbitale	6.64 ± 0.26	6.75 ± 0.16	6.89 ± 0.20	6.65 ± 0.24	6.91 ± 0.22	6.93 ± 0.16
Ot-Br	Otion to bregma	4.93 ± 0.16	5.02 ± 0.21	5.12 ± 0.18	4.91 ± 0.21	5.12 ± 0.23	5.01 ± 0.15
Mandible							
Gov-Cr	Mandibular height	7.73 ± 0.45	7.65 ± 0.35	7.91 ± 0.38	7.70 ± 0.29	7.90 ± 0.50	8.00 ± 0.46
Lm3-Lpm1	Mandibular length	5.63 ± 0.22	5.64 ± 0.24	5.56 ± 0.40	5.66 ± 0.24	5.64 ± 0.25	5.75 ± 0.23
Lpm1-Pa	Lower teeth row	9.87 ± 0.29	9.86 ± 0.36	10.01 ± 0.36	9.94 ± 0.31	10.12 ± 0.29	10.19 ± 0.27
Goc-Mt	Processus articulurus to lower 1st premolar	14.01 ± 0.46	14.39 ± 0.57	14.88 ± 0.54	14.65 ± 0.45	15.07 ± 0.52	14.97 ± 0.48

Table 3.14. Adequacy of classification results for DFA. Left column indicates the original group while the top row indicates the predicted group. Values are as percentage. Correct classifications are italicised.

	Norfolk	**Dorset/ Wilts**	**Carlisle/ Durham**	**Perth**	**Moray**	**Lancs**
Norfolk	***100.0***	0.0	0.0	0.0	0.0	0.0
Dorset/Wilts	0.0	***87.5***	0.0	12.5	0.0	0.0
Carlisle/Dur	0.0	5.6	***94.4***	0.0	0.0	0.0
Perth	0.0	6.3	0.0	***68.8***	18.8	6.3
Moray	0.0	0.0	0.0	8.3	***66.7***	25.0
Lancs	0.0	0.0	0.0	12.5	12.5	***75.0***

Table 3.15. Results of the Wilks' test

Discriminant function	**Wilks' λ**	X^2	**df**	***p*-value**
1	0.02	308.62	75	< 0.001
2	0.13	155.12	56	< 0.001

DFA allowed successful classification rates to be calculated for each of the 6 sub-populations (Table 3.14). Overall, 83.9 % of original grouped cases were correctly classified and 60.9 % cross validated cases were correctly classified. Within populations, Norfolk has the highest (100 %) correct classification, suggesting that this population is highly distinct. Both discriminant functions 1 and 2 were significant (Table 3.15). This shows that the populations were distinguishable based on skull morphology for both of these functions.

Table 3.16. Pooled within group correlations between discriminating variables and standardised canonical discriminant functions. Variables ordered hierarchically by absolute size of correlation within function. The largest absolute correlations with either discriminant function are shown in bold.

	Function	
	1	2
Um3a-3b	**0.65**	0.16
Lpm1-Pa	0.16	**-0.45**
Gov-Cr	0.12	**-0.30**
Goc-Mt	-0.03	-0.13
Ot-Ent	0.12	-0.19
N-Rh	0.00	-0.07
Um3b-Um1	0.18	-0.41
Um3b-Upm1	0.15	-0.32
Lm3-Lpm1	0.15	-0.34
Ot-Br	0.15	-0.14
Ot-Ab Orb	0.15	-0.31
J-M	0.03	-0.03
Zyg-Zyg	0.10	-0.16
J-Upm1	0.03	-0.16
Um3b-M	0.14	-0.06

Table 3.16 shows the relationship between each trait and the discriminant functions as a correlation index. The higher the correlation index the more that trait contributed to the separation between populations. For discriminant function 1 the main component was Um3a-3b which is a measure of the upper third molar length, this showed a positive loading. For discriminant function 2 the main components were Lpml- pa and Gov-Cr which were measures of mandibular length and height respectively, these showed negative loadings. This indicated that roe deer with long upper third molars would score highly at discriminant function 1 and roe deer with small mandibles would score highly at discriminant 2 (as shown in Figure 3.8). Overall, discriminant function 1 and 2 explained 67.4% and 20.4% of the among population variance respectively (cumulative variance 87.8 %).

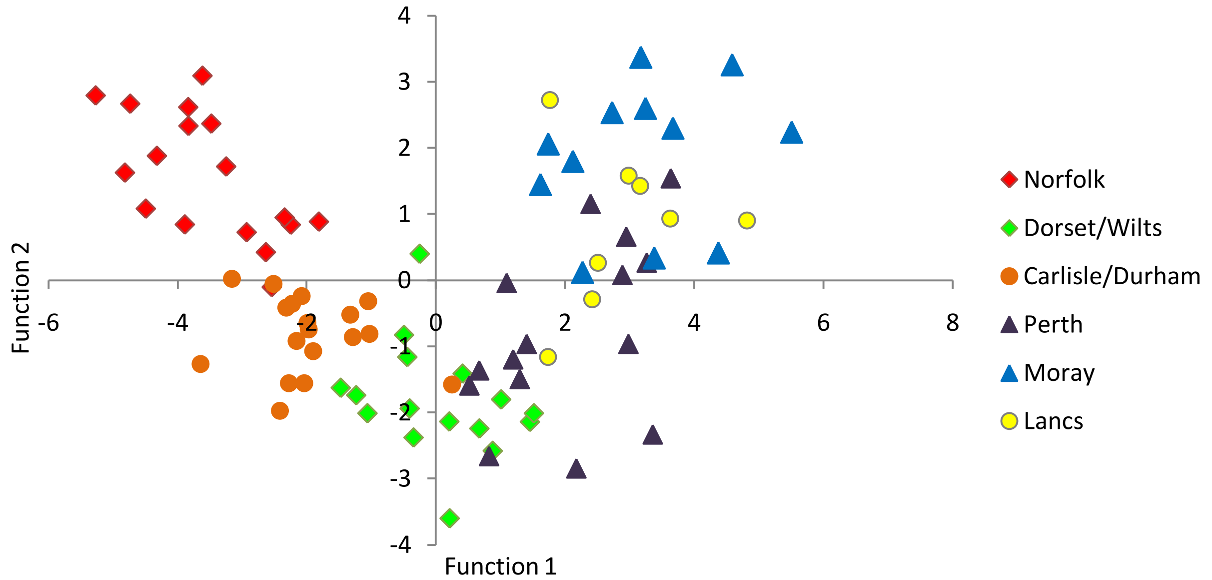


Figure 3.8. Plot of the discriminant function scores for roe deer from the UK based on skull morphology.

Discriminant function scores from individuals in different populations are consistent with the discriminant functions given above (Figure 3.8). The implications for skull shape are that Norfolk roe have long upper third molars but overall small mandibles with short and narrow skulls. Contrastingly, Perth and Dorset roe have short upper third molars but overall large mandibles with long and wide skulls. Moray roe were shown to have short upper third molars with small mandibles and short and relatively narrow skulls.

Table 3.17. Population centroids for each of the discriminant functions.

	Function	
	1	**2**
Norfolk	-3.53	1.57
Dorset/Wilts	0.05	-1.82
Carlisle/Dur	-1.95	-0.83
Perth	2.06	-0.72
Moray	3.20	1.87
Lancs	2.88	0.80

3.4 Discussion

3.4.1 Genetic diversity – microsatellites and mt-DNA

Genetic variability based on microsatellites diminished from north to south within the UK (range in Scotland: He = 0.72-0.76; middle England: 0.65-0.68; southern England: 0.57-0.64). The pattern for mt-DNA was broadly similar, especially with respect to high diversity in Scotland, though mt-DNA diversity was lower in middle than in southern England in some cases. Overall, this pattern is broadly consistent with the history of bottlenecking in these populations (for more detail, see Chapter 4).

The lowest levels of genetic diversity in UK roe populations are likely to have arisen because of founder effects. In particular, evidence for this came from the south western roe (Dorset/Wiltshire, Somerset and Berks locations), which showed losses of microsatellite (Table 3.6) and mitochondrial (Table 3.9) diversity compared to its presumed source population (Perthshire). Specifically, for microsatellites there was a greater effect on allelic diversity than on heterozygosity. This is in line with both theory (Nei et al., 1975) and the general trend found in empirical studies (Taylor et al., 1994; Bouzat et al., 1998 b). Other studies have found that introduced populations show reduced microsatellite variability relative to known sources. For example, in populations of the Bennett's Wallaby, *Macropus rufogriseus rufogriseus,* a known introduction of three wallabies took place onto South Island, New Zealand. The founder population retained only 40% of the allelic diversity and 75% of the heterozygosity observed relative to the source population in Tasmania (Le Page et al., 2000). Similarly a range of other re-introduced populations have shown notable losses of diversity relative to source populations including fisher, *Martes pennanti (Williams et al., 2000)*, Alpine ibex, *Capra ibex* (Maudet et al., 2002; Biebach and Keller, 2009), white tailed deer , *Odocoileus virginianus* (Deyoung et al., 2003) and Pennyslvania elk, *Cervus elaphus* (Williams et al., 2002). For the other introduced southern population (Norfolk) low microsatellite and mitochondrial diversity relative other UK populations is also likely due to founder events (although this could not be tested, as samples were not obtained from the proposed source, Germany).

Microsatellite diversity across all UK populations was moderate to high in comparison to those found in other roe populations across Europe (He= 0.59-0.76; average of 10.06 alleles

per locus) (see Table 3.6). Lorenzini et al., (2002) reported lower levels of heterozygosity in 12 Italian roe populations (He 0.17-0.58; average of 7.7 alleles per locus). Kuehn et al., (2004) reported more moderate levels of heterozygosity in nine populations from Switzerland and Germany (He 0.63-0.66; average of 9.8 alleles per locus). Zachos et al., (2006) found higher levels of heterozygosity in five German roe populations (He; 0.74-0.79; 14.1 alleles per locus).

For mt-DNA diversity, excluding the two populations that did not exhibit any variation (Norfolk and Somerset) values of haplotype diversity (h) (0.21-0.82) and nucleotide diversity (π) (0.08-0.44%) were also well within the range reported for other roe populations in Europe (see Table 3.9). For example, based on the same sequence region of the mt-DNA d- loop Zachos et al., (2006) found h and π to range between 0.64 - 0.89 and 0.253- 1.53 % respectively, and Lorenzini et al., (2002) found h and π to range between 0.040 – 0.758 and 0.004% - 0.147% respectively.

Taken together, the amount of microsatellite and mitochondrial variability may be considered surprising given this species' known history of past bottleneck events. Specifically, for southern populations where bottlenecking, as a result of introduction, can be inferred to have been most intense (see Chapter 4), levels of microsatellite variability are fairly high (He = 0.59- 0.64). In other mammalian examples where bottlenecks have occurred, much lower genetic variability has been reported. Examples include a population of bighorn sheep, *Ovis canadensis,* founded by 12 individuals (He= 0.43; Forbes et al., 1995) and an insular population of koalas, *Phascolarctos cinereus,* founded by 18 individuals (He= 0.33; Houlden et al., 1996).

In contrast to these examples, and similar to this study, populations of the white tailed deer were found to have maintained relatively high levels of microsatellite variability despite a history of severe bottlenecking. The authors attributed the maintenance of appreciable levels of genetic variability to the ability of the species to expand very quickly (DeYoung et al., 2003). Indeed, populations that experience rapid growth are less likely to lose genetic diversity than small populations which are subject to drift, inbreeding and bottlenecks (Gilpin and Soule, 1981).

Similar to the white tailed deer, the roe deer also had the potential to expand very quickly. This is for a number of reasons. The first reason is to do with habitat availability. Although roe are highly ecologically adaptable and able to exploit newly available habitat quickly (Putman and Langbein, 2003), many habitats into which introductions took place could be considered 'ideal'. Habitats encompassed newly forested sites (free available habitat) which would have enabled populations to thrive (Prior, 1995). Habitat has been inferred to be an important component that will aid a successful translocation (Sarrazin and Barbault, 1996) and, also, the amount of genetic diversity retained (Mock et al., 2004). The second reason roe could have expanded quickly is related to the species' great reproductive capacity. Roe deer are more 'r' selected than many other large mammals, as shown by the regular production of twins and an earlier age of first reproduction (Geist, 1998). The final reason may relate to lack of predators. This was one of the factors used to help explain rapid population growth of wallabies in New Zealand from a small number of colonising individuals (Le Page et al., 2000). In line with these hypotheses, following roe introduction into the south, it was noted that roe quickly spread taking advantage of available habitats (e.g. Prior, 1995; Whitehad 1964). However, clear expansion signals were not detected (at least, from neutrality tests, see Chapter 4).

Alternative reasons that levels of variability may be higher than expected in southern populations could be due to admixture amongst different founder groups. However, for at least the Norfolk population, this seems unlikely (see section 3.4.2).

One final reason for why microsatellite diversity is high, could be that marker polymorphism in loci used in our study was higher relative to those available for other studies (Amos and Harwood, 1998). Indeed, this is a potential drawback of comparing microsatellite variability across different studies.

3.4.2 Fine scale population structure – microsatellites and mitochondrial DNA

The amount of differentiation observed between populations using mitochondrial DNA (mt-DNA) was substantially higher compared to microsatellites. This can be explained by the differences in their properties; where mt-DNA is maternally inherited, nuclear DNA is biparentally inherited. Therefore, mt-DNA will show a low effective population size relative to the nuclear DNA and lead to the observed outcome (Prugnolle and de Meeus, 2002).

Furthermore, male-mediated gene flow between adjacent areas could also contribute to the differences detected between markers, because a higher number of microsatellite alleles than haplotypes will be introduced into a population. Indeed, most female ungulates are highly philopatric and sub-adult male roe deer are known to be the main dispersing sex (e.g. Kurt, 1999). Aside from these explained differences, genetic differentiation was generally found to be concordant between both mitochondrial and microsatellite data. For the most part, patterns were consistent and differentiation substantial.

Generally speaking, patterns of genetic structure are often related to length of reproductive isolation between populations. This could occur for all roe populations through female philopatry, which is well documented in central Europe (Linnell et al., 1998), and perhaps through habitat fragmentation (Lorenzini et al., 2004). These factors may be more prominent in certain populations than others (see discussion of the Norfolk population below). Another factor that could lead to reproductive isolation is distance between populations. This study demonstrated an isolation by distance model (IBD) across northern but not southern populations (Figures 3.3 and 3.4).

IBD was expected to play an important role in differentiating northern populations. In northern populations, re-introductions have seldom occurred and sufficient time, since historical bottlenecking, may have passed for relationships between gene flow and drift to be established. In addition to IBD, other factors, such as partial barriers to gene flow, may be enhancing differentiation between populations at the micro-geographic scale. For example, it may be that mountains or high altitude areas of land may be inhibiting gene flow between populations. This could explain the division of the Perthshire roe from Moray and, surprisingly, Glasgow (see map, Figure 3.1 and STRUCTURE result, Figure 3.6), where the mountainous mass of the Grampians and Monaliadths which are located in the northern region of Scotland follow an eastern to south western direction. Similarly, the division of the Durham population from Carlisle (evident from the SAMOVA, at least) could be partially explained by the Pennines which divide these areas. Mountains have been inferred to constitute potential barriers to gene flow in other ungulate species such as red deer in Norway (Haanes et al., 2010).

In southern England, roe populations did not show significant IBD. However, it is difficult to determine whether this was due to a lower number of sampling locations in the south (see

Figure 3.3). In any case, it was clear that southern English populations generally exhibited much higher levels of microsatellite differentiation (compared to northern populations).

For the two southern populations (Norfolk versus the south west) the high differentiation occurring between them is likely to be due to their divergent origins. Re-introduction records indicate that all the Norfolk roe descend from German stock introduced in 1884. The genetic data clearly support this, as the Norfolk population is highly distinct, both genetically (Figures 3.5, 3.6 and 3.7) and morphologically (Figure 3.8), from not only the south west but also all other UK populations. Re-introduction from distant sources has been known to enhance genetic differentiation and undermine isolation by distance patterns in other roe (Lorenzini et al., 2004) and red (Nussey et al., 2006) deer populations across Europe. The fact that differentiation is so high further suggests relatively little integration between the Norfolk and neighbouring populations has occurred. In line with this, the results imply that only very low levels of gene flow are occurring into the Norfolk population. In fact, the strongest evidence of gene flow into the population came from one outlying individual (see Figures 3.5 and 3.6). This individual could explain the heterozygote deficiency, high positive F_{IS} value and linkage disequilibrium (Table 3.6). Apart from this individual, overall integration into the Norfolk population appears limited. The relative isolation of the Norfolk population may have accentuated the genetic legacy of re-introduction as it has in other species e.g. in American wild turkey, *Meleagris gallopavo silvestris* (Latch and Rhodes, 2005) and Pronghorn antelope, *Antilocapra americana* (Stephen et al., 2005a). There are several reasons why the Norfolk population may have remained relatively isolated. The southern area of the UK (where the Norfolk population exists) is densely populated by humans. Wang and Schrieber (2001) showed that the proportion of urban areas (houses, villages and roads) between populations was significantly correlated with potential levels of gene flow among them. Alternatively, its relative isolation may be related to the dispersal nature of the roe deer. In general, roe tend to be philopatric and maintain small home ranges for many years (Kurt, 1999). When dispersal does occur, it tends to be male biased. Male roe have been found to be more likely to disperse with increasing population density and resource scarcity (Hewison and Gaillard, 1996). Dispersal distances of male roe seldom exceed 10 km (although they can travel further) (Danilkin, 1996). Therefore, it may be that suitable local conditions exist in the areas into which roe are born and so there is little requirement to

disperse long distances. Under this hypothesis, when populations begin to meet their carrying capacity the dispersal of roe will increase.

The other locations sampled in the south west (Dorset, Wiltshire, Berks and Somerset) showed high differentiation between relatively short distances (although STRUCTURE designated all these locations as only one cluster, and SAMOVA as two). For example, microsatellite differentiation between geographically close Somerset and Dorset was high (F_{ST} = 0.09). Several interpretations could explain the high amount of differentiation observed between south western populations.

Assuming all south western roe were founded by the same common population (Scotland: see Whitehead, 1964) then strong genetic drift could explain genetic differentiation. This interpretation has been used to explain divergence between populations of other introduced species. For example, Maudet et al., (2002) reported that populations of Alpine Ibex, *Capra ibex ibex*, within close geographical proximity (ten to several dozen km) showed high microsatellite differentiation (F_{ST} = 0.15) despite a common population history. Genetic drift could also explain the high levels of differentiation (Microsatellite F_{ST}; 0.20-0.23 and mt-F_{ST}; 0.19-0.76) evident between the south west and the proposed source (Perthshire) population. Even though these values were high they were still the lowest reported between any southern and northern population. Other studies have reported high differentiation between founder and source populations. Wisley et al., (2008) found that, after just 5-10 years of isolation, microsatellite divergences as high as 0.10 occurred between populations of the black footed ferret, *Mustela nigripes*, with a common source. Similarly, introduced nailtail wallabies, *Onychogalea fraenata*, apparently differentiated from their source after just four generations (Sigg, 2006)

One problem with the above interpretation is that strong genetic drift should cause large losses in genetic variability. However, as discussed (3.4.1), south western populations have maintained high levels of genetic diversity. Therefore, interpretations that can explain both the large genetic distances between, and levels of genetic diversity within, populations must be considered.

One explanation could be that introduced populations have more than one origin. Although it is generally considered that southern roe descended from introduced Scottish stock, which

would have been the most accessible (Hewison, 1995), this may not be accurate. Re-introductions involving roe of more divergent origins (e.g. from the continent) may have occurred and gone unrecorded. Alternatively, it may be that roe in southern England never went completely extinct. This scenario may have some foundation. Barclay (1932) considered that an ancient indigenous stock may have persisted in one southern English location (Petworth Park, Sussex). Evidence for this suggestion, which comes from local legends has, however, largely been dismissed given that recorded introduction events occurred into the park in the 1800s and 1890 (see Table 3.1). Elsewhere, folk tradition suggested very few roe still existed in the great woods of north Dorset and south Wilsthire (Prior, 1982, Prior pers. comm). A remnant population in this latter area could explain the appearance of the unique haplotype (Hap 1; see Figure 3.7) which is present in all south western locations but most abundant in a westerly direction (fixed in Somerset). The admixture of possible remnant populations with individuals transferred from elsewhere (e.g. in Dorset/Wilts and Berkshire) could then explain the higher genetic diversity.

Overall, the ancestry of southern populations appears more complex than previously assumed and further research should focus sampling efforts on south western populations to determine its true genetic ancestry.

3.4.3 Morphological differentiation

Data taken from skull measurements also revealed strong differentiation between populations and this was in line with the genetic results. The most distinct population was Norfolk (the DFA correctly classified 100 % of individuals; Table 3.14), providing further support that these deer are isolated and non-native (German) in origin. Other populations also showed high correct population assignment, indicating that populations showed strong morphological differentiation from one another (Table 3.14).

The cause of the overall morphological differentiation across these populations is unclear. It is often argued that morphological differences between populations are approximately proportional to genetic distance (Huson and Page, 1980; Pertoldi et al., 2006 b). The question here is whether the degree of divergence in neutral marker loci predicts the degree of divergence in genes coding for quantitative traits (Pertoldi et al., 2006 b). This question must, however, remain open because alternative explanations could explain the amount of

morphological divergence occurring across populations. Alternative explanations could be that an increase in inbreeding following bottlenecks or founder events has interrupted pleiotropic interactions (when an allele at one locus has multiple phenotypic effects). Inbred populations of Iberian lynx, *Lynx pardinus* (Pertoldi et al., 2006 a) and black footed ferret, *Mustela nigripes* (Wisely et al., 2008) have shown increases in levels of morphometric variation and decreases in character size. For the roe populations in this study it may be that inbreeding following a founder event from the Perthshire population caused a reduction in skull length for the Dorset population (Table 3.13). However, this hypothesis requires further investigation and is unlikely to explain variation in *all* populations.

Other explanations could be that craniometrical variation is due to environmental effects. Roe deer are generally known to exhibit a high degree of ecological and behavioural plasticity (Danilkin and Hewison, 1996). Across other populations in Europe, roe deer are known to show high variation in skull morphology. Aragon et al., (1998) argued that this variation is environmental rather than genetic in origin. Regional variation in available foods will determine feeding habits and this may be manifested in morphological differences among populations (Fandos and Reig, 2003). It was interesting to note that, in this study, the largest absolute correlations involved measurements taken from teeth (upper third molar; Um3a-3b) and mandibles (length; Lpm1-Pa and Gov-CR), suggesting that there is high variation in traits associated with feeding. In ruminants, such as the roe deer, it is essential that plant material is fully masticated in order that effective microbial digestion can occur (Nussey et al., 2007). Therefore, it might be expected that variations in morphology may be closely related to forage availability in different geographic areas. However, it did not seem that patterns of variation in skull morphology were consistent across parts of the UK. For example, in southern areas, roe showed both short (Norfolk) and long (Dorset) skulls where climate (and thus forage) may be similar. Overall, the mechanisms controlling morphological differentiation are unclear and require more thorough investigation.

3.5 Conclusion

The results of this chapter have direct implications for wildlife management, where re-introduction is an increasingly popular management tool (Lipsey and Child, 2007; Seddon and Armstrong, 2007). Re-introductions commonly cause losses in genetic diversity which, in turn, are a concern as they can impact on population fitness (see further in Chapter 5).

Nevertheless, it seems that for re-introduced roe deer, losses of diversity may have been ameliorated by rapid population expansion. The ability to rapidly expand could be related to factors such as this species' ecology and/or characteristics of habitat. This study reinforces that such factors should be carefully considered during any introduction event. An additional finding, relevant to management, is that re-introduction of roe deer clearly created spatial genetic structure across a vacant landscape over a short time scale. For those populations least impacted by re-introduction, this study enabled us to define population structure and the factors that driving that structure. Other studies of ungulates may reveal similar factors differentiating populations.

Chapter 4 : The impact of bottleneck events on British roe deer populations

4.1 Introduction

A population bottleneck occurs when populations experience severe, temporary reductions in size (Spencer et al., 2000). An increasing number of bottlenecks are impacting populations as a result of human induced disturbance such as habitat loss, fragmentation and over exploitation (Frankham, 1995). A particular kind of bottleneck, a founder event, occurs when a new population becomes established from a small number of individuals drawn from a large ancestral population (Templeton, 2008). Founder events may result from either natural or non-natural (human mediated) dispersals.

During the population bottleneck or founder event, losses of genetic diversity can occur because only a proportion of the original genetic variants survive or re-establish themselves (Nei et al., 1975). Further losses of diversity will result from genetic drift and inbreeding when a population remains small and isolated over many generations (Nei et al., 1975; Reed and Frankham, 2003). Losses of genetic diversity are a major concern to conservation biologists; diversity provides the raw material for evolution, is required for adaptation via natural selection (Fisher, 1930) and has been closely linked to population fitness (Frankham et al., 2002). Given the potentially serious impacts bottlenecks can have on a population, it is of paramount importance to identify bottlenecked populations.

Studies attempting to identify bottlenecked populations have often taken advantage of the straightforward concept that these populations should show reductions in genetic diversity (as considered above) (Wright, 1969; Nei et al., 1975; Lacy, 1987). Several studies have simply inferred that bottlenecks have occurred because small populations are accompanied by low genetic variability (Obrien et al., 1987; Ellegren et al., 1996). This assumption can be erroneous, however, firstly because alternative credible hypotheses can cause the same patterns (e.g. selective sweeps) (Bouzat et al., 1998 b) and, secondly, because not all bottlenecked populations show lowered levels of diversity (e.g. Bowling and Ryder, 1987; Kaeuffer et al., 2007). As a result, alternative methods to detect bottlenecks have been developed. Common methods employed to detect bottleneck events from contemporary

populations include looking for bottleneck ‘signatures’. Three bottleneck ‘signatures’ are: heterozygosity excess, modal shift distortions and a reduction in the Garza M ratio.

Heterozygosity excess was first introduced by Cornuet and Luikart (1996). They noted that when a population is significantly reduced there is a correlated and progressive reduction in the number of alleles and heterozygosity (Cornuet and Luikart, 1996). At this point, rare alleles, which contribute very little to overall heterozygosity, are lost rapidly. As a result allelic diversity is reduced faster than heterozygosity (Nei et al., 1975). Therefore, an excess of loci with an abundance of heterozygosity relative to the observed number of alleles is expected in a bottlenecked population.

Another proposed characteristic of bottlenecked populations is modal shift distortions in the distribution of allele frequencies for selectively neutral loci. Because alleles at low frequency are expected to be lost rapidly during a bottleneck (Nei et al., 1975; Allendorf, 1986;), modal shifts in the proportion of alleles in different frequency classes (with a relative deficit of rarer alleles) are expected (Luikart et al., 1998 b).

A final characteristic of bottlenecked populations is a reduction in the mean ratio of the number of alleles relative to the total size range. This occurs because there is random sampling of alleles of all sizes, so the number of alleles is reduced more than the range of allele sizes. This can be measured by the Garza- M ratio; where the value of ‘M’ will decrease as a population is reduced in size (Garza and Williamson, 2001).

A number of empirical examples based on bottlenecked populations show evidence of these signatures. Heterozygosity excesses have been observed in bottlenecked populations of Alpine ibex, *Capra ibex ibex* (Maudet et al., 2002) and Southern hairy nosed wombats, *Lasiwhinus zatipons* (Taylor et al., 1994). Modal shifts in allele size distributions have also been observed in bottlenecked populations of Northern hairy nosed wombats, *L. krefftii* ([186]Taylor et al., 1994) and Southern Australian koalas, *Phascolarctos cinereus* (Houlden et al., 1996)[192]. Gaps in allele size distributions have been detected in white beaked dolphins, *Lagenorhynchus albirostris* (Banguera-Hinestroza et al., 2010).

The extent to which genetic consequences of a bottleneck (reductions in genetic diversity and ‘signatures’) are retained in a population is dependent on a number of factors including

population recovery (e.g. Zenger et al., 2003), immigration (e.g. Keller et al., 2001; Sigg, 2006) and time since bottlenecking (Cornuet and Luikart, 1996).

This last factor, time, is the most important in determining whether a bottleneck will be detected (Nei et al., 1975). Following size reductions, distributions of genetic diversity will eventually return to equilibrium conditions (Cornuet and Luikart, 1996). Bottleneck 'signatures', specifically, are highly transient, although some more than others.

Different bottleneck signature methods are expected to exhibit different timescales for their detection (Abdelkrim et al., 2005). The bottleneck signature considered most likely to detect reductions in size over longer timescales is the M ratio method. This is because of the longer time needed for the M statistics (i.e. the ratio of the number of alleles to allele size range) to reach equilibrium (Garza and Williamson, 2001). Contrastingly, the other two bottleneck signature methods (heterozygosity excess and mode shift indicators) detect only relatively recent and acute bottlenecks (Luikart et al., 1998 b). As 'signatures' may only provide evidence for recent bottlenecks, important information on more historical bottlenecks can sometimes be missed. One way to get around this problem is to incorporate ancient DNA samples.

Ancient DNA, from samples pre-dating a presumed period of bottlenecking, can enable a direct examination of past variation. Ancient and contemporary populations can then be compared and losses of diversity directly assessed. The amount of diversity lost between time frames can even provide some indication of the magnitude of the demographic contraction.

Using ancient DNA direct connections between a reduction in population size and a considerable loss of genetic variation have been demonstrated in a range of species including birds (Bouzat et al., 1998 a), mammals (Larson et al., 2002) and insects (Harper et al., 2006). For many examples, losses of genetic diversity coincide with periods of heavy hunting pressure. For example, Hoelzel et al., (2002) showed that 7 mt-DNA haplotypes existed in just 22 pre-bottleneck Northern elephant seal (*Mirounga angustirostris*) samples which was a stark contrast to the 2 mt-DNA haplotypes found in over 185 post-bottleneck samples. This severe loss in haplotypes could be traced back to a period of severe hunting where the species was reduced to approximately 20 – 30 individuals (Hoelzel et al., 1993). In other examples

Nystrom et al., (2006) found that four out of seven (60%) historical haplotypes went extinct as the result of a period of hunting for fur from the Scandinavian arctic fox (*Alopex lagopus*).

Ancient DNA is not only useful for interpreting the impact of historical bottlenecks, but also in resolving whether a population is of native or non – native origin. This is especially useful for species that have been heavily influenced by re-introductions by humans. For example, a study based on the European rabbit which compared haplotypes from historical (11,000 - 3,000 years before present; YBP) and contemporary populations showed that a new mitochondrial lineage appeared suddenly after the middle ages (500 YBP) in most wild populations in France. Haplotypes from this lineage corresponded to those found in modern domestic rabbit breeds. It was concluded that modern rabbit communities resulted from a complex interaction between original populations and those introduced or manipulated by humans (Hardy et al., 1995).

4.1.2 The British Roe deer

Populations of the British roe deer are thought to have been subject to a number of bottleneck events. The first major bottleneck event may have occurred during the 14th century (persisting until at least the 18th century) due to overhunting and deforestation. In Scotland and potentially some of the northern parts of England, roe probably survived this period in remnant populations. Contrastingly, in southern England roe were believed to have gone extinct (Ritson, 1933; Whitehead, 1964).

By the early 19th century re-introduction events (mainly in the south) and re-planting of woodland (across all of UK) facilitated the rapid return of the roe. Recent founder events either through natural or non-natural (human introduction) dispersal into areas from which roe had been previously extirpated may have led to a bottleneck. This is because founder events typically involve the movement of small numbers of individuals.

In the south, where all populations were believed to have been completely extirpated and re-established through human introduction, the impact of recent founder events has probably been important. Conversely, for northern populations the impact of founder events may have been less important (depending on the extent to which populations survived initial declines).

In line with this, it may be that roe populations of the north most closely resemble historical roe populations.

The impacts of both the historical (medieval) and recent bottlenecks (founder events) may have left detectable traces on roe populations. These populations, therefore, offer the opportunity to examine evidence of bottlenecks using both signature methods and ancient DNA. Overall this study will address the following specific predictions:

1. Contemporary populations will show typical bottleneck signatures, most evidently in populations recently impacted by founder events.
2. Levels of genetic diversity will be higher in historic versus contemporary populations, reflecting medieval bottlenecking.
3. Northern roe populations will resemble historical levels of genetic diversity more closely than southern populations.

4.2 Materials and methods

4.2.1 Modern and ancient sample collection

Details of the samples obtained for modern genetic analysis are provided in Chapter 3 for modern DNA (mt-DNA and microsatellites) and chapter two for ancient DNA (mt-DNA).

4.2.2 Modern and ancient DNA extraction

DNA was extracted from modern samples and ancient samples as described in Chapter 2.

4.2.3 Microsatellites: amplification and genotyping

4.2.3.1 Modern microsatellites

Sixteen microsatellite loci were used to examine bottleneck signatures and levels of genetic diversity in modern populations. A subset of these sixteen (six loci; see below) were additionally used to determine relationships between ancient and modern populations. The amplification of these loci is described in Chapter 3.

4.2.3.2 Ancient microsatellites

Six microsatellite loci (MCM505, OarFCB 304, MAF70, NVHRT24, CSSM41 and NVHRT48) were used to determine relationships of ancient and modern populations (Table 4.1). Ancient DNA (aDNA) is often severely degraded to fragments of just a few hundred base pairs (Paabo et al., 2004), therefore the six microsatellites were specifically selected as they represented short amplicons (<200 bp). PCR set up and amplifications of these six loci were carried out exactly as described in Chapter 2. All microsatellites (modern and ancient) were genotyped as described in Chapter 3.

Ancient microsatellite data were replicated. The amplification of nuclear DNA from ancient samples is technologically difficult. This is because there are fewer copies per cell of nuclear DNA compared to mitochondrial DNA (Hoelzel, 2005). Additionally, in situations when nuclear DNA (specifically microsatellites) are amplified, the rate of genotyping errors is high (Wandeler et al., 2007). Two main types of genotyping errors are likely to occur: allelic dropout (the failure of one of the two alleles to amplify) and false alleles (artifacts of PCR

amplification due to slippage of taq polymerase). Generally, allelic dropout is the more common problem and, if unaccounted for, can lead to underestimations in levels of genetic diversity in past populations (Wandeler et al., 2007). As a result, all microsatellites were replicated at least three times. However, even with replication, results were dubious because many individuals were homozygotes. Therefore, these results were analysed using a program that could help to account for this error (see statistical analysis).

Table 4.1. Loci, primer sequences and additional characteristics of size microsatellites suitably selected to genotype ancient roe deer populations.

Locus	**Ta**	**Primer sequence**	**Dye**	**Size range**	**No. of alleles**
MCM505	56.5	ATC AGC ACC ATC TTA GGC CTA GA	HEX	110-134	9
		TGT AGA TTC CCT CAA TAT AAA AAT GGT			
OarFCB304	62	CCC TAG GAG CTT TCA ATA AAG AAT CGG	HEX	158-179	15
		CGC TGC TGT CAA CTG GGT CAG GG			
MAF70	62	CAC GGA GTC ACA AAG AGT CAG ACC	NED	129-173	10
		GCA GGA CTC TAC GGG GCC TTT GC			
NVHRT24	50	CGT GAA TCT TAA CCA GGT CT	FAM	147-155	5
		GGT CAG CTT CAT TTA GAA AC			
CSSM41	55.1	AAT TTC AAA GAA CCG TTA CAC AGC	HEX	122-124	3
		AAG GGA CTT GCA GGG ACT AAA ACA			
NVHRT48	55.1	CGT GAA TCT TAA CCA GGT CT	FAM	86-90	4
		GGT CAG CTT CAT TTA GAA AC			

4.2.4 Mt-DNA amplification and sequencing

4.2.4.1 Modern mt-DNA

For modern samples the entire mt-DNA control region, spanning 750 base pairs (bp), was amplified using the primer set; Lcap Pro and Hcap Phe (Randi et al., 1998). The primer set, amplification and sequencing methods are provided in Chapter 2. For calculations of modern population genetic diversity the full 750 bp region of the amplified mt-DNA was used for analysis. For all other analyses involving comparison with ancient DNA, only the consensus region (419 bp; see below) was used.

4.2.4.2 Ancient mt-DNA

For ancient samples, the first portion of the hypervariable region 1 (HVR-1) was amplified using the primer pairs Roe_1F with Roe_1R and Roe_2F with Roe_2R; these amplified fragments of 244 and 267 bp respectively (including primers). Together, these combined to give a total of 419 bp. Procedures to avoid contaminating ancient samples during DNA extraction and amplification of both mt-DNA and microsatellites were as described in Chapter 2.

4.2.5 Statistical analysis

4.2.5.1 Genotyping errors and tests for Hardy-Weinberg equilibrium

Genotyping errors and tests for H-W equilibrium were carried out for both ancient and modern samples as described in Chapter 3.

4.2.5.2 Modern populations; genetic diversity and bottleneck signatures

The program ARLEQUIN 2.000 (Schneider, 2000) was used to calculate observed and expected heterozygosity from16 microsatellites. The programme DNA sp 10.4.9 (Rozas et al., 2003) was used to calculate mitochondrial diversity in terms of number of unique haplotypes (h), haplotype diversity (H_d) and nucleotide diversity (π) for the full 750 bp region of the mt-DNA d-loop. In addition, this programme was used to generate tests of neutrality including Tajima's D (Tajima, 1989) and Fu's Fs test (Fu, 1997). These tests were conducted to detect traces of past population expansion or decline, yielding significantly positive values for contracting populations or significantly negative values for expanding populations.

Bottleneck signatures including heterozygosity excess, mode shift distortions in the distribution of allele frequencies and the Garza-M ratio were all examined using data based on 16 microsatellites (in H-W equilibrium; see Chapter 3) from contemporary populations. British roe deer were divided into 8 contemporary populations that had been previously defined by STRUCTURE analyses (Chapter 3).

The program Bottleneck (Cornuet and Luikart, 1996; Piry et al., 1999) was used to compute the distribution of gene diversity (H_{eq}) expected from the observed numbers of alleles (k), given the sample size (n). This works by simulating the coalescent process under three

different mutation models: the infinite allele model (IAM); the stepwise mutation model (SMM); and, finally, the two-phased mutation model (TPM). Each of the three mutation models was implemented because they assume slightly different mechanisms of mutation. The Infinite Allele Model (IAM) involves mutations of any number of tandem repeats and always results in an allele that is not already encountered in the population. The Stepwise Mutation Model (SMM) involves mutations by the gain or loss of a single tandem repeat and so the mutation may result in a copy of an allele already present in the population; consequently, alleles of very different sizes will be more distantly related than alleles of similar sizes (Shriver et al., 1993; Valdes et al., 1993; Estoup et al., 1995). The Two-Phase Mutation Model (TPM) assumes that most mutations result in an increase or decrease of a single tandem repeat but that larger mutations can also occur. TPM reflects a combination of both IAM and SMM and is suggested to be the best fit for microsatellite data (Di Rienzo, 1994). For the TPM model used for this study, 70% IAM and 30% SMM was implemented (Dirienzo et al., 1994; Spencer et al., 2000). Each of the mutational models were compared using three separate statistical tests: a sign test, a standardized differences test (Cornuet and Luikart, 1996) and a Wilcoxon sign-rank test (Luikart and Cornuet, 1998). For each of the tests using the different mutational models, 1000 replicates were performed.

Luikart and co-workers (1998) illustrated that populations at mutation-drift equilibrium have a large proportion of alleles at very low frequency. In contrast, recently bottlenecked populations characteristically show a mode-shift in the distribution of allele frequencies so that alleles of very low frequency (<0.1) are less numerous than alleles that occur more frequently. In contemporary roe populations, evidence of mode shift distortions was assessed using the program Bottleneck.

Finally, reductions in population size were also tested with the Garza M ratio statistic proposed by Garza and Williamson (2001). This statistic is calculated as the mean ratio of the number of alleles at a given locus to the range in allele size. Populations that experience a bottleneck are expected to lose rare alleles (Nei et al., 1975) creating allelic vacancies and therefore decreasing M. This statistic was calculated using the programme Arlequin v 3.1 (Excoffier et al., 2005).

4.2.6.3 Direct comparison of ancient and modern populations

Mt-DNA

Ancient and modern UK sequences were first aligned with CLUSTAL X (Larkin et al., 2007) and all subsequent analyses were carried out on a 419 bp region of the mt-DNA d loop.

The purpose of using both ancient and modern samples in this analysis was to detect the impact of history on these populations. Population genetic summary statistics were therefore calculated on aligned samples from ancient and modern samples individually and then later combined. This enabled a direct comparison to be made between time periods. All population genetic summary statistics were computed in DNA sp 10.4.9 (Rozas et al., 2003) including: number of segregating (polymorphic) sites (S); number of unique haplotypes (h); haplotype diversity (H_d); average number of pairwise nucleotide differences (k); and nucleotide diversity (π) (the average number of nucleotide differences per site between two sequences).

In order to compare phylogenies between modern and ancient haplotypes, a median joining network was created in NETWORK 4.5.1.6 (http://www.fluxus-engineering.com). This method was chosen because, relative to phylogenetic methods, it is better suited to inferring haplotype genealogies at the population level. This is because networks explicitly allow for extant ancestral sequences and alternative connections (Bandelt et al., 1999). Three different networks were constructed: ancient UK; modern UK; and both ancient and modern UK samples combined. Inferences about possible native and non-native haplotypes were made using the combined network.

Population differentiation; mt-DNA and microsatellites

Whether northern populations best represent native UK populations was examined by investigating the relationship between the UK roe from the eight contemporary populations and the ancient population (assuming that ancient populations best represent native populations). The ancient population had to be considered as one single population due to restrictions of small sample sizes per area.

Pairwise F_{ST} values (Weir and Cockerham, 1984) were calculated using an analysis of molecular variance (AMOVA) with 1000 permutations for both mitochondrial and microsatellite markers. As the AMOVA involves using multiple tests, Bonferroni correction was applied.

For the mitochondrial DNA the AMOVA was performed in Arlequin 3.0 (Excoffier et al., 2005) and was based on the 419 base pair region (as above). For the microsatellites the AMOVA was performed in FreeNA (Chapuis and Estoup, 2007). This programme estimates F_{ST} from microsatellite datasets (i.e. ancient samples) that are known to harbour null alleles or suffer from allelic dropout. The method used for such estimation is known as the ENA method. In brief, this method restricts Weir's (1996) F_{ST} to the computation of visible states by ignoring null alleles and not adjusting allele frequencies to 1. This is feasible because F_{ST}-estimates at a given locus are the appropriate combination of single allele estimates of F_{ST} (Chapuis and Estoup, 2007).

4.3 Results

4.3.1 Genotyping errors and tests for Hardy Weinberg equilibrium in modern and ancient samples

Results from the programme Micro-checker and Hardy Weinberg analyses for modern samples are presented in Chapter 3. For ancient samples micro-checker revealed that 4 out of the 6 microsatellite loci used in this analysis suffered from allelic dropout or null alleles. These same loci also showed significant heterozygote deficiency (see appendix 4). This result may have been due to the Wahlund effect (i.e. sub-structuring within ancient populations); however, due to the low sample sizes it was not possible to further sub-divide samples. In any case, many samples were homozygotes which probably resulted from errors generated from low quality DNA. Therefore, analyses based on these microsatellite data were limited to the programme FreeNA, which accounts for such errors (see methods).

4.3.2 Modern populations: genetic diversity and bottleneck signatures

Levels of genetic diversity measured from both microsatellites and mt-DNA varied across populations. Overall, the lowest levels of diversity were observed in the Norfolk population, with highest diversity in the Perth and Moray populations.

Genetic variability based on microsatellites diminished from North to South within the UK (range of He: Scotland, 0.72-0.76; middle England, 0.65-0.68; southern England, 0.57-0.64) (see Table 4.2). The pattern for mt-DNA was broadly similar, especially with respect to high diversity in Scotland, although mt-DNA diversity was lower in middle than in southern England in some cases.

Neutrality tests revealed positive (but non-significant) values for 4 out of the 7 tested populations showing some evidence for contractions. Evidence of expansion was apparent in one of the populations (Carlisle) which showed a significantly negative value for both Tajima's D and Fu's Fs test.

Table 4.2. Microsatellite and mt-DNA diversity and neutrality test results for the 8 populations of roe in the UK.

	Microsatellite diversity			**Mt-DNA diversity**				
Population	**H o**	**H e**	**P**	**h**	**hd**	**π**	**Tajima's D**	**Fu's Fs**
Norfolk	0.49	0.57	<0.001	1	0	0	-	-
Berks/Wilts/Dorset/Somerset	0.59	0.64	<0.001	4	0.58	0.0025	0.63	1.27
Durham/N York	0.59	0.61	0.01	4	0.42	0.0068	0.69	-0.03
Carlisle	0.67	0.65	0.92	4	0.21	0.0008	**-2.10**	**-2.80**
Perth	0.69	0.72	0.01	7	0.78	0.0044	0.71	0.92
Moray	0.66	0.71	0.0015	9	0.81	0.0044	-0.12	1.06
Ayr	0.64	0.65	0.004	7	0.36	0.0024	-0.99	0.4
Lancs	0.63	0.66	0.14	5	0.82	0.0050	0.72	0.58

Detection of the apparent heterozygote excess is highly dependent on the underlying model of microsatellite evolution (Table 4.3). The infinite allele model (IAM) showed heterozygote excess under all three tests (Signed, Standardised difference and Wilcoxon rank) in nearly all 8 populations. The stepwise mutation model (SMM), however, showed evidence for heterozygote excess only for the sign difference test in the N York/Durham and Moray/Glasgow population. Finally, under the two phase mutation (TPM) model heterozygote excess was detected only for the Norfolk and Carlisle population using the Wilcoxon rank test.

For each population in this study the allele frequency distribution was found to be normally L-shaped (Table 4.3) as expected under mutation-drift equilibrium. There was no evidence of a mode-shift.

The Garza M ratio statistic ranged from 0.63-0.75 in roe UK populations with a mean of 0.68 (Table 4.4). Case studies suggest that values between 0.6-0.7 are indicative of reduced population size whereas values > 0.8 indicate unperturbed populations (Garza and Williamson 2001).

4.3.3 Direct comparison of ancient and modern DNA

4.3.3.1 Ancient UK samples

168 samples were obtained from museum, archaeological services and university collections. The location of the archaeological site, approximate date (from stratigraphy) and period from which roe were sampled are shown in Table 2.1 (Chapter 2). Of the 168 samples extracted, 126 (75%) were successfully amplified for the full 419 base pair region. For a number of additional samples, half of the target region (244 or 267 bp) was amplified; however, these were not used in further analyses.

The 125 samples obtained for the full 419 base pair range were analysed in DNA sp to determine the numbers of haplotypes. A total of 34 haplotypes were identified from ancient UK samples (Table 4.5). Ten of the haplotypes came from one particular site from London, Moor House. This amount of diversity was completely unexpected given this site's location and age. Furthermore, subsequent discovery of a report based on the site drew attention to the fact that the samples may not be of British origin (Armitage and Butler, 2005). The site was unique, in that large groups of roe deer antlers (86 in total) were found at this medieval site, despite the fact that roe were believed to be very scarce at that time. The site may have been a tannery where roe were imported from the continent. These samples warrant their own study to examine likely origin. A network was drawn both including and excluding the Moor House samples. However, to avoid confounding interpretation, for all direct comparisons between ancient and modern UK populations, Moor House samples were excluded.

Table 4.3. Results of bottleneck detection tests; sign, standardised difference, Wilcoxon rank. Each test was carried out under the mutation models; I.A.M, S.M.M. and T.P.M. P = probability difference was significant where – P >0.05, * P<0.05, ** P<0.01, *** P significant after Bonferroni correction. Mode shift distortion results are also shown where L represents a normal distribution.

Test	Mutation model	No. of loci with expected heterozygosity excess	Norfolk	Dorset/Wilt/ Berks/ Somerset	N york/ Durham	Carlisle	Perth	Moray/ Glasgow	Ayrshire	Lancs
Sign	I.A.M	Expected	8.66	9.03	9.06	9.04	9.35	9.23	9.04	9.13
		Actual	15	15	14	14	15	14	14	13
		P	***	***	**	**	***	**	**	*
	S.M.M	Expected	9.33	9.48	9.54	9.53	9.46	9.59	9.51	9.38
		Actual	7	5	6		9	7	8	7
		P	-	*	-	-	-	-	-	-
	T.P.M	Expected	9.15	9.34	9.37	9.34	9.47	9.46	9.37	9.36
		Actual	14	10	10	11	12	11	12	11
		P	*	-	-	-	-	-	-	-
Standardised difference	I.A.M	T2	3.475	3.266	2.97	3.495	3.408	3.604	3.615	2.848
		P	***	***	**	***	***	***	***	**
	S.M.M	T2	-0.39	-2.891	-2.326	-0.376	-0.925	-2.89	-1.65	-0.123
		P	-	***	**	-	-	***	*	-
	T.P.M	T2	1.954	1.182	0.972	1.99	1.756	1.339	1.624	1.567
		P	*	-	-	*	*	-	-	-
Wilcoxon rank	I.A.M	P (one tail for H excess)	***	***	***	***	***	***	***	***
		P (two tails H excess or deficiency)	***	***	**	***	***	***	***	***
	S.M.M	P (one tail for H excess)	-	-	-	-	-	-	-	-
		P (two tails H excess or deficiency)	-	-	-	-	-	-	-	-
	T.P.M	P (one tail for H excess)	***	-	-	*	*	-	*	*
		P (two tails H excess or deficiency)	*	-	-	***	-	-	-	-
Mode shift			L	L	L	L	L	L	L	L

Table 4.4. Calculation of Garza M statistic; ‘M’ statistic for each of the 8 populations.

Population	‘M’ statistic	s.d.
1. Norfolk	0.63	0.24
2. Dorset/Wilts/Berks/Somerset	0.70	0.24
3. N York/Durham	0.63	0.23
4. Carlisle	0.66	0.24
5. Perth	0.68	0.21
6. Moray	0.75	0.17
7. Ayr	0.69	0.24
8. Lancashire	0.69	0.23
Mean	0.68	0.23

The ancient DNA median joining network (including Moor House samples) shows the relationship of 34 haplotypes (Figure 4.1). The two most common haplotypes (a1 and a2) are found at the centre of the network with all other haplotypes derived. This star-shaped network suggests population expansion and the two central haplotypes can be considered ancestral. This same pattern is maintained when Moor House samples are excluded (Figure 4.2 a).

4.3.3.2 Modern UK samples

314 sequences were obtained and analysed using DNA sp, revealing 12 haplotypes. This contrasts with the 24 found in ancient populations. Haplotype m4 is the most common and widespread haplotype found in contemporary populations (Table 4.6). Some haplotypes were only sampled at very low frequencies and in isolated populations (e.g. m7, m8 and m11). Haplotype m3 is the only haplotype found in the Norfolk population and is unique to this area.

4.3.3.3 Direct comparison of ancient and modern populations

From ancient to modern periods haplotype number, haplotype diversity, nucleotide diversity and K were all reduced (Table 4.7, Figure 4.2a,b). The reduction in nucleotide diversity was not as marked as that of haplotypic diversity.

Table 4.5. List of ancient haplotypes (a1-134) and the locations and archaeological site (for site codes see Chapter 2; Table 2.1) in which they originated from.

H	N	Location and archaeological site of ancient haplotypes
a1	31	London (MH,FEH,WOO,UP,BGH),Derbyshire (CC),Sussex (FIS),Kent (BIS),Hampshire (FAC) and Oxfordshire (BAN)
a2	28	London (MH,WOO,LBI,KWS,BGH),Derbyshire (CC),Sussex (FIS),Kent (BIS),Hampshire (FAC),Hereford (GAO),Wiltshire (DW),Durham (ARB)
a3	8	London (MH, MRG), Durham (ARB), Hampshire (FAC)
a4	1	London (MH)
a5	1	London (MH)
a6	2	London (MH)
a7	2	London (MH)
a8	1	London (MH)
a9	1	London (MH)
a10	1	London (MH)
a11	3	London (MH)
a12	1	London (MH)
a13	3	London (MH), Hereford (GAO)
a14	1	London (MH)
a15	1	Sussex (FIS)
a16	2	Sussex (FIS)
a17	4	Sussex (FIS) and Kent (BIS)
a18	3	London (BAX, LBI) and Sussex (FIS)
a19	1	Sussex (FIS)
a20	2	Sussex (FIS) and Hampshire (FAC)
a21	3	London (FEH), Hampshire (FAC) and Oxfordshire (BAN)
a22	1	London (BAX)
a23	3	London (MRG), Perthshire (HC), Durham (BAR)
a24	1	Hereford (CH)
a25	1	Chester (CHE)
a26	11	Derbyshire (CC) and Lincolnshire (WQ)
a27	1	Perthshire (HC)
a28	1	Wiltshire (DW)
a29	1	Yorkshire (STP)
a30	1	Hampshire (FAC)
a31	1	Hampshire (FAC)
a32	1	Oxfordshire (BAN)
a33	1	Oxfordshire (BAN)
a34	1	Gloucestershire (SAL)

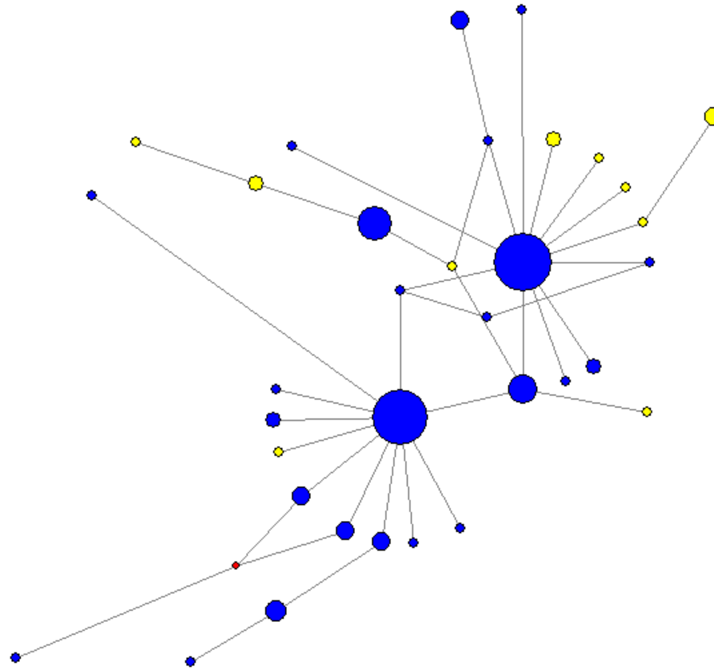


Figure 4.1. Median joining network of phylogenetic relationships among all ancient samples where dark blue (●) indicates ancient UK and yellow (○) indicates haplotypes unique to the Moor house site. The size of the circle indicates relative frequency of the haplotype.

Table 4.6. Modern haplotypes based on 419 bp.

H	N	Location
m1	51	Hampshire, Dorset, Perth, Moray, Durham, N Yorkshire, Lancashire
m2	66	Hampshire, Dorset, Somerset, Berkshire, Moray
m3	41	Norfolk
m4	121	Berkshire, Perth, Durham, N Yorkshire, Carlisle, Moray, Ayr, Lancashire
m5	13	Perth, Moray
m6	4	Lancashire
m7	1	Lancashire
m8	1	Perth
m9	9	Perth, Moray, Ayr
m10	3	Moray
m11	1	N Yorkshire
m12	3	Ayr

Table 4.7 showing estimates of gene diversity at mt-DNA control region in roe deer from ancient, ancient excluding Moor House samples (excl. MH), modern and modern excluding Norfolk samples (excl. Norfolk) British roe populations. Bp = number of base pairs used in analysis, n= number of sequenced individuals, n*h*= number of observed mt-DNA haplotypes, hd = haplotype diversity, π = nucleotide diversity, K= average pairwise sequence divergence.

	Bp	N	Nh	hd ± s.dev	Π	K
Ancient UK	419	124	34	0.88±0.0003	0.00567	2.38
Ancient UK (excl. MH)	419	86	24	0.88±0.0004	0.00581	2.47
Modern UK	419	320	12	0.77±0.0002	0.00536	2.25
Modern UK (excl. Norfolk)	419	278	11	0.71±0.0003	0.00406	1.70

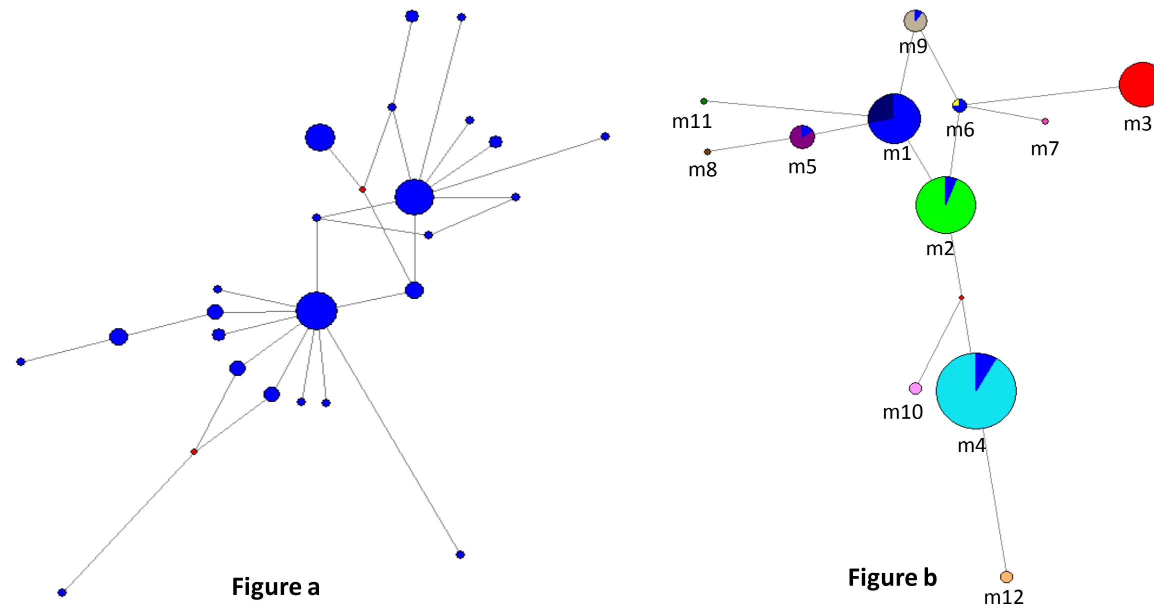


Figure 4.2. A direct comparison of ancient (Figure a) versus modern (Figure b) median joining networks computed in NETWORK. In Figure a dark blue circles (●) indicate all ancient haplotypes (n= 24). In Figure b the modern haplotypes (n=12; m1-12; see Table 4.6) are labelled and coloured; shared colours represent haplotypes also found in ancient populations.

A combined network produced from ancient and modern data showed which haplotypes were shared between the two periods (Figure 4.3 a). A total of 30 haplotypes could be detected (Figure 4.3 a). Six haplotypes were shared between ancient and modern periods, indicating that these were 'native' (Figure 4.2 b and Figure 4.3 a). In order to see whether some of the 'novel' haplotypes present in modern samples (but not found in ancient samples) resulted from known points of re-introduction, modern haplotype distributions were mapped (Figure 4.3b). The most notable finding was the appearance of the 'novel' haplotype (m3) located in the Norfolk population (see Figure 4.3b; haplotype coloured red).

An analysis of molecular variance was conducted to determine which contemporary populations were most closely related to ancient UK populations. Levels of differentiation for both mitochondrial and microsatellite loci between all ancient and UK contemporary populations were nearly all significant (Table 4.8). However, slightly different results were found for the mitochondrial compared to the microsatellite data. The contemporary populations most closely related to ancient UK populations were Lancashire (based on mitochondrial data; the only non-significant difference) or Moray (based on microsatellite data). In general, both mitochondrial and microsatellite data showed that contemporary populations found in the north were more closely related to ancient UK populations than those found in the south. Specifically, the southern population of Norfolk was most differentiated from ancient populations. However, these results should be treated with some caution as the ancient sample is unlikely to represent a single population.

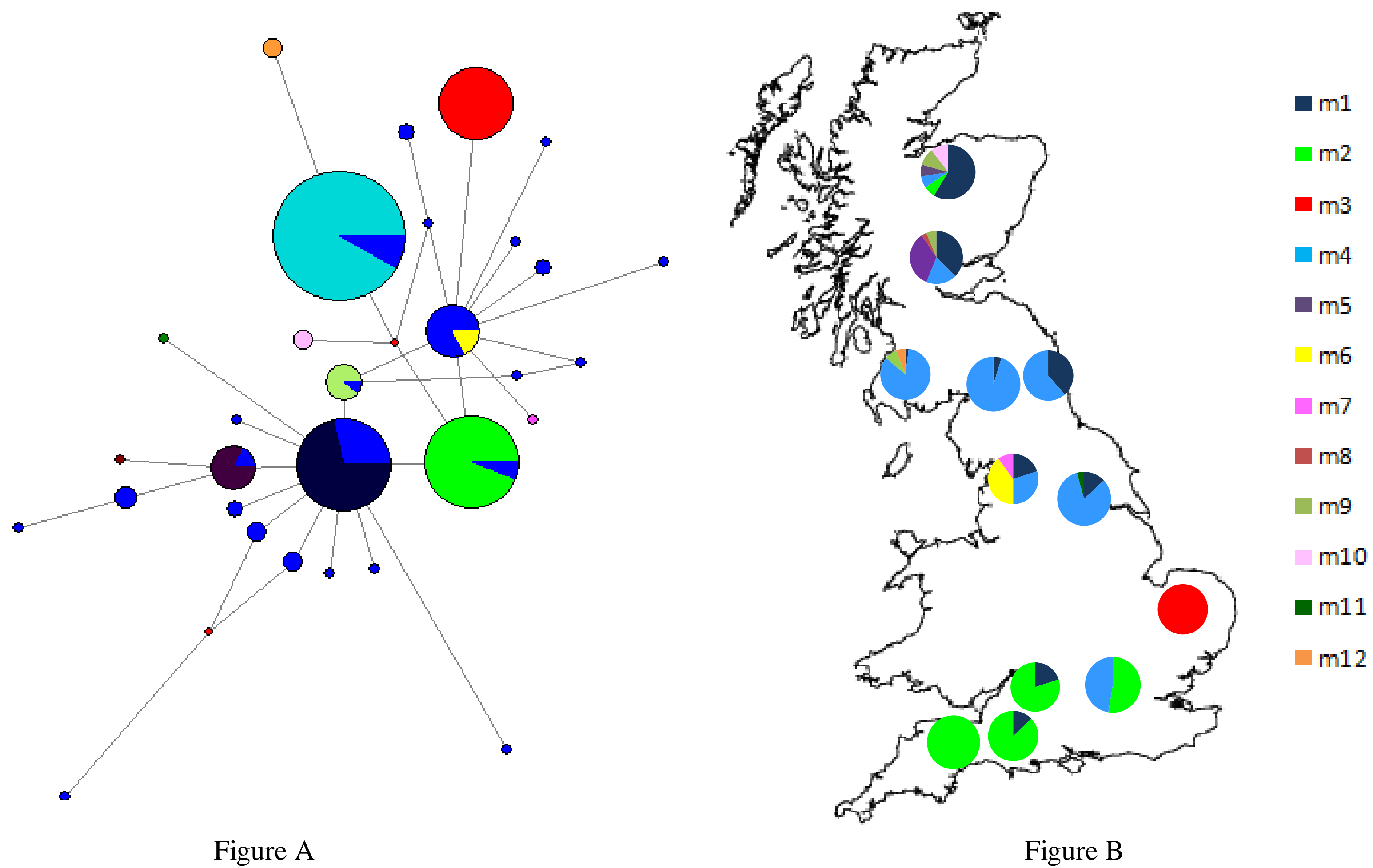


Figure 4.3 a) Median joining network of phylogenetic relationships among ancient and modern mitochondrial haplotypes where the size of the circle indicates relative frequency of the haplotype where dark blue (●) indicates ancient haplotypes and all other colours are modern haplotypes. b) The 12 modern haplotypes (based on 399 base pairs; m1-m12) and their distributions across the UK.

Table 4.8. Modern and ancient UK population pairwise F_{ST} values for 6 polymorphic microsatellites (below diagonal) and 419 bps of the mt-DNA d loop (above diagonal). Values in bold indicate significance after Bonferroni adjustment.

Population	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1. Norfolk	-	**0.89**	**0.88**	**0.98**	**0.84**	**0.86**	**0.90**	**0.87**	**0.62**
2. Hamps,Wilts,Dorset,Somerset	**0.24**	-	**0.53**	**0.73**	**0.43**	**0.37**	**0.64**	**0.29**	**0.16**
3. Durham, N york	**0.23**	**0.24**	-	0.07	**0.45**	**0.47**	0.04	**0.31**	**0.32**
4. Carlisle	**0.23**	**0.20**	**0.04**	-	**0.63**	**0.68**	-0.01	**0.56**	**0.44**
5. Perth	**0.26**	**0.19**	**0.19**	**0.13**	-	0.05	**0.58**	**0.27**	**0.09**
6. Moray	**0.20**	**0.17**	**0.12**	**0.06**	**0.05**	-	**0.61**	**0.24**	**0.05**
7. Ayr	**0.22**	**0.22**	**0.06**	**0.06**	**0.13**	**0.07**	-	**0.44**	**0.43**
8. Lanc	**0.14**	**0.22**	**0.11**	**0.09**	**0.18**	**0.10**	**0.11**	-	0.02
9. Ancient UK	**0.20**	**0.17**	**0.18**	**0.14**	**0.11**	**0.10**	**0.14**	**0.14**	-

4.4 Discussion

4.4.1 The use of modern DNA to detect bottlenecks

As expected, evidence for reductions in population size depended on the bottleneck 'signature' test used. The bottleneck 'signature' often considered most robust for detecting evidence for historical reductions in population size is Garza's M (Spear et al., 2006; Hundertmark and Van Daele, 2010). This method is able to detect bottlenecks for a longer number of subsequent generations (125-500) than the other signature methods considered below (Garza and Williamson, 2001). Indeed, UK populations showed the Garza M statistic was relatively low in all populations. All values were lower than the threshold (0.82) suggested by Garza and Williamson (2001) indicative of unperturbed populations. The average value was 0.68, which is the value put forward as the recommended limit expected for bottlenecked populations (Garza and Williamson, 2001). This suggested that all UK populations have been impacted by some reductions in size, although some populations (e.g. Norfolk, N York/Durham) appear to have been impacted to a greater extent than others (e.g. Moray) (see Table 4.4).

The other bottleneck signatures (heterozygosity excess and mode shift indicator) are highly transient and were only expected in populations that had experienced recent and acute genetic bottlenecks (Cornuet and Luikart, 1996; Luikart et al., 1998 b; Ramstad et al., 2004). There was some evidence of bottlenecks from heterozygosity excess, although patterns were inconsistent and seemed to be highly dependent on the mutation model assumed. When an IAM model of evolution was assumed, all populations showed evidence of heterozygosity excess (most readily under the standardised difference and Wilcoxon rank test). However, when an SMM mutation model was assumed, few populations showed evidence of heterozygosity excess after Bonferroni correction (Table 4.3). Previous studies have also shown a greater propensity for significant results under the IAM than the SMM model (Cornuet and Luikart, 1996; Le Page et al., 2000; Harper et al., 2006; Busch et al., 2007), even though microsatellite loci may be expected to evolve by stepwise mutation. One possible explanation is that microsatellite allele size distributions often depart from the SMM model (Le Page et al., 2000; Harper et al., 2006). It is generally considered that the SMM model may be too conservative and that the IAM model may be more sensitive for detecting subtle bottlenecks (Busch et al., 2007). However, results from the IAM model should also be

interpreted with caution as it can identify heterozygosity excess in non – bottlenecked populations (Luikart and Cornuet, 1998). It may, therefore, be most appropriate to consider the results of the TPM model which combines both SMM and IAM in varying amounts and is suggested to be the most realistic model of microsatellite mutational events (Dirienzo et al., 1994; Piry et al., 1999). Here, the results of the TPM showed significant heterozygote excess for only one population (under the Wilcoxon rank test for the Norfolk population). The mode shift indicator was unable to detect bottlenecks in any of the populations. A number of studies have previously commented on the relative insensitivity of this test (Swatdipong et al., 2010) which only detects reductions that have occurred within a maximum of a few dozen generations (Cornuet and Luikart, 1996). It is likely that bottlenecks impacting most UK populations have occurred over too long a timescale for reliable detection using this method.

Overall, bottleneck signatures revealed the strongest evidence for bottlenecking in the Norfolk population. The Norfolk population showed the lowest Garza's M values (Table 4.4); the only significant value for heterozygosity excess under TPM (Table 4.3); and the lowest levels of genetic variability (Table 4.2). These results probably reflect the known founder event, documented to have occurred in 1884 when 12 roe were introduced from Europe. The founder effect is likely to have been strong (and thus easily detectable) for a number of reasons. Firstly, the event occurred relatively recently (42 generations ago, assuming a generation time of 3 years; Randi et al., 2004). This is known to be within the time frame detectable for most bottleneck signature methods (Luikart and Cornuet, 1998; Luikart et al., 1998 b). Secondly, only a small number of founders were involved. Other studies with similar founder event characteristics (similar timescale and numbers of founders) have also left detectable bottleneck signatures. For example, a population of Alpine ibex founded by 15 individuals 100 years ago showed similar levels of significant heterozygosity excess (Maudet et al., 2002). Finally, one other factor contributing to the strength of the founder event may be related to the insularity of this population. Since the re-establishment of Norfolk roe took place, the population seems to have remained relatively isolated (see Figure 4.3 and Chapter 3, STRUCTURE results). The possible reasons for this were discussed in Chapter 3. Busch et al., (2007) consider that only when populations are sufficiently isolated will bottleneck detection methods identify genetic signatures.

It was surprising that signature methods did not detect strong evidence of bottlenecking in the other southern population (Dorset/Wilts/Somerset/Berks). This population showed no significant heterozygosity excess (based on TPM) and a high Garza's M value (relative to other populations; see Table 4.4). Furthermore, this population retained appreciable levels of diversity (Table 4.2). The weaker signal for bottlenecking in spite of recent founder events could be due to a number of factors. Firstly, the last founder event recorded for this population (1825) may have occurred too long ago for the signal to be detected using the BOTTLENECK method (62 generations have elapsed). Secondly, introduction information indicates that more than one translocation event has occurred into this population (see Chapter 3). Admixture between different founder individuals may have ameliorated the signals for bottlenecking. Finally, bottleneck signatures may have been weakened by the grouping of samples from all of the south western locations into a single population, although samples from these regions all group together in the FCA and STRUCTURE analyses shown in Chapter 3 (Figures 3.5 and 3.6), suggesting that they represent a single population. For the other UK populations, the overall evidence for bottlenecks using signature methods was weak.

4.4.2 The use of ancient DNA to detect bottlenecks

The use of ancient DNA revealed that a significant change has occurred between historic and contemporary time points, leading to the extinction of 18 of the unique historical haplotypes. The cause of this change may be a period of bottlenecking caused by over hunting and deforestation between the late 14th and 18th centuries which appears to have impacted *all* populations.

Populations in the most northerly parts of the UK (e.g. Perth/Moray/Glasgow) showed the least impact of bottlenecking. These populations have retained the highest numbers of native haplotypes (i.e. those found in historical populations; see Figure 4.3) and show close relationships with historical populations (Table 4.8). This result is consistent with the historical record, which claimed that roe never went extinct in this region and may have retained appreciable numbers (Whitehead, 1964).

Northern English populations (e.g. Carlisle, Durham/North York) show more evidence of bottlenecking, as only low numbers of native haplotypes were detected in these populations

(Figure 4.3). This result is consistent with Whitehead (1964) who claimed that south of the Glasgow – Edinburgh road, roe became increasingly scarce during the medieval period. They may have even become extinct in these areas (Bewick, 1790) and re-established through expansion. That possibility is consistent with the signal detected for this from modern genetic data (see Table 4.2).

The other northern English population, Lancashire, harboured three native haplotypes. Interestingly, one of these native haplotypes (m6) was unique to this location, which may imply that not all native roe in this area went extinct. Indeed, Whitehead (1964) suggested that a population of roe may have always inhabited 'the rough wooded valleys of Furness Fells.' Also found at this location was a further haplotype (m7) which appeared to be novel (not found in historical populations). This haplotype may represent relicts of an introduction event which occurred when 12 Austrian roe were introduced into this population in 1913 to 'improve the local breed' (Whitehead, 1964; Prior, 1995), though it is also possible that it was present in the ancient population, but not detected.

The Norfolk population showed no evidence for the retention of any native haplotypes. This population exhibited a single novel haplotype, unique to this location (m3; Figure 4.3). Furthermore, relative to other modern UK populations, this population exhibited the highest microsatellite and mitochondrial F_{ST} values (0.20/0.62) when compared against the ancient UK sample set. This finding supports the likely role of a founder event involving the introduction of non-native (German) roe into this location (as discussed above).

Roe in south western locations (Dorset/Wilts/Somerset/Berks) are often assumed to have gone extinct as a result of medieval bottlenecking and later re-established through Scottish re-introductions (Whitehead, 1964). Indeed, the appearance of the native haplotypes (m1, m2 and m4; see Figure 4.3) which are common to both populations may be consistent with this scenario.

Overall, the use of ancient DNA has been especially useful in this study for detecting probable bottlenecks and re-introductions into populations. Nevertheless, there are potential pitfalls. Firstly, while a bottleneck may lead to lost haplotypes, haplotypes could also be lost by temporal genetic drift. Furthermore, in the absence of data on historical population structure, we had to treat the historical samples as a single group for comparison with modern

populations. However, modern roe are highly structured (Chapter 3) and although some of this has to do with a series of recent introductions, there was likely some level of population structure in historical times as well. The strong deviations detected from Hardy-Weinberg equilibrium in ancient populations (appendix 4) may result from allelic dropout, but may also be due in part to a spatial or temporal Wahlund effect. Using more extensive ancient sample sets with comprehensive spatial and temporal distribution would likely help resolve these issues. Unfortunately, this is hard to obtain, given the limited availability of ancient samples.

4.5 Conclusions

Bottlenecks have been shown to increase extinction risk in natural populations (Newman and Pilson, 1997; Saccheri et al., 1998) and, therefore, the ability to detect them is central to conservation genetics. This study used modern data to identify bottleneck 'signatures' resulting from past size reductions. However, as found in various other studies, the power of these signatures to detect bottlenecks was weak and confounded by uncertainties regarding the relative roles of power and sensitivity. The use of ancient DNA, however, provided further valuable insight for detecting historical bottlenecks and for differentiating between native and re-introduced populations (often another priority for conservation biologists). In the case of roe deer, the combination of analyses undertaken provided evidence for a pattern of historical bottlenecks and reintroductions that tracked well with the historical data. Furthermore, it was evident that the ancient samples also showed evidence of expansion (based on the star-shaped phylogeny), consistent with an earlier founder event when retreating ice released habitat after the last glacial period (see Chapter 2 for further discussion).

Chapter 5 : The impacts of past population history on the fitness of roe populations

5.1 Introduction

Many populations have undergone periods of demographic size reductions (bottlenecking). The immediate impact of a bottleneck is losses of genetic diversity. Even during population recovery, further losses of genetic diversity may occur through inbreeding because survivors are forced to mate with close relatives.

Loss of genetic diversity is frequently correlated with a loss of individual fitness in wild populations (commonly termed inbreeding depression) (Crnokrak and Roff, 1999; Coltman and Slate, 2003). Genetically depauperate individuals have been shown to have a lowered reproductive output (e.g. red deer, Cervus elaphus, Slate et al., 2000) increased susceptibility to disease (e.g. Soay sheep, Ovis aries, Coltman et al., 1999; naked mole rat, Heterocephalus glaber, Ross-Gillespie et al., 2007) and reduced probability of survival (e.g. juvenile red deer, Coulson et al., 1999). Lowered genetic diversity has also been shown to reduce fitness at the population level (e.g. Newman and Pilson, 1997). As a result, the overall effect of reduced genetic variability can be an increased risk of extinction (Saccheri et al., 1998; Ross-Gillespie et al., 2007).

Several studies have, however, shown that genetically depauperate populations showing signs of lowered fitness can be quickly ‘rescued’ by the introduction of only a small number of divergent migrants (reviewed in Tallmon et al., 2004). Such ‘rescued’ populations may show evidence of enhanced fitness, otherwise known as heterosis. Heterosis may occur via two mechanisms. First, matings between individuals of divergent origin can produce highly heterozygous offspring which may be favoured by natural selection. Second, immigrant alleles can mask deleterious recessive alleles that, prior to immigration, may have been locally prevalent due to genetic drift (Crow, 1948). Recent literature suggests the latter mechanism is the most frequent mechanism of heterosis (Tallmon et al., 2004). Given the potential this ‘genetic rescue’ has for reversing detrimental impacts of inbreeding depression it has received much attention as a potential management tool (Hedrick and Fredrickson, 2010).

Populations of British roe deer are well known to have undergone periods of past bottlenecking. Bottlenecks impacting roe populations have resulted from medieval deforestation and over-hunting and from founder events during re-establishment (Chapter 4). Although bottlenecks have impacted all UK populations, some populations have been more severely impacted than others (Chapter 4). Under theoretical expectations, populations that have undergone the most severe bottlenecks and lost the most genetic variability may show the lowest levels of fitness. Such populations generally occur in Southern UK where, following extirpation during the medieval period, founder events occurred (Chapter 3 & 4). One example population where a founder event has been particularly strong is Norfolk. The Norfolk population was believed to have descended from the introduction of only 12 German roe introduced in 1884 (Whitehead, 1964). Since this introduction it seems that this population has remained semi –isolated, although there may be evidence for low levels of gene flow (Chapter 3). Overall, Norfolk roe show the lowest genetic diversity and the strongest evidence for bottlenecking (Chapter 3 & 4); this population may, therefore, show the lowest overall fitness. However, this assumption could be complicated by this population's high divergence from all other populations, due to its non-native origins (Chapter 3). Therefore, even low levels of gene flow with neighbouring (native) populations could enhance fitness through heterosis. For the Northern UK populations, that have been less severely impacted by bottlenecks (thus retaining the most genetic diversity), it may be expected that fitness would be highest (see Chapter 3 & 4). In order to determine whether levels of fitness do vary across populations, developmental homeostasis will be examined.

Developmental homeostasis is the ability of an organism to withstand environmental and genetic disturbances encountered during development, to produce a genetically predetermined phenotype (Lerner, 1954). Under optimal conditions, organisms are able precisely to express genetically determined developmental pathways; any small random perturbations resulting from stochastic processes (developmental noise) are corrected by developmental stability mechanisms. However, when stressful conditions prevail, increases in energy expenditure mean that resources available to developmental pathways become reduced (Hendrickx et al., 2003). Stress which can increase developmental instability can be defined as either intrinsic (genetic) or extrinsic (environmental) in origin. It is hypothesised that when intrinsic (genetic) stress is greater (i.e. for less genetically variable individuals) developmental instability prevails (Lerner, 1954). When extrinsic (environmental) stress is

greater, those genotypes in more stressful environmental conditions show increased developmental instability compared to identical genotypes in less stressful conditions (for reviews, see Zakharov 1989, 1992). Under both the types of stress, the efficiency of the stability mechanisms are affected and, as a result, developmental pathways may deviate from expected trajectories, producing aberrant phenotypes (Clarke, 1995). The efficiency of this stability mechanism is considered to be an integral component of an individual's fitness (Clarke, 1995). Measures of developmental stability can, therefore, provide important information on the fitness of individuals and populations.

The most commonly used method for measuring developmental stability or instability is fluctuating asymmetry (FA) (Palmer, 1994). FA refers to the small random deviations from bilateral symmetry in a morphological trait, normally distributed around a mean of 0 (Van Valen, 1962). It is based on the idea that both sides of an organism presumably share the same genes and, under homogenous environments, external effects on development are also the same on both sides (Klingenberg, 2003). During development, developmental noise acts locally to impact a small part of one body side. Therefore, any unsigned deviation from symmetry, to which the FA typically refers (Palmer and Strobeck, 2003), can be interpreted as evidence of developmental instability.

The use of FA as a measure of fitness in populations has many advantages. Firstly, there is no other morphological or physiological trait that reflects fitness reliably (Dongen, 2006). Secondly, FA is one of the few morphological attributes for which the norm, i.e. perfect symmetry, is known (Palmer, 1996). Thirdly, the sample sizes (minimum of 30) (Clarke, 1995) generally required to perform valid statistical analyses are easily obtainable. Fourthly, fluctuating asymmetry does not require expensive equipment and can be assessed non – destructively (Lens et al., 2002). Finally, FA is more sensitive in detecting reduced fitness than other, more traditional, measures (e.g. survival, Clarke and McKenzie, 1992).

Despite these advantages, the use of FA as an indicator of fitness has been controversial (e.g. Rasmuson, 2002). This controversy centres on whether FA consistently correlates with genetic variability. Although a number of studies have shown the expected associations between fluctuating asymmetry and genetic stress (Leary et al., 1983; Mitton, 1993), many have not (e.g.Mitton, 1978; Gilligan et al., 2000; Kruuk et al., 2003; Fessehaye et al., 2007).

Overall, using meta-analytic approaches (where the results of a large number of studies are analysed) it was found that correlations between heterozygosity and FA were only very weak (Britten, 1996; Vollestad et al., 1999).

Studies based on mammalian populations have generally found expected negative correlations between genetic variability and FA. Examples include those based on tamarin, *Saguinus* (Hutchison and Cheverud, 1995); brown hare, *Lepus europaeus* (Hartl et al., 1995) German roe deer, *Caperolus capreolus* (Zachos et al., 2007) and common shrew, *Sorex araneus* (White and Searle, 2008). Although these examples support the use of FA as an indicator of fitness, the associations reported have tended to only be weak and non-significant, echoing the results of meta-analytic studies.

This chapter will determine whether patterns of FA among populations reveal any information on the fitness consequences of demographic and genetic processes that have impacted populations. Furthermore, relationships between genetic diversity and FA within, across and among all UK roe deer populations will also be examined. Specifically, the following predictions are addressed:

1. Levels of FA will be highest in populations that have been impacted by recent and strong bottlenecks.
2. Levels of FA for individuals within, across and among all populations will be negatively correlated with genetic variability or positively correlated with inbreeding.

5.2 Methods

5.2.1 Collection of samples

Tissue (for DNA) and skull samples were collected from female roe deer older than 2 years of age only. Females were selected to avoid possible inter–sex variation. In addition, individuals aged over 2 were chosen as this is when roe skulls are completely developed (Sokolov et al., 1985). Once heads had been collected they were stored at -20 ^{o}C until ready for preparation. One hundred and nineteen deer were used in this study, collected from nine different locations incorporating six putative populations (see Figure 5.1 and Table 5.1). Individuals were assigned to these populations based on the statistical analyses carried out on both genetics and morphology in Chapter 3 (e.g. *a priori* knowledge). These particular populations were chosen for this study because these were considered to cover varying levels of diversity.

Figure 5.1. Map of the UK showing the eight different locations across six different populations from which doe heads were collected. Populations are represented as follows; 1. Moray, 2. Perth, 3. Carlisle/Durham, 4. Lancashire, 5. Norfolk and 6. Wiltshire and Dorset.

Population number and name		**N skulls**
1.	Moray	15
2.	Perth	15
3.	Carlisle	8
3.	Durham	19
4.	Lancashire	10
5.	Norfolk	23
6.	Wiltshire	4
6.	Dorset	25
	Total N	**119**

Table 5.1. Number of skulls collected from each of the locations and the populations these locations belong to.

5.2.2 Skull preparation and measurements

All roe heads were prepared as described in Chapter 3.

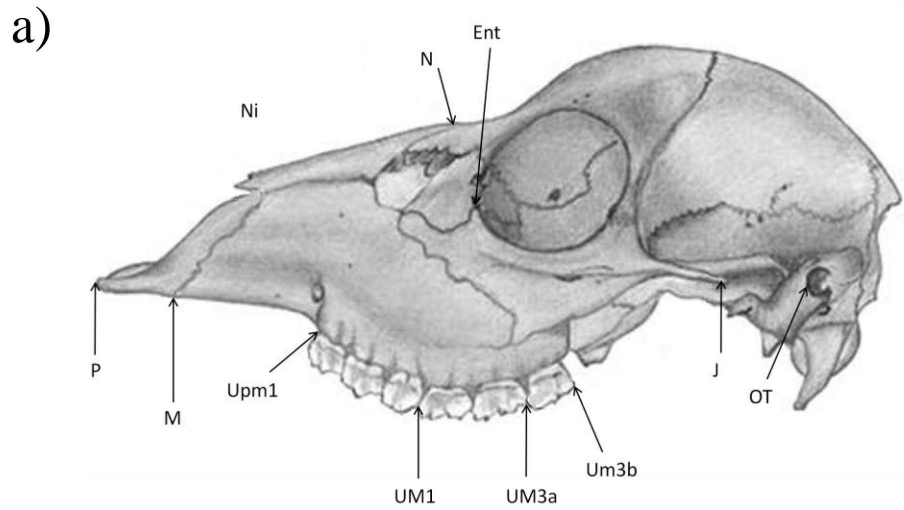
A total of 16 bilateral metric skull and mandible characters were used for assessing asymmetry (see Figure 5.2; Table 5.2). Measurements were mainly derived from previous studies that have investigated fluctuating asymmetry from cranial traits (e.g. Hartl et al., 1995; Lovatt, 2007; Zachos et al., 2007).

Each of the bilateral traits was measured by the same person (KHB) on the left and then the right side with vernier callipers. All measurements were taken twice to the nearest 0.01mm, and then averaged (with a subset measured three times to assess measurement error, see below). Repeated measurements were always taken 'blindly' that is with the measurer having no knowledge of the previous measurements. Several days elapsed between each complete set of measurements on each skull as this has been shown to give the most reliable estimate of measurement error (Palmer, 1994). No measurements were attempted on broken or worn parts of the skulls, and therefore some skulls have missing values.

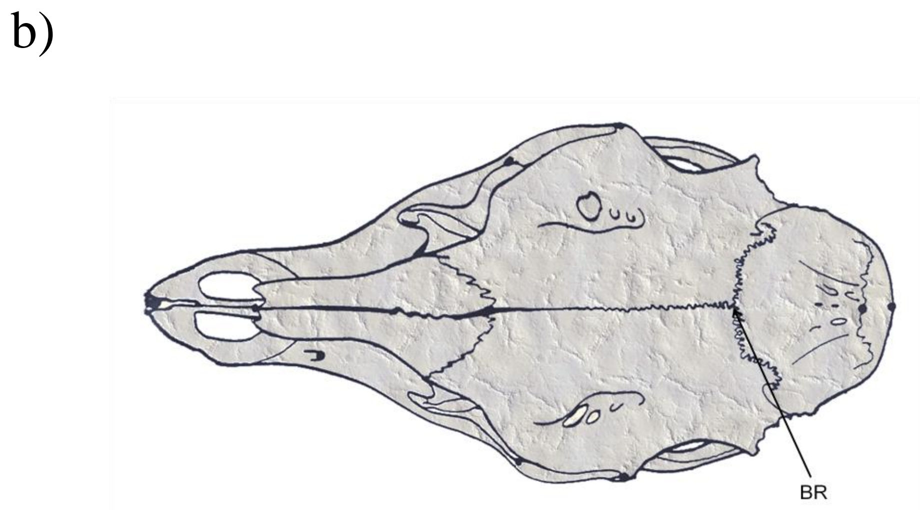
5.2.3 Microsatellite data

In order to measure various indices of genetic diversity, 16 microsatellites were incorporated into this study. These were as described in Chapter 3.

a)



b)



c)

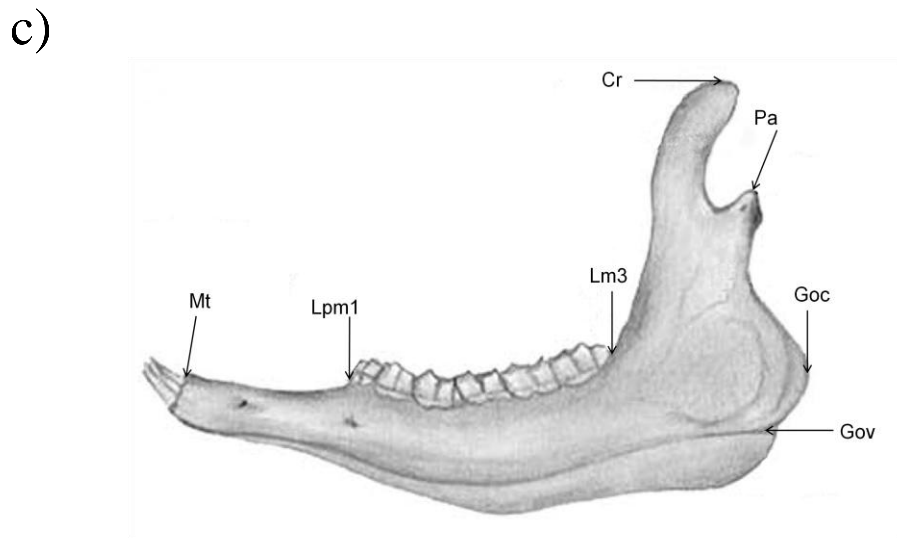


Figure 5.2. Traits measured in the skull of the roe deer a) Lateral view of skull b) Dorsal view of skull c) Lateral view of mandible

Table 5.2 a) Codes and definitions of the measuring points taken on the cranium b) Measurements and their respective descriptions that were taken on both cranium and mandible.

a)

Definitions of measuring points of the cranium	
Code	**Point on cranium**
Rh	Rhinion
N	Nasion
Ent	Entorbitale
Ni	Nasointermaxillare
Ot	Otion
Po	Palatinoorale
St	Staphylion
Vom	Vomar notch
Br	Bregma
Upm 1	Upper premolar 1
J	Jugal
M	Maxillary tip
Um 3a and 3b	Upper molar 3
Um 1	Upper molar 1
Gov	Gonion ventrale
Cr	Coronion
Goc	Gonion caudale
Pa	Processus articulus
Mt	Tip of mandible
Lpm 1	Lower premolar 1
Lm3	Lower molar 3

b)

Bilateral measurements of the cranium	**Description**
Um3b-M	Upper third molar to tip of maxillary
Um3b-Upm1	Upper tooth row length
Um3b-Um1	Upper molar length
Um3a-3b	Length of 3rd molar
J-Upm1	Jugal to 1st premolar
J-M	Jugal to tip of maxillary
Ni-P	Premaxillary length
N-Rh	Nasal length
Ot-Br	Otion to bregma
St-po	Staphylion to palatinoorale
Ent-P	Entorbitale to prosthion
Vom-Po	Vomar notch to palatinoorale
Bilateral measurements of the mandible	**Description**
Gov-Cr	Dental height
Goc-Mt	mandibular length
Lm3-Lpm1	Lower teeth row
Lpm1-Pa	Processus articulurus to lower 1st premolar

5.2.4 Statistical analyses

All statistical analyses were performed using SPSS (version 15.0). Siginificance was examined at the 0.05 level. It was also examined after sequential Bonferroni adjustment, which uses the nominal of 0.05 to correct critical values for multiple tests, and reduces the possibility of making Type 1 errors (Rice, 1989).

5.2.4.1 Outliers and measurement error

As suggested by Palmer and Strobeck (2003), all traits were individually inspected for outliers by scatter plots. Possible outliers were removed and the measurement was taken again.

The estimation of measurement error in fluctuating asymmetry studies is indispensable. This is because, just like FA, measurement error is often small and normally distributed around a mean of zero (Merilä and Björklund, 1995). Measurement error can artificially inflate estimates of fluctuating asymmetry or completely obscure its detection (Palmer and Strobeck, 1994) and therefore, without its removal, a valid assessment of FA cannot be made.

In order to determine the relative contribution of measurement error the following steps were taken. Each measurement was repeated three times on each side in 40 individuals. On the basis of the resulting dataset of 240 measurements for each trait (two sides x three repeated measurements x 40 individuals) two-way mixed model ANOVAs were carried out (Palmer and Strobeck, 1986) for each trait with the factors ‘sides’ (S) and ‘repeat’ (R) as fixed and ‘individual’ (I) as random.
Repeatability was then calculated by the following equation:

$$\frac{\text{MS of } I \times S}{\text{Combined MS of } I \times S \times R + I \times R}$$

The combined MS (mean square) of I x S x R + I x R was calculated by summing the relevant sum of squares (SS) and dividing by the degrees of freedom (df).
The resulting value from the above equation provided an F test that determined whether between individual variation in estimated asymmetry was significantly greater than

measurement error (Palmer and Strobeck, 1986). When values were shown to be significant, traits were considered repeatable (Moller and Swaddle, 1997).

Three measurement error indices (ME2, ME3 and ME5) were calculated for each trait (Palmer, 1994; Palmer and Strobeck, 2003). The two way mixed model ANOVA (sides = fixed and individuals as random) was used to generate MSm and MSi values (where MSm is the error mean square of sides × individuals and MSi the mean squares interaction of sides × individuals). MSi and MSm values were then used to calculate the measurement error indices from the calculations below:

$$\mathbf{ME2} = \sqrt{MS_m}$$

$$\mathbf{ME3} = 100 \times MSm/MSi$$

$$\mathbf{ME5} = (MSi - MSm)/\ (MSi + (n\text{-}1)\ MSm)$$

ME2 represents the standard deviation of repeated measurements. ME3 expresses the average difference between repeat measurements as a percentage of the average difference between sides. Finally, ME5 is a repeatability measure which expresses variation due to asymmetry as a proportion of the total between sides variation (which includes ME) (Palmer and Strobeck, 2003). The larger the repeatability, the smaller ME is relative to FA.

5.2.4.2 Fluctuating, directional and antisymmetry

Departures from bilateral symmetry take three forms: fluctuating (FA), directional (DA) and antisymmetry (AS).

Fluctuating asymmetry has a pattern where the differences in variation between the right and left sides (R - L) are normally distributed about a mean of zero (mean = 0, normal). Directional asymmetry has a pattern of variation of (R - L) where variation is normally distributed about a mean that is significantly different from zero. Antisymmetry has a pattern of variation of (R - L) where the variation is distributed about a mean of zero, but the frequency distribution departs from normality in the direction of platykurtosis or bimodality (mean = 0, platykurtic or bimodal) (Palmer and Strobeck, 2003).

Both directional and antisymmetry will inflate fluctuating asymmetry estimates, complicating interpretation; therefore, it is essential to check for both of these alternate forms of asymmetry.

Directional asymmetry was tested for in two ways. Firstly, the same two way mixed ANOVA described in section 5.2.4.1 was used to obtain values for MSs (mean square of sides) and MSi (mean squares of side x individual). Directional asymmetry was then calculated (MSs/MSi) and the resulting value was used in an F test to determine if there were significant differences between the mean of the right and left sides relative to the mean within side variation. Secondly, a two-tailed one-sample t – test was used to test for departure of mean (R-L) from an expected mean of zero.

Evidence of antisymmetry was examined by simply plotting the frequency of observations of the subtraction of right minus left (R-L) on a scatter graph. Antisymmetry displays platykurtic or bimodal curves on R-L scatter plots. In addition, the Kolomogorov- Smirnow test was used to look for departure from normality (Palmer and Strobeck, 1986).

5.2.4.3 Size dependency

Variation of the magnitude of asymmetry of populations can exist due to the difference in size of a trait (Palmer, 1994). Therefore, asymmetry of each trait had to be tested for size dependency. This was achieved by carrying out a Spearman rank correlation between absolute FA (R-L) and the average of both sides (R+L/2). The Spearman rank correlation is suitable as it is a non- parametric test that does not assume homogeneity of variance and is not influenced by a few extreme observations (Palmer, 1994).

5.2.4.4 Fluctuating asymmetry (FA); single and composite traits

The traits used to examine fluctuating asymmetry were not significantly confounded by measurement error, showed no true evidence of directional or antisymmetry, and did not require corrections for size dependency.

Single traits

The FA1 index defined by Palmer and Strobeck (1986) was applied and calculated as the absolute mean difference in length between right and left sides (mean [R-L]). As each pair of measurements was repeated twice, averages of the two estimates were used. FA1 was calculated both for individuals within each of the populations and across all individuals.

Composite traits

It has been argued that analyses that combine fluctuating asymmetry estimates over many traits should be more reliable detectors than those relying on single traits. Leung et al., (2000) therefore proposed the use of 'composite' measures to detect fluctuating asymmetry more robustly.

Here, CFA-1 (Leung et al., 2000) (also referred to as index 11 in Palmer and Strobeck 2003) was calculated by summing the mean absolute values of FA in all traits. This was calculated again at the level of the individual within each of their populations, across all individuals and finally among populations.

5.2.4.5 Genetic diversity indices

As previously discussed, all roe deer were typed for up to sixteen highly variable microsatellite markers (see Chapter 3). Four genetic variables were calculated from microsatellite data.

The measures of genetic diversity used in this study were: multi-locus heterozygosity (H), standardised heterozygosity (H_S), mean d^2, standardised mean d^2, internal relatedness (IR) and heterozygosity weighted by locus (HL). All these measures were calculated using the excel macro IR macroN3 (Amos et al., 2001).

Multilocus heterozygosity (H) was calculated as the proportion of typed loci for which an individual was heterozygous. In addition to this, standardised heterozygosity was used. This measure ensures that all individuals are measured on an identical scale by avoiding potential bias that could be introduced by individuals being untyped at particular loci (Coltman et al.,

1999). This measure is calculated as the proportion of heterozygous typed loci divided by mean heterozygosity at loci typed:

$$H_S = \frac{H}{H_{li}}$$

Where l is locus at individual i.

Mean d^2 was also used. Mean d^2 is the squared difference in repeat units between two alleles at a locus averaged over all typed loci:

$$\text{Mean } d^2 = \sum_{i=1}^{n} \left[\frac{(i_a - i_b)^2}{n}\right]$$

Where i_a and i_b are the length in repeat units of alleles a and b at locus i and n is the number of typed loci.

Under the stepwise mutation model it is expected that d^2 is a linear function of the time since coalescence of the two alleles (Valdes et al., 1993; Goldstein et al., 1995); therefore, mean d^2 is expected to reflect the genetic distance between the two parental gametes. Thus, a positive correlation between fitness and mean d^2 would suggest that individuals with dissimilar parents have greater fitness.

One problem with mean d^2 is that some loci may contribute more than others to the measure. In order to overcome this, Coulson, et al. (1999) suggested the use of standardised mean d^2 which divides the d^2 values by the maximum observed value at that locus and averages the results across loci, as below:

$$\text{Standardised } d^2 = \sum_{i=1}^{n} \left[\frac{\left(\frac{(i_a - i_b)^2}{\sigma_i^2}\right)}{n}\right]$$

Internal relatedness (IR) was also used:

$$\mathrm{IR} = \left[\frac{(2\mathrm{H} - \sum \mathrm{f_i})}{(2\mathrm{N} - \sum \mathrm{f_i})}\right]$$

Where H is the number of loci that are homozygous, N is the number of loci and f_i is the frequency of the ith allele contained in the genotype.

Internal relatedness (IR) is currently the most widely used index and its main attribute is that allele frequency is incorporated into the measure. The problem with IR, however, is that it underestimates heterozygosity of individuals carrying rare alleles. It is for this reason that an alternative index, homozygosity by loci (hereafter HL) was proposed. Homozygosity by loci weights the contribution of each locus to the homozygosity index depending on its allelic variability (Aparicio et al., 2006):

$$\mathrm{HL} = \frac{\sum \mathrm{E_h}}{\sum \mathrm{E_h} + \sum \mathrm{E_J}}$$

Where $\mathrm{E_h}$ and $\mathrm{E_J}$ are the expected heterozygosities of the loci that an individual bears in homozygosis (***h***) and in heterozygosis (***j***) respectively.

Differences in genetic diversity indices among the different populations were tested using ANOVA. Relationships among the different measures of genetic diversity were investigated using Pearson correlations.

5.2.4.6 Correlations between FA and genetic diversity

Relationships between genetic diversity (based on 16 microsatellites) as measured by the genetic diversity indices and both individual (FA-1) and composite (CFA-1) measures of fluctuating asymmetry were investigated within, across and among all populations. For all correlations it was expected that levels of FA would be positively correlated with heterozygosity, standardised heterozygosity and standardised d^2 and positively correlated with IR and HL. Pearson correlations were performed to examine most relationships; however, when outliers were detected a Spearman’s rank correlation was selectively used.

5.3 Results

5.3.1 Measurement error

The average value for the standard deviation of repeated measurements, ME2, was 0.59 (range 0.3 -0.9). The average value for the difference between repeat measurements as a percentage of difference between sides, ME3, was 24% (range 5% - 46%). The average value for the measure of repeatability (ME5) which expresses variation due to asymmetry as a proportion of total between-side variation was 0.62 (range 0.44 - 0.9).

The results of the two-way ANOVA were used to test whether between sides variation was significantly greater than variation due to measurement error. Every trait was tested and, in most cases, the result was highly significant ($P<0.001$) which suggests asymmetries were highly repeatable. However, traits Um3a-3b and Um3b-M did not show significant results ($P = 0.55$ and $P = 0.18$ respectively).

5.3.2 Directional asymmetry (DA), antisymmetry (AS) and size dependence

Table 5.3 shows the results from both the *t* test and ANOVA which were carried out to test for directional asymmetry. A number of the measurements were shown to contain significant amounts of directional asymmetry from both *t* tests and two way ANOVAs; after sequential Bonferroni correction was applied: Um3a-3b, Um3b-M, J-M, Lm3-Lpm1 and N-Rh were shown to be affected.

The Kolmogorov-Smirnov test for departures from normality was used as an indication of antisymmetry. The results from the K-S test showed that traits Um3b-Um1, Um3a-3b and Lm3-Lpm1 deviated significantly from normality after Bonferroni adjustment (see Table 5.3). Scatter plots of observation frequency versus right minus left in these same traits also revealed evidence of platykurtosis or kurtosis, further supporting that these traits were being influenced by antisymmetry.

None of the traits showed size dependence of fluctuating asymmetry using the Spearman bivariate rank correlation. This means that the different sides (R-L) did not depend on trait size (R+L/2) (see Table 5.3).

5.3.3 Traits used to measure fluctuating asymmetry (FA)

Traits that showed evidence of being influenced by measurement error (Um3a-3b and Um3b-M), directional asymmetry (Um3a-3b, Um3b-M, J-M, Lm3-Lpm1, N-Rh) or antisymmetry (Um3b-Um1, Um3a-3b and Lm3-Lpm1) were excluded from further analyses. The remaining 10 traits used for further analyses of FA were: Um3b-Upm1, J-Upm1, J-M, Ni-P, Vom-Po, St-Po, Ent-P, Ot-Br, Gov-Cr, Lpm1-Pa, Goc-Mt.

Table 5.3. Results from statistical analyses used to detect directional asymmetry, antisymmetry and size dependence in each trait.

Effect tested	Statistic	Trait							
		Um3b-M	Um3b-Upm1	Um3b-Um1	Um3a-3b	J-Upm1	J-M	Ni-P	N-Rh
	n	117	133	132	131	131	116	90	109
Directional asymmetry	Mean	0.025	-0.009	0.004	-0.011	-0.013	-0.034	-0.009	-0.031
	T test (t)	2.72	-1.65	0.92	-3.85	-1.5	-3.29	-1.07	-2.07
	Sig. 2 tailed	**	ns	ns	***	ns	**	ns	ns
	ANOVA F	7.04	0.06	0.15	16.2	0.87	11.88	0.57	4.82
	P value	**	ns	ns	***	ns	***	ns	ns
Antisymmetry	K-S test	0.71	1.03	2.07	2.38	0.95	0.77	1.26	0.68
	Sig.	ns	ns	***	***	ns	ns	ns	ns
Size dependence	Spearman's rho	ns	ns	ns	ns	ns	ns	ns	ns

Effect tested	Statistic	Trait							
		Vom-Po	St-Po	Ent-P	Ot-Br	Gov-Cr	Lm3-Lpm1	Lpm1-Pa	Goc-Mt
	n	117	124	82	131	128	129	129	130
Directional asymmetry	Mean	-0.021	-0.026	0.014	-0.016	-0.009	0.034	-0.022	-0.017
	T test (t)	-1.26	-1.44	1.48	-2.08	-0.82	4.11	-2.22	-1.54
	Sig. 2 tailed	ns	ns	ns	ns	ns	***	ns	ns
	ANOVA F	2.47	2.16	1.39	4.5	1.1	18.79	4.92	1.88
	P value	ns	ns	ns	ns	ns	***	ns	0.017
Antisymmetry	K-S test	1.04	1.1	1.29	1.49	1.44	1.65	1.12	0.99
	Sig.	ns	ns	ns	ns	ns	**	ns	ns
Size dependence	Spearman's rho	ns	ns	ns	ns	ns	ns	ns	ns

5.3.4 Fluctuating asymmetry (FA) among populations

Basic statistics showing levels of FA in each trait (FA-1) and across all traits averaged (CFA-1) for each of the populations are shown below in Table 5.4. This table shows that the amount of FA varied across single traits. Highest levels of FA are apparent in the traits St- Po and Vom-Po, whilst lowest levels were found in the trait Um3b-Upm1. Additionally, this table shows that the amount of FA varies among populations.

Based on single traits (FA-1), highest levels of FA were found in the Dorset population with three out of the ten traits showing the highest values. The Norfolk population exhibited the second highest levels of FA with two out of ten traits showing highest values. The Lancashire population showed the third highest levels whist the Perth, Moray and Durham populations all showed comparable low levels. Similar patterns were found when all traits were averaged (CFA-1) for each population.

Table 5.4. Basic statistics of fluctuating asymmetry represented by single FA-1 for each trait in each of the six designated populations.

	POPULATION											
	Norfolk		**Dorset**		**Durham/Carlisle**		**Perth**		**Moray**		**Lancashire**	
Trait	**Mean FA-1**	**± s.d.**	**Mean FA-1**	**± s.d.**	**Mean FA-1**	**± s.d.**	**Mean FA-1**	**± s.d.**	**Mean FA-1**	**± s.d.**	**Mean FA-1**	**± s.d.**
Um3b-Upm1	0.061	0.054	0.051	0.040	0.049	0.046	0.047	0.036	0.045	0.035	0.019	0.016
J-Upm1	0.067	0.059	0.093	0.065	0.076	0.049	0.079	0.062	0.072	0.047	0.111	0.068
Ni-P	0.044	0.043	0.078	0.055	0.049	0.036	0.065	0.070	0.053	0.047	0.092	0.046
Vom-Po	0.125	0.082	0.147	0.161	0.111	0.098	0.129	0.139	0.099	0.093	0.130	0.147
St-Po	0.133	0.093	0.174	0.163	0.122	0.103	0.173	0.169	0.144	0.130	0.123	0.126
Ent-p	0.064	0.053	0.070	0.059	0.046	0.073	0.059	0.041	0.040	0.041	0.063	0.119
Ot-Br	0.064	0.058	0.073	0.051	0.049	0.053	0.084	0.090	0.037	0.038	0.083	0.049
Gov-Cr	0.118	0.172	0.064	0.059	0.080	0.071	0.048	0.044	0.082	0.054	0.048	0.027
Lpm1-Pa	0.065	0.063	0.095	0.087	0.077	0.070	0.051	0.050	0.120	0.116	0.072	0.078
Goc-Mt	0.089	0.119	0.089	0.063	0.106	0.089	0.092	0.087	0.080	0.058	0.085	0.080
CFA-1	0.083	0.035	0.093	0.039	0.077	0.029	0.076	0.022	0.077	0.652	0.083	0.034

5.3.5 Genetic diversity among populations

Tables 5.5 and 5.7 show the results of the different indices of genetic diversity for the six separate populations studied. Table 5.5 only presents this data for individuals for which cranial measurements are available, whereas Table 5.7 presents data based on all individuals genotyped in each population. Both tables show that the Norfolk population exhibited the lowest levels of heterozygosity and highest levels of inbreeding whereas the Scottish, Perth and Moray populations, showed the highest levels of heterozygosity and lowest levels of inbreeding. The differences in genetic diversity across populations were shown to be significant (Table 5.6 and 5.8).

Table 5.9 shows the Pearson correlations for the indices of genetic diversity. As expected, strong positive correlations existed between both standardised heterozygosity and heterozygosity. Standardised d^2 also shows a significant positive correlation to both of these measures; however, the correlation is not as strong (see Table 5.9). Heterozygosity by locus and internal relatedness show a significantly positive strong correlation. Both of these measures also show significantly strong negative correlations with both standardised heterozygosity and heterozygosity. Relationships with mean d^2 are again significant but not as strong as for other associations. Similar degrees of correlation between these genetic indices have been found in other studies based on other species (e.g. Amos et al., 2001). This demonstrates that genetic diversity indices are not independent of one another.

Table 5.5. Basic statistics of mean genetic diversity indices for individuals within each of the six populations from which skulls were sampled.

Population	Samples	Genetic diversity indices				
	N	Heterozygosity	Standardised heterozygosity	Standardised d^2	IR	HL
Norfolk	23	0.482 ± 0.126	0.812 ± 0.212	0.080 ± 0.040	0.365 ± 0.161	0.513 ± 0.118
Dorset	31	0.546 ± 0.140	0.921 ± 0.236	0.128 ± 0.051	0.277 ± 0.182	0.453 ± 0.137
Carlisle and Durham	28	0.618 ± 0.136	1.042 ± 0.230	0.127 ± 0.039	0.151 ± 0.188	0.337 ± 0.134
Lancashire	10	0.594 ± 0.090	1.001 ± 0.151	0.115 ± 0.054	0.214 ± 0.112	0.391 ± 0.088
Perth	15	0.654 ± 0.164	1.102 ± 0.274	0.163 ± 0.052	0.140 ± 0.210	0.340 ± 0.162
Moray	15	0.652 ± 0.128	1.098 ± 0.216	0.165 ± 0.052	0.142 ± 0.161	0.338 ± 0.124

Table 5.6. Results from ANOVA testing for differences in genetic diversity levels for individuals from which skulls were obtained among the six populations.

	Genetic diversity indices				
	Heterozygosity	Standardised heterozygosity	Standardised d^2	IR	HL
F (5, 114)	4.476	4.456	6.342	5.285	5.512
Significance	0.001	0.001	<0.001	<0.001	<0.001

Table 5.7. Basic statistics of genetic diversity indices for each of the six populations including all individuals sampled from populations (irrespective of whether a skull was collected).

Population	**Samples**	**Genetic diversity indices**				
	N	**Heterozygosity**	**Standardised heterozygosity**	**Standardised d^2**	**IR**	**HL**
Norfolk	45	0.485 ± 0.143	0.797 ± 0.234	0.071 ± 0.033	0.351 ± 0.185	0.510 ± 0.141
Dorset	47	0.588 ± 0.136	0.968 ± 0.224	0.128 ± 0.054	0.230 ± 0.175	0.407 ± 0.135
Carlisle and Durham	49	0.641 ± 0.125	1.052 ± 0.205	0.123 ± 0.046	0.119 ± 0.171	0.352 ± 0.124
Lancashire	17	0.620 ± 0.102	1.018 ± 0.166	0.094 ± 0.046	0.175 ± 0.127	0.364 ± 0.099
Perth	32	0.684 ± 0.134	1.121 ± 0.218	0.148 ± 0.053	0.102 ± 0.170	0.306 ± 0.132
Moray	30	0.649 ± 0.124	1.062 ± 0.203	0.137 ± 0.053	0.148 ± 0.159	0.343 ± 0.122

Table 5.8. Results from ANOVA testing for differences in genetic diversity levels among the five populations for all individuals from populations (irrespective of whether a skull was collected).

	Genetic diversity indices				
	Heterozygosity	**Standardised heterozygosity**	**Standardised d^2**	**IR**	**HL**
F (5, 238)	11.84	11.792	13.66	12.65	12.79
Significance	<0.001	<0.001	<0.001	<0.001	<0.001

Table 5.9. Relationships between measures of genetic diversity.

	Heterozygosity		Standardised heterozygosity		Standardised d^2		IR	
	Pearson's	P.	Pearson's	P.	Pearson's	P.	Pearson's	Sig.
Standardised heterozygosity	1	<0.001						
Standardised d2	0.661	<0.001	0.649	<0.001				
IR	-0.985	<0.001	-0.985	<0.001	-0.63	<0.001		
HL	-0.991	<0.001	-0.988	<0.001	-0.639	<0.001	0.983	<0.001

5.3.6 Correlations between FA and genetic diversity

5.3.6.1 Individual traits (FA-1) and genetic diversity: individuals within and across all populations

Most single traits (FA-1), except Vom-Po in the Norfolk population (Table 5.10), did not show significant correlations between genetic diversity and FA when considered at either within or across all population levels. However, nearly all traits (with the exception of Um3b-Upm1 and Ent-P) showed relationships existing in the expected directions.

5.3.6.2 Composite measures (CFA-1) and genetic diversity: individuals within and across all populations

Using average FA calculated across all traits (CFA-1), further relationships with genetic diversity were examined. When individuals were considered within populations there was no evidence of significant associations with genetic diversity (Table 5.10). The Norfolk population was, however, approaching a level of significance in all indices (apart from standardised d^2). All other populations showed relationships in expected directions.

When all populations were collated (individuals across populations); the correlation between CFA-1 and genetic diversity indices (except once again for standardised d^2) became significant at the 0.05 level, but not after Bonferroni correction (Table 5.10, Figure 2).

Three outlying individuals made relationships between genetic diversity indices and fluctuating asymmetry significant (Figure 5.3). One of these individuals came from the Norfolk population and the other two from the Dorset population. When these three individuals were removed from the analyses significant relationships could no longer be detected (Table 5.10; see Figure 5.4 for graphic representation); however, the trends remained in the same directions.

That significant associations, in general, were observed only when all individuals across populations were grouped together suggests that statistical power is low (a result of small sample sizes). However, it is also possible that the collected population result is only detecting differences among populations. Therefore, the most distinct population (Norfolk) with respect to levels of diversity and differentiation from other populations (see Chapter 2) was omitted and the correlations tested again (both with and without the outlier samples; Table 5.10; Appendix 5 and 6). The results of this test and the tight clustering of most data points suggest that power is the main factor.

Table 5.10. Pearson correlations between asymmetry and genetic variability measured as heterozygosity, standardised heterozygosity, standardised d^2, IR (internal relatedness) and HL (heterozygosity by locus) across individuals within populations and across all individuals in all populations for the trait Vom-Po and for average fluctuating asymmetry (CFA-1) where * significance at the 0.05 level and ~ denotes all populations excluding 3 outliers.

		GENETIC DIVERSITY INDICES									
		Heterozygosity		**Standardised heterozygosity**		**Standardised d^2**		**IR**		**HL**	
Trait	**Population**	Pearson's	Sig.	Pearson's	Sig.	Pearson's	Sig.	Pearson's	Sig.	Pearson's	Sig.
Vom-po	Norfolk	-0.518	0.019*	-0.519	0.019*	-0.204	0.389	0.515	0.020*	0.520	0.019*
	Dorset	-0.045	0.819	-0.040	0.841	-0.051	0.802	0.033	0.871	0.027	0.893
	Durham/Carlisle	-0.279	0.176	-0.281	0.174	-0.156	0.457	0.270	0.191	0.277	0.181
	Lancashire	-0.421	0.225	-0.421	0.225	-0.162	0.655	0.422	0.224	0.424	0.222
	Perth	-0.032	0.918	-0.043	0.889	0.330	0.270	0.059	0.848	0.020	0.950
	Moray	-0.262	0.411	-0.257	0.419	-0.358	0.253	0.212	0.508	0.243	0.447
	All populations	-0.186	0.052	-0.188	0.053	-0.075	0.444	0.184	0.057	0.178	0.066
CFA-1	Norfolk	-0.377	0.095	-0.353	0.098	-0.209	0.337	0.347	0.105	0.344	0.108
	Dorset	-0.790	0.678	-0.069	0.720	-0.088	0.650	0.071	0.712	0.058	0.766
	Durham/Carlisle	-0.228	0.243	-0.229	0.241	-0.209	0.337	0.198	0.313	0.216	0.270
	Lancashire	-0.268	0.455	-0.268	0.455	-0.217	0.547	0.249	0.487	0.267	0.455
	Perth	-0.197	0.500	-0.202	0.488	0.075	0.798	0.256	0.378	0.187	0.523
	Moray	-0.221	0.428	-0.217	0.436	0.095	0.737	0.216	0.440	0.230	0.410
	All populations	-0.230	0.011*	-0.225	0.011*	-0.093	0.312	0.226	0.013*	0.225	0.012*
	~ All populations ~	-0.148	0.112	-0.147	0.116	0.021	0.823	0.155	0.096	0.142	0.129
	All populations (excl. Norfolk)	-0.216	0.033*	-0.216	0.035*	-0.086	0.405	0.222	0.030*	0.214	0.036*
	~ All populations (excl. Norfolk) ~	-0.166	0.109	-0.165	0.113	-0.014	0.892	0.177	0.088	0.158	0.128

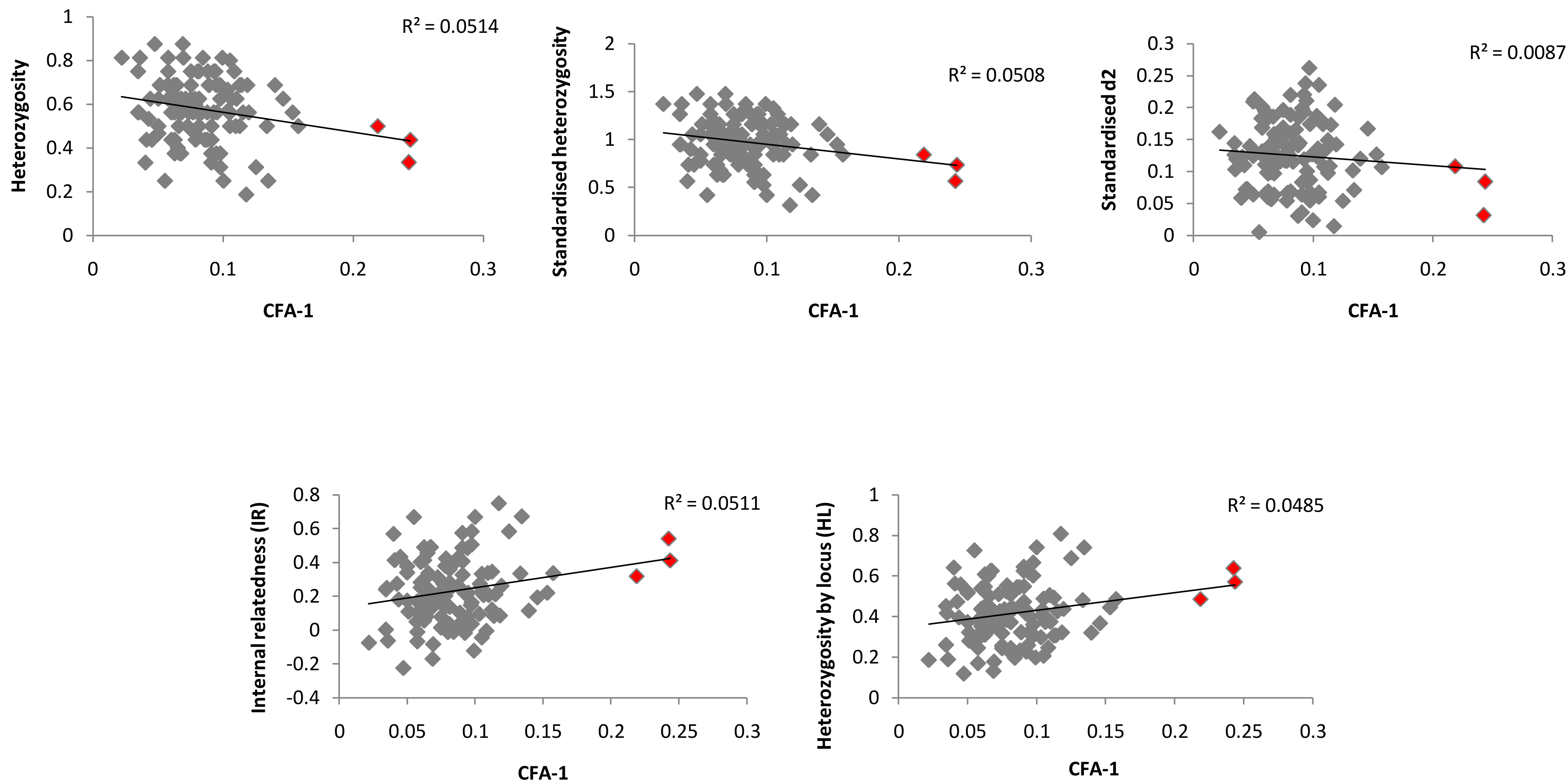


Figure 5.3. Significant correlations between CFA-1 and genetic diversity for all individuals from all UK populations. Three outlying individuals are marked in red.

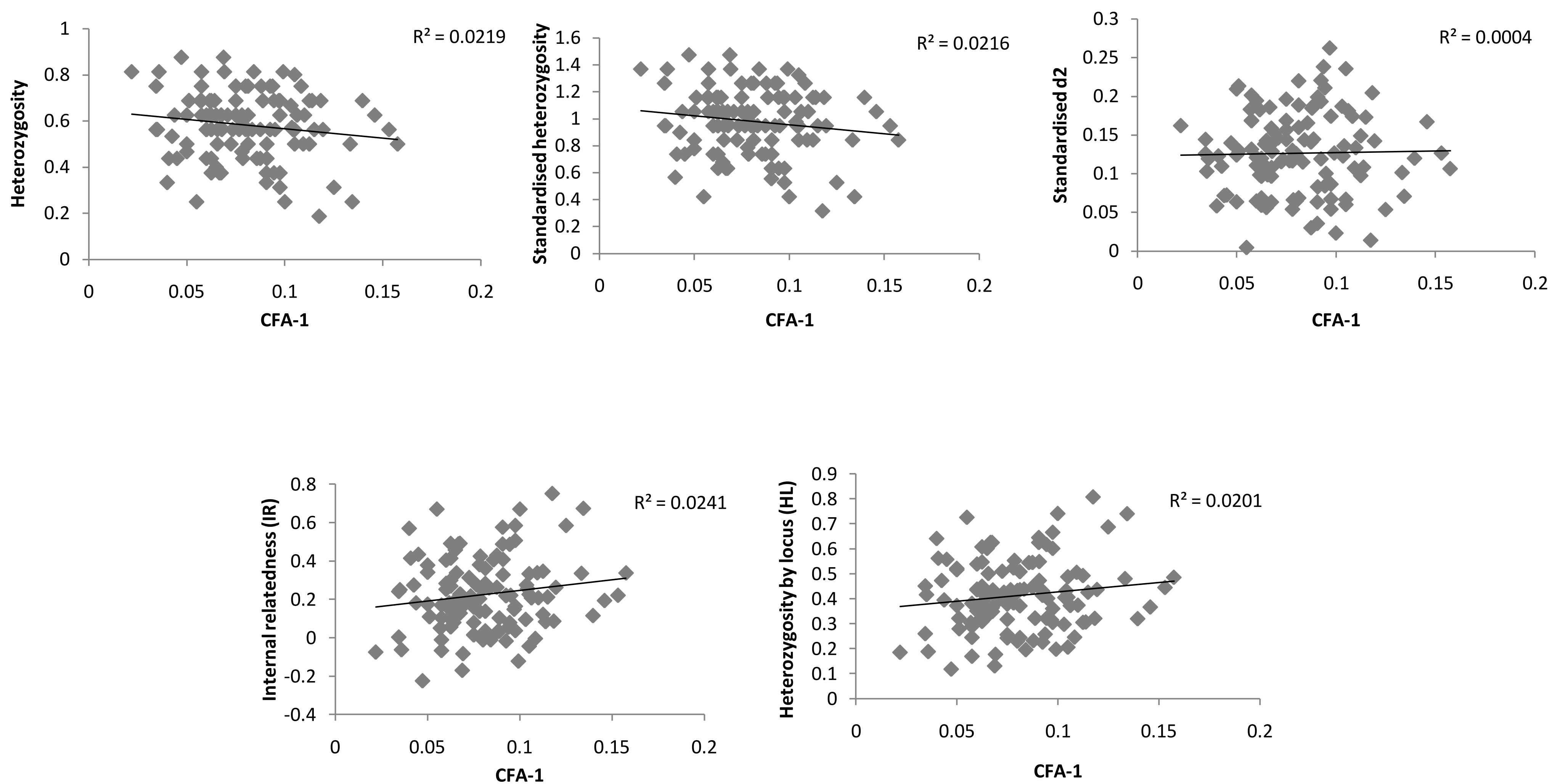


Figure 5.4. Correlations between CFA-1 and genetic diversity indices for all individuals (except the three outlying individuals shown in Figure 5.3) from all UK populations.

5.3.6.3 Composite measures (CFA-1) and genetic diversity: among populations

Correlations between population mean values of CFA-1 (from Table 5.4) and population mean values of the indices for genetic diversity (from table 5.5) were examined. Table 5.11 shows the direction and relative strength of the trends. Although correlations existed in expected directions, these were not statistically significant (due to Norfolk being an outlier; Table 5.11 and Figure 5.5). Furthermore, there was no evidence that CFA-1 differed significantly among populations (ANOVA: $F_{5,114} = 1.562$, $P > 0.05$).

Table 5.11. Pearson correlations between CFA-1 and genetic diversity indices among populations.

		Population CFA-1	**Population CFA-1 (excl. Norfolk)**
H	Pearson	-0.618	-0.923
	P	0.191	0.025*
Standardised H	Pearson	-0.615	-0.932
	P	0.193	0.021*
Standardised d²	Pearson	-0.364	-0.478
	P	0.479	0.415
IR	Pearson	0.628	0.968
	P	0.182	0.007*
HL	Pearson	0.633	0.942
	P	0.177	0.017*

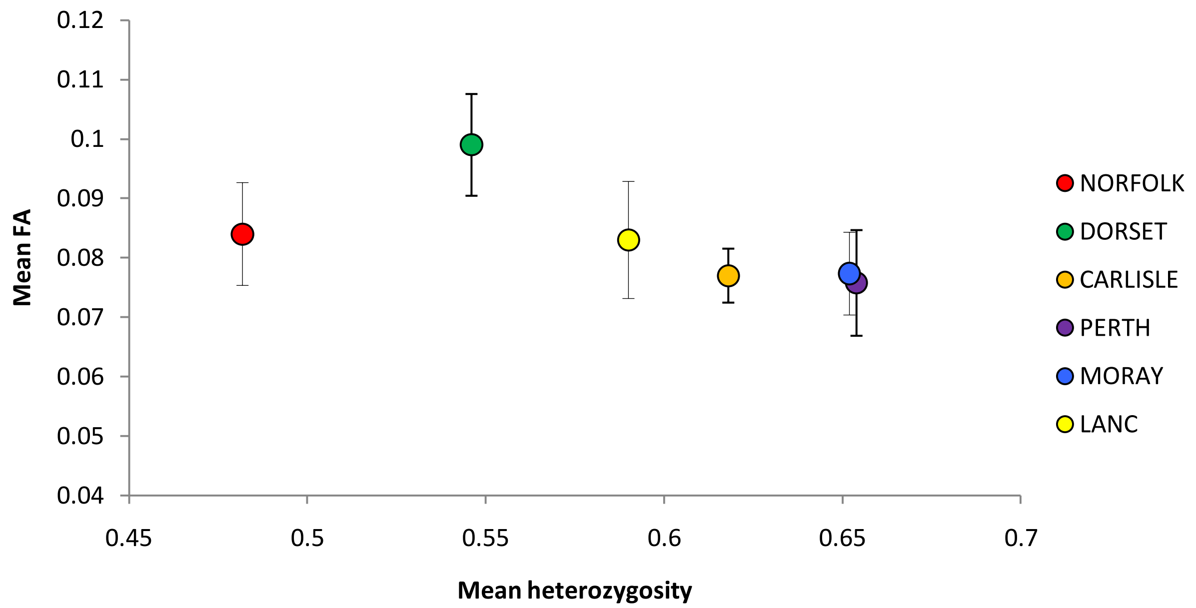


Figure 5.5. Relationship between genetic diversity (measured as heterozygosity) and fluctuating asymmetry in six populations of roe deer. Error bars represent standard error.

5.4 Discussion

5.4.1 Fluctuating asymmetry (FA)

5.4.1.1 Single trait FA

The levels of asymmetry, as measured by FA1 (absolute right minus left), varied across traits and populations. The trait that showed the least amount of FA was Um3b-Upm1 (average across all populations = 0.045). This trait might show low FA because it is of functional importance. It has been proposed by various authors (e.g. Palmer and Strobeck, 1986; Stearns, 1992) that functionally important traits are subject to stronger stabilising selection. In the case of bilaterally symmetric traits, this can result in lower levels of asymmetry. This has been supported by several studies; for example, Karnoven et al., (2003) found that levels of FA in greenfinches differed depending on the character chosen in analysis; specifically, levels were lowest for functionally important traits and highest for less functionally important traits. Here, the trait Um3b-Upm1 is a measurement of the lower row of teeth. Traits associated with teeth may be expected to be under stronger selection compared to other traits, given their high importance for feeding. Conversely, the traits that exhibited highest amounts of fluctuating asymmetry (St-Po and Vom-Po) may be less functionally important. These results support previous studies which have suggested that some characters or traits may be better predictors of fluctuating asymmetry than others (Suchentrunk, 1993; Palmer and Strobeck, 1997) and that organism-wide asymmetry (the tendency that an individual which is more asymmetrical for one trait is more asymmetrical for other traits too) (Dufour and Weatherhead, 1996) seldom exists. Thus, the indicator ability of fluctuating asymmetry may well depend on choosing the 'right' (i.e. most sensitive) trait (Palmer, 1994).

5.4.1.2 FA levels among populations

In relation to population FA-1 and CFA-1 highest values were found in the Dorset population whilst lowest values were found in the Perth, Moray and Durham/Carlisle populations. This pattern was *generally* consistent with the prediction that populations impacted by recent and strong bottlenecks (Southern populations; Dorset and Norfolk) would exhibit higher FA relative to populations that had been less impacted by strong bottlenecks (Northern populations; Perth and Moray) (see Chapters 3 and 4). However, interpretations are

somewhat confounded by the finding that FA did not significantly differ between populations. Therefore, it is difficult to make any conclusions based on this result.

5.4.3 Correlations between FA and genetic diversity

5.4.3.1 Performance of genetic diversity indices

This study used five different measures to examine relationships between genetic diversity and FA. Mean d^2 was outperformed by all other genetic diversity indices, as it did not show any correlation with fluctuating asymmetry at any level of the analyses. Although mean d^2 has been shown to be positively associated with fitness-related traits in a number of other wild populations (Hansson et al., 2001; Rossiter et al., 2001), results here indicated it was a less effective measure.

There has been considerable criticism of the efficacy of mean d^2 in comparison to other indices (e.g. Coltman et al., 1998; Slate et al., 2000; Tsitrone et al., 2001; Slate and Pemberton, 2002). Possible reasons for reduced efficacy of mean d^2 compared to other indices could be that this measure is most suited to detecting events deep in an individual's ancestry (for example, population admixture; (Coulson et al., 1998; Pemberton, 1999). Hedrick et al., (2001) found that only when a population arose by admixture of two large, divergent subpopulations was mean d^2 more highly correlated with fitness than other genetic diversity measures. Under virtually all other conditions, other measures of genetic diversity outperformed mean d^2 (Hedrick et al., 2001). Results from Chapter 3 suggest that there is little evidence of roe admixture across populations and, therefore, mean d^2 may be less suited to this study.

5.4.3.2 FA-1 and genetic diversity

When measures of FA-1 and genetic diversity were measured over single traits only one significant correlation was detected (for the trait Vom-Po within the Norfolk population; see Table 5.10). In a previous study on FA in a cervid species (reindeer, *Rangifer tarandus*), FA was also high for this trait (Lovatt, 2007) suggesting a potential use in future cervid FA studies.

Consistent with earlier studies, these results show that single trait asymmetry only weakly reflects the underlying instability of development. One problem is stochastic error associated with sampling effects (Whitlock, 1996). This is especially a problem in this study due to small sample sizes, and therefore the focus here will be on the composite measure (CFA-1), argued by some to be a more reliable indicator of developmental instability or stress (Leung et al., 2000).

5.4.3.3 CFA-1 and genetic diversity: individuals within and across all populations

None of the populations showed significant associations between CFA-1 and genetic diversity for individuals within populations. However, all associations occurred in expected directions. Furthermore, for the Norfolk population correlations were approaching significance at the 0.05 level. This may suggest that the trends are real, but that power was insufficient to provide a statistical assessment. Slate and Pemberton (2002) argued that correlations between microsatellite heterozygosity and fitness-related traits are seldom observed because at least 20 loci in samples of more than 100 individuals are needed to attain sufficient statistical power to detect them. Therefore, it is perhaps not surprising that we found no correlation between heterozygosity and FA at the individual level.

Nevertheless, this study was still able to detect significant relationships (prior to Bonferroni correction) between genetic diversity indices (except for mean d^2) and CFA-1 for individuals across all populations. This probably reflected either an increased power associated with the increased sample size, a relationship among populations that is distinct from any relationship within populations, or a combination of the two. The relatively tight cluster of individual values (not clustered separately by population), and the retention of the pattern when the most differentiated population was removed (appendix 5 & 6), suggests that power is more important in this case than intrinsic differences among populations.

Interestingly, the result obtained for the Pearson correlation between heterozygosity and CFA-1 across individuals (Pearson = -0.230, P= 0.011) was strikingly similar to a result (Pearson = -0.236, P =0.008) based on the same analyses reported in another study using 105 individuals from five German roe deer populations genotyped at 8 loci (Zachos et al., 2007). Furthermore, consistent with the study here, Zachos et al., (2007) failed to find any relationship between CFA-1 and mean d^2. The striking similarity of my results with those of

Zachos et al., (2007) could arise because many of the same traits were used or because heterozygosity and mean d^2 calculations were both calculated from nuclear DNA (in some instances using the same microsatellite loci). Alternatively, it may be due to the similarly low sample sizes used in both studies. Finally, it may be that in both cases levels of heterozygosity were not low enough to detect strong correlations. It was notable that levels of expected heterozygosity (He) in the five German populations studied (range; He 0.55-0.64) were very similar to those stated in my study (range; He 0.59-0.72). Previous studies have suggested that when the mean and variance in inbreeding are low in a population, heterozygosity-fitness correlations may be very weak or undetectable (Overall, 2005).

5.4.3.4 Mean CFA-1 and genetic diversity: among populations

For the final part of the analyses the relationship between mean CFA-1 and genetic diversity among populations was examined. For five of the six populations there was a tendency for FA to increase as genetic diversity decreased (Figure 5.5, Table 5.11). The sixth population (Norfolk), however, did not follow the same trend. Various factors intrinsic to a particular population may explain a lack of linearity in this relationship, and this has been seen in other studies as well (e.g. Zachos et al., 2007 for European roe deer and Hartl et al., 1995 for brown hare, *Lepus europaeus*). In a study of common shrew (*Sorex araneus*) (White and Searle, 2008) there was an apparently consistent trend, but the relationship was driven by one small and very inbred population with high levels of FA. When that population was removed from the analysis the correlation was lost.

One problem with examining CFA-1 among populations in this study was that there were only 6 populations examined (thus 5 degrees of freedom). With so few data points, it is perhaps unsurprising that significance was not detected.

5.5 Conclusion

Results from this study were inconclusive in determining whether individual past population histories have impacted levels of fitness (as measured through fluctuating asymmetry). Furthermore, analyses did not reveal any straightforward link between genetic diversity and fluctuating asymmetry. When individuals were assessed within each of their respective populations, significant correlations were rarely found, probably owing to limited sample

sizes. Undoubtedly, the collection of further skulls would allow better statistical power in the comparison of indirect measures of fitness and measures of genetic diversity at this level. This should, therefore, be carefully considered in future studies. In fact, the increased sample size when all individuals were examined across all populations was likely to have produced the significant result detected at this level. Overall, these results support the weakly significant negative associations found between measures of heterozygosity and fluctuating asymmetry in previous meta analyses (Britten, 1996; Vollestad et al., 1999).

Chapter 6: General Conclusions

6.1 Overview:

6.1.1 Phylogeography and population expansions

In line with my initial hypothesis, the UK roe were shown to be part of the mt-DNA Central lineage; which has previously been found to be the most widely spread across Northern Europe (Randi et al., 2004; Lorenzini and Lovari, 2006). This result provided insight into the possible re-colonisation route taken by roe into the UK which is likely to have occurred from an eastern refuge, although the exact location remains unknown (see Randi et al., 2004; Royo et al., 2007). It is less clear to what extent roe deer represented by this lineage had been forced into a refuge during the last glacial maximum (LGM). Studies of mammals, reptiles and amphibians have identified divergent lineages in the Carpathians, many of which predate the LGM, providing evidence that a major north eastern refuge may have existed (Provan and Bennett, 2008). The fossil record indicates that roe deer were one species existing around this region at the time (Sommer and Zachos, 2009). Proving whether the Carpathians constituted a possible refuge for the Central lineage of roe could follow an approach used in a previous study based on the bank vole (*Clethrionomys glareolus*) (Kotlik et al., 2006). Kotlik et al. (2006) collected mt-DNA sequences of bank voles from both the Carpathians and adjacent regions and used an IM analysis approach, with a range of substitution rates, to date the population splitting times. The results showed that the splitting time of the Carpathian population most likely occurred 22,000 years ago during the height of the last glacial maximum thus supporting the existence of a population in the region. Furthermore, following the end of the glaciation, the IM analysis indicated that gene flow occurred out of the Carpathians demonstrating a clear contribution to European re-colonisation (Kotlik et al., 2006). This study provided the first direct evidence of Carpathian refugia for a species. If this area also acted as a refuge for other species, such as the roe deer, our view on the role of northern refugia in the post-glacial recolonisation process would change (Kotlik et al., 2006).

The roe deer substitution rate calculated by direct calibration using ancient DNA (Chapter 2) provided a plausible estimate of a population split between UK and European populations which occurred not long after the land bridge split. In addition, this substitution rate was used to assess expansion events of European roe populations. European populations were shown to have undergone expansion events which occurred since the end of the last glacial maximum

and were interpreted to reflect an improvement in environmental conditions. In some instances, these results were corroborated by the fossil record (see Sommer et al., 2009; Sommer and Zachos, 2009). Overall, the expansion events calculated, using the substitution rate in this study, occurred over a much more recent time frame than previously put forward; thus adjusting our view on how the past shaped populations (see Randi et al., 2004; Lorenzini and Lovari, 2006). This was because the substitution rate obtained was much faster than previously published 'phylogenetic rates'. However, the substitution rate and, therefore, inference about the timing of these events are highly plausible, given that the rate was uniquely validated by the accurate dating of the known split between Perthshire and Dorset roe deer (Chapter 2).

6.1.2 Population structure in the British Isles

Northern populations in England and in Scotland showed higher levels of genetic diversity than populations in southern England. Patterns of isolation by distance (IBD) were also detected which indicate that since medieval size reductions occurred (see Chapter 4) there has been a relationship between gene flow and drift. Therefore, the overall pattern suggested restricted gene flow over long distances, consistent with this species dispersal capability (Danilkin, 1996).

Future studies could examine the dispersal behaviour of northern UK roe to address questions related to dispersal distances and whether dispersal is male biased as was suggested from some of the results reported here (see Chapter 3). Knowledge of such behaviour can be important in the development of management strategies to achieve sporting or population management objectives (Shaw et al., 2006; Perez-Espona et al., 2010). Other future work could investigate the extent to which certain landscape features impact gene flow. Previous studies have shown that movement of roe can be influenced by fragmented woodlands (Coulon et al., 2004) highways, rivers and canals (Coulon et al., 2006). In order to carry out such work, denser sampling across areas of interest would be required.

In southern populations, both reduced levels of genetic diversity (relative to the northern populations) and, to some extent non-significant IBD (Chapter 3), was consistent with the inference that all populations had been impacted by introductions involving small numbers of individuals involving both native and non-native stock (Chapter 4).

In reference to the southern population of Dorset, Berks, Somerset, Wiltshire, the re-introduction record (Chapter 2; Whitehead 1964) suggests that these roe are native stock translocated from Scotland. The shared haplotypes which were common to both to the proposed source (Perth; Scotland) and introduced populations (Chapter 2 & 4) may provide some support for this. Furthermore, IM analysis accurately dated a recorded introduction event involving the movement of Perthshire deer into at least one location; Milton Abbas, Dorset (Chapter 2). Assuming that all southern roe were translocated from Scotland then the amount of genetic and morphological differentiation that has subsequently occurred between populations is substantial (Chapter 3). Consistent with this study, it has previously been reported that over comparable timescales substantial differentiation has occurred between introduced and source populations of reindeer (Lovatt, 2007), elephant (Whitehouse and Harley, 2001) moose (Broders et al., 1999) and koala (Houlden et al., 1996). This study, along with others, may therefore demonstrate the powerful effect of genetic drift in causing divergence following re-introduction. However, one problem with assuming a single founder event was that genetic diversity in this southern population was still high. Although considerable diversity can be retained after founder events when recovery is rapid (e.g. Lovatt, 2007), as discussed in Chapter 2, the possibility of a more complex introduction history should be further investigated.

For the other southern populations examined in this study (Norfolk) all evidence suggested that it was of non-native origin. This population was shown to be both genetically and morphologically distinct (Chapter 3). Furthermore, the unique haplotype found in this population was not detected in any of the historical samples, suggesting it had been introduced (Chapter 4). Indeed, the re-introduction record had suggested this population descended solely from the introduction of a small founder group from Germany in 1884. Future studies could aim to sample DNA from the original German roe (Württemberg) population from which those in Norfolk are believed to have descended. This would further confirm the origins of these deer whilst enabling an additional evaluation to be made on the role of genetic drift in founder populations. Overall, it seems that the southern populations studied may be made up of both native and non-native re-introduced populations.

6.1.3 The impacts of bottlenecks events

British roe deer populations are understood to have been impacted by past bottlenecks which occurred as a result of both overhunting (during the medieval period) and founder events induced by natural or non-natural dispersal (during the re-establishment of several populations). Evidence for past bottlenecks was, therefore, investigated by examining both bottleneck signatures and ancient DNA.

Bottleneck signature methods were considered to have revealed strong evidence of recent size reductions when populations showed heterozygosity excess under a TPM model of evolution, and low Garza's M ratio values. The southern population of Norfolk showed the strongest evidence of bottlenecking which was consistent with a recent history of introduction involving a small founder population size (as discussed in Chapter 3). It was expected that the other recently founded southern population (located in the south west) may have exhibited similarly strong evidence of bottlenecking; however, this was not the case. Possible explanations may have been related to the admixture between different founder groups or too long a time frame having passed since founding for detection. Although all populations showed *some* evidence of past bottlenecking; signals generally appeared weak, which in common with a number of other recent studies (e.g. Whitehouse and Harley, 2001; Harley et al., 2005), highlights the limitations of employing bottleneck signatures.

The use of ancient DNA, as an alternative method for detecting bottlenecks, was shown to be particularly useful in this study. Direct comparison of DNA from before (up to 5,000 ybp) and after (modern) the medieval period showed that considerable genetic diversity had been lost over that time frame. The results of this part of the study was consistent with historical evidence that suggested over-hunting and deforestation led to severe population size reductions for roe deer during the medieval period (Whitehead, 1964), which could have caused the observed losses in diversity. However, given the time frame over which samples were collected, it is also possible that declines occurring before the medieval period could also have contributed to losses in diversity. For example, declines may have occurred between Roman and Norman periods when both the human population and agriculture were expanding and areas of woodland (i.e. preferred deer roe habitat; Putman and Langbein 2003) were contracting (Phillips et al., 2010). Future studies could therefore obtain samples over more concentrated time frames to more carefully examine historical population changes in

diversity. Ancient DNA analyses also showed that historical populations were generally more closely related to those currently found in the north than those in the south. This finding was consistent with historical records which suggested that in northern UK remnant populations may have survived the medieval bottleneck, unlike populations in southern UK which were thought to have been extirpated. The fact that levels of genetic diversity were also generally lower in southern compared to northern populations (Chapters 3&4) also supports this interpretation.

6.1.4 Fitness

Chapter 5 aimed to investigate whether the population history of the roe deer described in previous chapters had influenced fitness. In order to examine whether this was the case, first correlations between FA (as a measure of fitness) and genetic diversity were examined for both individuals within and across all populations. All correlations between FA and genetic diversity were found to exist in the expected negative directions, and were strongest for composite measures of FA (CFA-1) rather than single trait FA (FA-1). Nevertheless, even when CFA-1 was compared with genetic diversity, correlations were found to be weak.

No significant relationship was found between genetic diversity and CFA-1 when individuals were considered within each of their populations. However, when all individuals across populations were considered together, significant associations were found to exist at the 0.05 level. This latter result was considered to reflect an increased power associated with increased sample size. Indeed, sample size is likely to have a strong influence on whether relationships between fitness and genetic diversity are found. Previous studies that have found significant relationships have used much larger sample sizes than those used here; for example in studies where 650 red deer calves, 275 harbour seal pups (Pemberton, 1999) and 356 harbour porpoises (de-Luna-Lopez, 2005) were used. In the latter study, the author had only found significant relationships for the population where largest number of samples (356) had been obtained. For other populations examined in this same study where sample sizes were ten-fold smaller, no significant relationships were found and the author attributed this to statistical power.

6.2 Further implications

6.2.1 Impacts of future climatic change; looking backwards to look forwards

The use of ancient DNA for direct calibration of substitution rates and thus potentially more accurate assessments on past events is currently a particularly valuable area of research. The consensus is that the world is abnormally rapidly warming due to the anthropogenic production of greenhouse gases (IPCC, 2007). Therefore, understanding the response of populations, species and communities to past climatic change could help to make predictions for possible future environmental perturbations (Van Tuinen et al., 2004; Hadly and Barnosky, 2009).

A number of studies have begun to use this approach to make future predictions. One recent study provided a stark warning of the potential impacts of warming. A study of a key species of the arctic biota, the collared lemming (*Dicrostonyx torquatus*), demonstrated that previous warming events had strong influences on genetic diversity and population sizes. It was suggested that future climate change could completely abolish the remaining genetic diversity in this population which could lead to local extinctions. Extinctions would have severe effects on the arctic ecosystem, as collared lemmings are a principal prey resource for local predators. Therefore, warming effects were implicated to have major impacts on the trophic interactions and ecosystem processes in the Arctic (Prost et al., 2010).

For temperate species, such as the European roe, it is likely that increases in temperature will enable distributional expansions. These expansions are likely to occur in northerly directions where the roe's distribution is currently restricted by cold winters, short growing seasons and high snow accumulation (Holand et al., 1998). Indeed, numerous investigations are already documenting that roe are surviving well and increasing their range and densities in northern environments (review in Holand et al., 1998). One reason for this could be that an increased frequency of warmer winters is reducing calf mortality rates (Phillips et al., 2010). In contrast, in the most southerly parts of the roe's distribution (Mediterranean) suitable habitat may become limited as increased temperatures will lower water availability, a key requirement for roe (Wallach et al., 2007).

For mainland Europe, such changes in roe abundance and distribution could have a knock on effect for other species. This is because roe have been proposed to be keystone species

(Cederlund et al., 1998) supporting a variety of large carnivores (Aanes et al., 1998). For the UK, increased temperatures will also enable roe population sizes to increase; however, lack of natural predators may mean population rises may go unchecked. Increases in deer numbers across both Europe and the UK can lead to a number of associated problems (as outlined at the end of this discussion).

6.2.2 The management and conservation of roe deer

The sound management of any species necessitates knowing the scales at which its natural diversity is structured. In Chapter 3, the structure of UK roe populations was defined and the amount of genetic diversity within them quantified. This information can contribute to the planning of sustainable roe deer management. Roe deer management plans often involve defining Deer Management groups which include several estates or other land holdings sharing a population of deer (Mayle, 1999). Genetic data from both Chapters 3&4 may also prove useful for defining any such groups. These chapters revealed that northern populations have been least impacted by human activity and may have retained important historical genetic information potentially providing the best record regarding this species' evolutionary history. Contrastingly, southern populations have been most strongly influenced by human activity; and are made up of both recently translocated native and non-native populations. Conservation management is generally concerned with the preservation of genetically divergent populations that harbour potentially important local adaptation (i.e. those that are native; (Rhymer and Simberloff, 1996; Allendorf et al., 2001; Hansen and Taylor, 2008). With this in mind, the results of this study suggest that the most important populations that future management should act to conserve are populations found in northern parts of the UK. Translocated native populations may also be considered important to conserve; although they represent only a very small portion of original native genetic diversity. Contrastingly, the clear recognition of the Norfolk roe as non-native (German) and less genetically variable (Chapters 3&4) implies that this population may be of less concern to conservation as a separate management unit (though it too may retain novel variation from its source population).

6.2.3 Hybridisation between native and non – native stock

The existence of the non-native Norfolk roe within the UK and its proximity to nearby native populations raises issues with regards to potential integration with native populations. Conservational guidelines often suggest that anthropogenic hybridisation events should be avoided (Allendorf et al., 2001) because such events can result in outbreeding depression. However, it may be that such hybridisation events may yield positive consequences where fitness traits are enhanced (heterosis); Chapter 5; (Keller and Waller, 2002).

Here, it may be argued that heterosis is more likely for integration of German and native roe. The negative consequences of hybridisation generally only occur for populations that have been isolated for very long periods of time (reviewed in Edmands, 2002). However, all UK populations, including those of non-native origin, were found to belong to the same phylogeographic (central) lineage (Chapter 2). UK roe from native and non-native populations may have therefore only been isolated from one another since the land bridge split (Chapter 2). This amount of time may be insufficient to result in a significant loss of reproductive fitness. Evidence for this comes from continental roe (from Austria; central lineage) which may have already fully admixed with native UK (Lancashire) populations (Chapter 3&4). This population may already be exhibiting the positive effects of admixture as levels of genetic diversity are high relative to other populations and evidence of bottlenecking low (Chapters 3, 4 & 5 respectively).

Previous studies based on red deer (*Cervus elaphus*) have shown that following inter-breeding between distantly related individuals heterosis effects were observed; as lifetime reproduction and calf weight both increased (Coulson et al., 1998; Slate et al., 2000). Similarly, other studies based on Norwegian red deer, *Cervus elaphus* (Haanes et al., 2008) and white tailed deer, *Odocoileus virginianus* (DeYoung et al., 2003) suggested hybridisation between native and non- native deer stocks has led to increased population growth. Hybridisation between non-native and native stock of roe in the UK could result in similar positive effects for population growth, and future work should investigate this possibility in more detail.

6.2.4 The use of genetic rescue as a management tool

The implication that admixture between non-native and native deer may result in heterosis is highly relevant to the field of conservation genetics. Conservation genetics is increasingly interested in management options that can help overcome the impacts of inbreeding depression in small, endangered or fragmented populations (Hedrick and Fredrickson, 2010). One such management option is the use of genetic rescue which seeks to increase population fitness by introducing immigrants with new alleles (Tallmon et al., 2004). The results from this study may support the use genetic rescue as a tool for potentially promoting fitness; however, further work is required to fully examine this hypothesis.

Aims of a re-introduction programme should be to maximise the levels of diversity introduced and retained within a population. In order to achieve this, large numbers of founders from genetically divergent source populations or alternatively from several different stocks should be used (see Schwartz and May, 2008). However, care should be taken not to over-compensate by introducing stock that are too divergent, to avoid outbreeding depression. When any introduction does take place further consideration is required to minimise the impacts of genetic drift. Introduced populations should therefore be strongly inter-connected with neighbouring populations to facilitate gene flow (Latch and Rhodes, 2005; Hicks et al., 2007; Vonholdt et al., 2008), but this also depends on the behaviour of the subject species, and for roe deer dispersal range is evidently small (see Chapter 3).

6.2.5 Future expansion of the roe deer population

The roe is currently the most widespread and abundant Cervid species found within the British Isles (Ward, 2005). Recent studies have suggested that the roe population continues to grow at a rapid rate; with a recent study suggesting 5.2 % per year which is projected to continue, unless anthropogenic changes are severe enough to lower capacity (Ward et al., 2008). The implications of increased roe populations raise a number of concerns related to crop damage, road traffic accidents and nature conservation (Phillips et al., 2010). Some now believe that wild deer management in Britain is the least well regulated than in any other European country (Apollonio et al., 2009) and that deer numbers are now too high in some places to achieve desired social, ecological and economic objectives (Hunt, 2003) (see Phillips, et al. 2009). Deer managers should aim to minimise such negative public perceptions

by careful control of deer numbers. The results of this study are important as they show that roe deer have highly structured populations (both for molecular genetic and morphological phenotypic characteristics). Although some populations have retained diversity despite historical bottlenecks, others are relatively depauperate. Management should consider the partitioning of diversity among apparently insular populations, and the persistence of regional non-native populations.

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Appendix

Appendix 1: Mismatch analyses of individual European clades and the sub –species *C. c. italicus*. Figures show mismatch distributions for the two non-significant P values (>0.05) East (Figure a) and *C. c. italicus* (Figure b).

Clade/Sub species	SSD	P	R	P	Tau
East	**0.01**	**0.10**	**0.04**	**0.12**	**3.838**
West	0.05	0.01	0.12	0.03	
Central	0.01	0.00	0.05	0.03	
C.c. Italicus	**0.064**	**0.06**	**0.62**	**0.52**	**3.359**

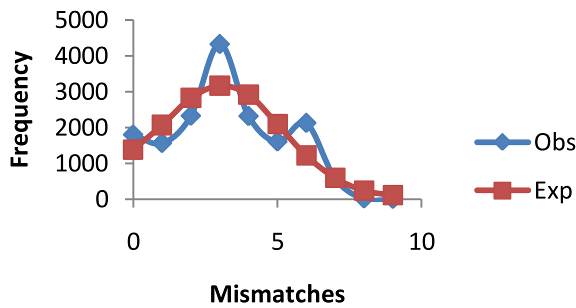


Figure a

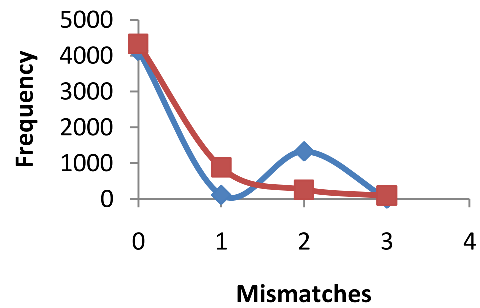


Figure b

Appendix 2: Estimated substitution rates obtained from other studies for comparison for the rate obtained from this study, where SES is the southern elephant seal.

	Estimated Substitution rate (Subs/site/year)	HPD interval		Reference
		Lo	**Hi**	
Adelie penguin	9.60E-07	5.30E-07	1.43E-06	Lambert et al (2002)
Aurochs	7.72E-07	1.82E-07	1.39E-06	Edwards et al (2007)
Bison	3.20E-07	2.30E-07	4.20E-07	Shapiro et al (2004)
Boar	2.90E-06	1.60E-06	4.40E-06	Ho et al (2007)
Bowhead whale	1.59E-07	5.10E-08	2.72E-07	Ho et al (2008)
Brown bear	3.00E-07	1.30E-07	4.80E-07	Saarma et al (2007)
Cave bear	2.60E-07	1.00E-07	5.30E-07	Saarma et al (2007)
Cave lion	2.02E-07	3.15E-08	4.04E-07	Ho et al (2007)
Grey wolf	2.90E-06	4.90E-07	6.40E-06	Pilot et al (2010)
Horse	1.11E-07	2.00E-08	3.08E-07	Ho et al (2007)
Roe deer	3.73E-07	1.82E-07	5.82E-07	This study
SES	9.80E-07	1.67E-09	2.06E-06	Debruyn et al (2009)

Appendix 3: Hardy Weinberg test results for individual populations and loci. Ho = observed heterozygosity; He = expected heterozygosity; * indicate P values significant at the 0.05 level; ** significant after Bonferroni adjustment.

Norfolk			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.356	0.431	0.129
CSSM41	0.455	0.456	0.095
BM757	0.500	0.685	0.004*
CSSM43	0.366	0.471	0.328
HUJ117	0.615	0.782	0.037
BMC1009	0.341	0.474	0.102
RT1	0.581	0.729	0.016*
CSSM39	0.714	0.729	0.988
IDVGA 8	0.349	0.471	0.00002**
BM1706	0.415	0.455	0.046*
OARFCB304	0.439	0.550	0.139
MAF70	0.409	0.520	0.302
BM848	0.474	0.534	0.220
NVHRT24	0.674	0.649	0.953
MCM505	0.614	0.759	0.057
ILSTO11	0.605	0.684	0.189

Berks			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.550	0.642	0.421
CSSM41	0.650	0.673	0.255
BM757	1.000	0.849	0.114
CSSM43	0.750	0.678	1.000
HUJ117	0.600	0.658	0.327
BMC1009	0.650	0.583	0.745
RT1	0.550	0.556	0.911
CSSM39	0.250	0.276	1.000
IDVGA 8	0.350	0.387	0.579
BM1706	0.550	0.490	0.555
OARFCB304	0.842	0.767	0.117
MAF70	0.750	0.800	0.786
BM848	0.600	0.647	0.348
NVHRT24	0.850	0.715	0.112
MCM505	0.650	0.633	1.000
ILSTO11	0.737	0.562	0.282

Dorset			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.658	0.632	0.919
CSSM41	0.605	0.647	0.319
BM757	0.842	0.765	0.080
CSSM43	0.079	0.149	0.131
HUJ117	0.605	0.480	0.270
BMC1009	0.684	0.587	0.043*
RT1	0.711	0.718	0.718
CSSM39	0.568	0.686	0.266
IDVGA 8	0.579	0.535	0.855
BM1706	0.622	0.625	0.521
OARFCB304	0.800	0.703	0.351
MAF70	0.514	0.656	0.016
BM848	0.632	0.766	0.095
NVHRT24	0.632	0.636	1.000
MCM505	0.297	0.373	0.016*
ILSTO11	0.759	0.680	0.877

Wiltshire			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.444	0.595	0.431
CSSM41	0.556	0.660	0.121
BM757	0.778	0.712	0.812
CSSM43	0.333	0.314	1.000
HUJ117	0.333	0.621	0.034
BMC1009	0.778	0.850	0.237
RT1	0.556	0.686	0.100
CSSM39	0.889	0.739	0.694
IDVGA 8	0.333	0.529	0.528
BM1706	1.000	0.765	0.445
OARFCB304	0.625	0.683	0.243
MAF70	0.333	0.706	0.023*
BM848	0.667	0.712	1.000
NVHRT24	0.667	0.660	0.510
MCM505	0.333	0.294	1.000
ILSTO11	0.500	0.650	0.438

Somerset			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.600	0.645	0.615
CSSM41	0.400	0.649	0.110
BM757	0.526	0.578	0.481
CSSM43	0.632	0.622	0.040*
HUJ117	0.474	0.519	0.464
BMC1009	0.300	0.459	0.194
RT1	0.684	0.717	0.065
CSSM39	0.400	0.581	0.030*
IDVGA 8	0.600	0.703	0.092
BM1706	0.722	0.595	0.244
OARFCB304	0.882	0.670	0.096
MAF70	0.550	0.559	0.594
BM848	0.211	0.246	1.000
NVHRT24	0.529	0.606	0.041*
MCM505	0.250	0.273	1.000
ILSTO11	0.529	0.635	0.001

Durham			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.579	0.457	0.449
CSSM41	0.588	0.667	0.319
BM757	0.737	0.751	0.963
CSSM43	0.500	0.546	0.268
HUJ117	0.556	0.589	1.000
BMC1009	0.579	0.572	0.606
RT1	0.684	0.630	0.910
CSSM39	0.778	0.746	0.574
IDVGA 8	0.737	0.750	0.708
BM1706	0.421	0.615	0.258
OARFCB304	0.737	0.856	0.124
MAF70	0.526	0.546	1.000
BM848	0.444	0.759	0.029*
NVHRT24	0.526	0.616	0.419
MCM505	0.474	0.504	0.408
ILSTO11	0.647	0.804	0.645

N york			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.400	0.469	0.809
CSSM41	0.300	0.501	0.057
BM757	0.700	0.680	0.895
CSSM43	0.467	0.556	0.570
HUJ117	0.667	0.587	0.872
BMC1009	0.333	0.390	0.007
RT1	0.433	0.512	0.206
CSSM39	0.800	0.731	0.523
IDVGA 8	0.733	0.637	0.392
BM1706	0.733	0.674	0.000
OARFCB304	0.567	0.682	0.065
MAF70	0.655	0.513	0.059
BM848	0.667	0.738	0.461
NVHRT24	0.571	0.577	0.211
MCM505	0.533	0.625	0.522
ILSTO11	0.720	0.780	0.016*

Carlisle			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.533	0.625	0.642
CSSM41	0.567	0.521	0.441
BM757	0.929	0.786	0.328
CSSM43	0.733	0.560	0.184
HUJ117	0.533	0.598	0.548
BMC1009	0.633	0.529	0.427
RT1	0.667	0.676	0.409
CSSM39	0.667	0.694	0.295
IDVGA 8	0.733	0.752	0.938
BM1706	0.700	0.660	0.197
OARFCB304	0.900	0.816	0.885
MAF70	0.600	0.634	0.462
BM848	0.633	0.639	0.856
NVHRT24	0.393	0.486	0.121
MCM505	0.800	0.753	0.625
ILSTO11	0.714	0.714	0.509

Perth			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.500	0.620	0.405
CSSM41	0.448	0.482	0.852
BM757	0.964	0.908	0.020*
CSSM43	0.759	0.690	0.338
HUJ117	0.724	0.741	0.065
BMC1009	0.567	0.646	0.082
RT1	0.667	0.853	0.103
CSSM39	0.633	0.677	0.070
IDVGA 8	0.733	0.767	0.071
BM1706	0.733	0.765	0.886
OARFCB304	0.929	0.838	0.339
MAF70	0.567	0.617	0.965
BM848	0.733	0.701	0.930
NVHRT24	0.633	0.650	0.451
MCM505	0.733	0.811	0.367
ILSTO11	0.655	0.685	0.097

Moray			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.517	0.685	0.248
CSSM41	0.467	0.420	0.040*
BM757	0.933	0.767	0.317
CSSM43	0.414	0.525	0.626
HUJ117	0.655	0.580	0.193
BMC1009	0.533	0.555	0.064
RT1	0.700	0.793	0.122
CSSM39	0.633	0.668	0.801
IDVGA 8	0.767	0.769	0.290
BM1706	0.733	0.788	0.908
OARFCB304	0.700	0.863	0.073
MAF70	0.517	0.670	0.173
BM848	0.600	0.771	0.108
NVHRT24	0.733	0.763	0.505
MCM505	0.600	0.776	0.066
ILSTO11	0.767	0.758	0.003**

Aviemore			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.600	0.691	0.164
CSSM41	0.368	0.472	0.425
BM757	0.750	0.815	0.062
CSSM43	0.579	0.684	0.103
HUJ117	0.579	0.649	0.652
BMC1009	0.650	0.695	0.967
RT1	0.850	0.850	0.506
CSSM39	0.789	0.755	0.819
IDVGA 8	0.667	0.782	0.190
BM1706	0.619	0.695	0.171
OARFCB304	0.700	0.838	0.084
MAF70	0.571	0.647	0.271
BM848	0.722	0.737	0.311
NVHRT24	0.800	0.749	0.373
MCM505	0.500	0.750	0.127
ILSTO11	0.684	0.637	0.932

Glasgow			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.600	0.642	0.600
CSSM41	0.444	0.745	0.538
BM757	1.000	0.900	0.617
CSSM43	0.556	0.680	0.575
HUJ117	0.600	0.784	0.887
BMC1009	0.800	0.847	0.344
RT1	1.000	0.911	0.871
CSSM39	0.800	0.805	0.973
IDVGA 8	0.700	0.642	0.765
BM1706	0.800	0.795	0.208
OARFCB304	0.900	0.932	0.810
MAF70	0.600	0.616	1.000
BM848	0.500	0.642	0.692
NVHRT24	0.778	0.699	1.000
MCM505	1.000	0.800	0.430
ILSTO11	0.778	0.725	0.190

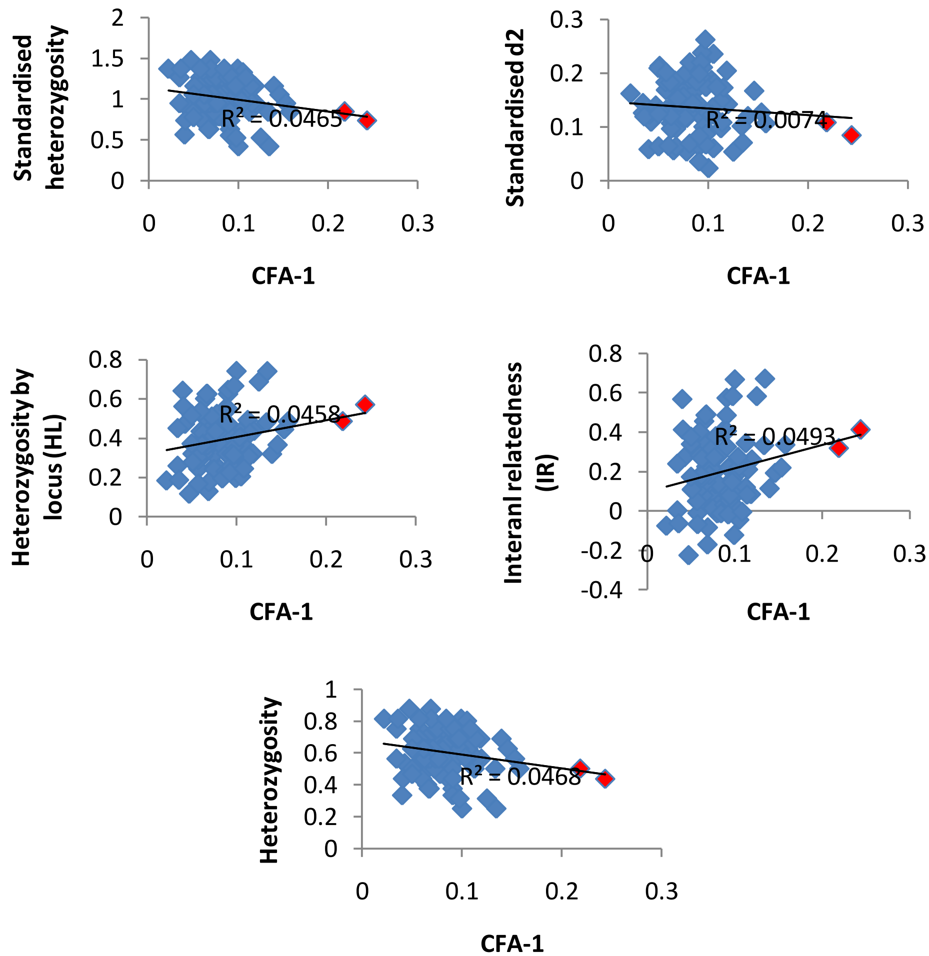
Ayr			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.596	0.602	0.797
CSSM41	0.635	0.635	0.465
BM757	0.846	0.846	0.698
CSSM43	0.588	0.567	0.147
HUJ117	0.520	0.648	0.011*
BMC1009	0.420	0.494	0.191
RT1	0.660	0.710	0.799
CSSM39	0.596	0.701	0.278
IDVGA 8	0.706	0.733	0.088
BM1706	0.608	0.687	0.236
OARFCB304	0.863	0.871	0.884
MAF70	0.569	0.557	0.702
BM848	0.580	0.520	0.702
NVHRT24	0.720	0.689	0.021 *
MCM505	0.540	0.571	0.350
ILSTO11	0.796	0.757	0.783

Lancashire			
Locus	**Ho**	**He**	**P value**
NVHRT48	0.235	0.266	1.000
CSSM41	0.294	0.442	0.040 *
BM757	0.941	0.788	0.868
CSSM43	0.353	0.506	0.169
HUJ117	0.765	0.804	0.625
BMC1009	0.529	0.535	0.892
RT1	0.529	0.718	0.176
CSSM39	0.765	0.736	0.639
IDVGA 8	0.688	0.639	1.000
BM1706	0.813	0.736	0.659
OARFCB304	0.625	0.806	0.034
MAF70	0.625	0.778	0.464
BM848	0.563	0.728	0.609
NVHRT24	0.625	0.726	0.385
MCM505	0.786	0.881	0.140
ILSTO11	0.933	0.791	0.823

Appendix 4: Hardy Weinberg test results for the individual ancient population and loci. Ho = observed heterozygosity; He = expected heterozygosity; * indicate P values significant at the 0.05 level; ** significant after Bonferroni adjustment.

Ancient Locus	**Ho**	**He**	**P Value**
NVHRT48	0.54	0.84	<0.0001**
CSSM41	0.48	0.811	<0.0001 **
OAR	0.54	0.92	<0.0001 *
MAF70	0.8	0.92	0.43
NVHRT24	0.77	0.91	<0.0001 **
MCM505	0.9	0.91	0.35
Mean	**0.67**	**0.89**	**0**

Appendix 5: Graphic representation of the correlations found between FA and genetic diversity for all individuals from all UK populations (except Norfolk) including 2 recognised outliers (marked in red).



Appendix 6: Graphic representation of the correlations found between FA and genetic diversity for all individuals from all UK populations (except Norfolk) excluding 2 recognised outliers.