CONSERVATION GENETICS OF THE COMMON DORMOUSE *MUSCARDINUS AVELLANARIUS* IN UK.

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by

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ABSTRACT

As anthropogenic factors have threaten species worldwide, conservation of a species through *ex situ* (*i.e.* captive breeding, reintroduction) provides one of the most powerful tools for species conservation. However, baseline genetic data prior to reintroduction of captive-bred individuals is essential for guiding such efforts, but this has not been gathered previously in the common dormouse, *Muscardinus avellanarius*. Thus, this thesis provides the first comprehensive study of spatial and temporal patterns of genetic diversity of populations of *M. avellanarius* in UK, with specific reference to investigate the breeding structure and contemporary and historical patterns of gene flow, both in natural and reintroduced populations. Additionally, this thesis analysed patterns of variation at two regions of mitochondrial DNA (mtDNA) to broader geographic divergence among UK populations of *M. avellanarius*. The main findings in this thesis are: (1) reproductive behaviour of reintroduced *M. avellanarius* population was retained as in the wild population despite enforced bottleneck during captivity that could change behaviour of a species, (2) both ecological and molecular genetic data provide broadly congruent estimates about the dispersal characteristics of *M. avellanarius* in a large, continuous habitat. A significant isolation-by-distance (IBD) pattern at a fine scale (less than 1 km) was apparent within continuous populations with males more mobile than females (male-biased dispersal), (3) gene flow was generally restricted among separate populations (*i.e.* between habitat patches) at a scale of 1-10 km, (4) using mtDNA sequence data, three divergent phylogenetic lineages (Northwestern, Central and Southern) were recognized in the UK, implying colonization of the UK from separate refugia (*e.g.* continental Europe), that probably diverged during the Pleistocene period but prior to the last Ice Age. Interestingly, genealogical evidence revealed that the source populations of captive bred *M. avellanarius* that were released in Wych (northern England) are from the southern UK, thus highlighting the use of genetics for conservation. The results of these studies will not only contribute to the understanding of dispersal characteristics and how this process has structured the populations at small and large scales, but also add significantly to biological and evolutionary understanding on *M. avellanarius*, which can be directly applied to the ongoing conservation and management of this species.

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Chapter 1: General Introduction

1.0 Biodiversity conservation

Accelerated expansion of human activities in detriment of the Earth's natural environments is causing severe, and probably irreversible, biodiversity losses world-wide (Ehrlich 1994; Pimm et al. 1995; Ceballos and Ehrlich 2002; Ehrlich 2002; Luck et al. 2003; Ceballos et al. 2005; Ehrlich and Pringle 2008). Major threats to the future of biodiversity are habitat conversion and direct exploitation of wildlife (Seabloom et al. 2002; Avise et al. 2008; Ehrlich and Pringle 2008). More recently, the ever-growing atmospheric input of the greenhouse gas carbon dioxide from the burning of fossil fuels is causing climate change that is expected to amplify these existing threats, and to subsequently add new ones, both regionally and globally (McLaughlin et al. 2002; May 2010). Consequently, while the World Conservation Monitoring Centre (1992) reported that extinction rates of organisms range from 1-5 % to 8-11 % (reviewed in Frankham 1999), extinction rates are now, and for the next few decades, are estimated to be in the order of 100s to 1,000s of times faster than the background rate (Pimm 2009). This major loss of biodiversity, it has been argued, represents a sixth mass extinction episode (Leakey and Lewin 1995; Glavin 2007; reviewed in Avise et al. 2008).

Perhaps the single largest direct driver of extinction is loss and fragmentation of habitat (Gaines et al. 1996; Meffe and Carroll 1997; Burkey and Reed 2006; Prugh et al. 2008; May 2010); habitat fragmentation is defined as a subdivision of a continuous habitat into smaller pieces (Franklin et al. 2002). Globally, approximately 40 % of land has been anthropogenically converted for human use (Foley 2005) and formerly diverse regions such as eastern United States, the Philippines and Ghana have lost more than 90 % of their natural habitat (Prugh et al. 2008). Broadly, the deleterious effects of habitat fragmentation are apparent with nearly a quarter (22.2 %) of 5,487 mammal species listed in IUCN Red List (2009) are globally threatened or extinct. Habitat fragmentation diminishes the landscape's capacity to sustain healthy populations or metapopulations in four primary ways: (1) loss of the original habitat, (2) reduction in habitat patch size (3) increased edge

effects and (4) increasing isolation of habitat patches, that lead to reduced connectivity between areas and reductions in population size (reviewed in Saunders et al. 1991; Andrén 1994; Gaines et al. 1996; Fahrig 2003). These factors subsequently may have a very strong impact on the demographic (Lande 1988) and genetic (Frankham 1995, 1998, 2005) characteristics of a population, with the strength of their effects largely determined by the population size (Reed 2004).

Demographic stochasticity caused by random independent events of individual survival and reproduction, particularly in small populations, produces random fluctuation in per capita growth rate of the population inversely proportional to population size (Lande 1993; 1998). A decrease in population growth rate may occur because of low density (an Allee effect), for example due to reduction in cooperative interactions among individuals (Lande 1993; Stephens and Sutherland 1999; Dennis 2002). Such populations with fluctuating demographies and a possible Allee effect are especially vulnerable to extinction as the fluctuations may drive their densities below a critical threshold of population size that is necessary for survival (Stephens and Sutherland 1999; Engen et al. 2003). Indeed, models of populations exhibiting this effect have been observed in several taxa, including vertebrates (Grenfell et al. 1992; Turchin and Taylor 1992; Turchin 1993).

Species with reduced population size can suffer also from loss of genetic diversity (Frankham 1998; Frankham et al. 2002; Reed et al. 2002; Reed 2004). Typically, maintenance of genetic diversity is considered important as it is correlated with rates of survival and reproduction linked to inbreeding (Saccheri et al. 1998; Ebert et al. 2002; Reed 2005); it is also argued that genetic diversity will determine a population's ability to cope with changing environments (Frankel and Soulé 1981; Lacy 1997; Pertoldi et al. 2007). While future events are hard (if not impossible) to predict, inbreeding depression is regarded to be an immediate and potentially damaging effect, as it affects all components of species' life cycles (Keller 1998; Frankham et al. 2002; Villas et al. 2006). Inbreeding depression has been noted in both wild and reintroduced populations (Madsen et al. 1996; O'Grady et al. 2006), commonly where populations have experienced a reduction in numbers. Typically, the effects of inbreeding depression are more severe in more stressful wild environments than in more benign captive populations (Crnokrak and Roff 1999; Armbruster and Reed 2005); thus, where captive breeding programmes are used to limit

population extinction, the consequences of inbreeding may go undetected until population reintroductions are attempted. Loss and fragmentation of suitable habitat are expected to result in genetic erosion, which can increase a population's probability of extinction (Saccheri et al. 1998; Westemeier et al. 1998). Clearly therefore, efforts to conserve plant and animal populations should take into account the genetic consequences of habitat fragmentation.

Since loss of genetic diversity is correlated with extinction, there is much concern for the survival of many species/populations as they are apparently losing genetic diversity as a consequence of small population size, typically associated with loss of habitat (*e.g.* Dixon et al. 2007; Bergl et al. 2008; Mitrovski et al. 2008; see Dallas et al. 1995; Hale et al. 2001; Hale and Lurz 2003; Redeker et al. 2006; Lampila et al. 2009 for case studies in small mammals). However, in addition to the effects of (effective) population size, the rate and pattern of dispersal (*i.e.* gene flow) determines the rate of genetic erosion as it is a clear mechanism that allows the introduction (or not, if dispersal is prevented) of new polymorphisms; dispersal can limit loss of genetic diversity through genetic drift in small populations, and can provide essential diversity upon which selection can act. Hence dispersal is a key evolutionary trait and has been studied widely (see *e.g.* Clobert et al. 2001; Broquet and Petit 2009 for synthesis and reviews). Indeed, with the advent of relatively inexpensive and powerful molecular-genetic techniques, a vast number of studies have quantified the extent of gene flow (*i.e.* dispersal) and patterns of spatial genetic structure among natural populations (as evidenced by numerous studies - see reviews by Slatkin 1987; Bohonak 1999; Kokko & López-Sepulcre 2006; Broquet and Petit 2009; Burney and Brumfield 2009), although the functional significance of any genetic divergence is typically unknown.

1.1 Population structure and genetic diversity

Establishing links between the ecology and the evolution of a species generally involves quantifying the relationship between dispersal and effective population size, since these parameters control demography and the rate of genetic divergence (reviewed by Bohonak 1999; Frankham et al. 2002). From an applied perspective, such estimation of population differentiation is believed to be crucial in conservation biology, where it is often necessary to understand whether populations are genetically isolated from each other, and if so, to

what extent (Bohonak 1999; Surridge et al. 1999; Frankham et al. 2002). Numerous factors, including, species' life histories (*e.g.* their dispersal capability, mating system, *etc.*), environmental barriers and historical processes, may all, to some extent, shape the genetic structure of populations (see *e.g.* Almeida et al. 2005; Loew et al. 2005; Miranda et al. 2007; Busch et al. 2009; Brouat et al. 2009; Kozakiewicz et al. 2009a; Nicolas et al. 2009; Spaeth et al. 2009). Of particular relevance for conservation are studies that have inextricably linked dispersal with population persistence (*e.g.* Dossantos et al. 1995; Sommer et al. 2002; Gauffre et al. 2008).

Dispersal is recognised to be a key life history trait that has effects on both the dynamics and genetics of species, and accordingly has been a major concern in evolutionary biology (Clobert et al. 2001; Prugnolle and de Meeus 2002; Kokko & López-Sepulcre 2006; Broquet and Petit 2009). A wide variety of proximate triggers for dispersal in mammals, particularly rodents, have been reported and some of the proposed potential triggers include: (1) reproductive opportunities, (2) inbreeding avoidance, (3) improve access to environmental resources and (4) other social interactions (conspecific attraction) (Clobert et al. 2001; Heise and Rozenfeld 2002; Solomon 2003; Bowler et al. 2005; Fahrig 2007; Nunes 2007). Clearly, the various functions of dispersal are related to survival and reproduction, and a better understanding of how movements are affected by the landscape structure is needed (Wiens et al. 1993; Selonen and Hanski 2004; Fahrig 2007).

The structure of the landscape occupied by a species will influence patterns of movement among habitat patches (Selonen and Hanski 2004; Fahrig 2007). Patch size and shape, nature of the matrix habitat, changes in landscape structure and heterogeneity can affect path direction and colonization success because different cover types in the landscape present different levels of risk and benefit (Johnson et al. 2002; Phillips et al. 2004; Selonen and Hanski 2004; Hernandez and Laundre 2005; Fahrig 2007). Moreover, the dispersal process depends on the interactions between species attributes and landscape structure, or connectivity, that is thought to be one of the most important features affecting patch colonisation (Ewers and Didham 2006; Fahrig 2007; Dover and Settele 2009; Kadoya 2009). Highly mobile animals may perceive landscape patchiness at different scales than do more sedentary animals by responding in a less-sensitive manner to patch edges (Selonen and Hanski 2004). Accordingly, among animals, there are normally large differences

between sexes in terms of distances travelled during dispersal and/or dispersal rates (*i.e.* sex-biased dispersal) (Handley and Perrin 2007).

When the balance of dispersal differs between genders, it is expected that dispersal is sex-biased (Gauffre et al. 2009). In particular, when males defend female resources and exhibited male-male competition, dispersal is expected to be male-biased (Gauffre et al. 2009). Greenwood (1980) reviewed mating systems and dispersal strategies and concluded that dispersal was predominantly male-biased (*i.e.* restricted dispersal – philopatry – by females) in mammals, whereas birds tended to exhibit female-biased dispersal and male philopatry (reviewed by Handley and Perrin 2007). This pattern of dispersal has received high attention in field and theoretical studies during the past decades (Prugnolle and de Meeus 2002) as it have important effects on population demography, social structure and genetic composition (Ji et al. 2001). Two key elements appear to play a role; (1) sex biased dispersal will lead to inbreeding avoidance, which means the chance that mating between close relatives will occur is small, and (2) dispersal costs may be different between the sexes (Beebee and Rowe 2004). This observation raised a hypothesis that mating system is closely linked to the direction of dispersal, with female-defence polygyny, a common breeding system of mammals, leading to a male-biased dispersal, and resource-defence monogamy, which is common in birds, resulting in the reverse pattern (Greenwood 1980; see *e.g.* Busch et al. 2009; Gauffre et al. 2009; Perez-Gonzalez and Carranza 2009; Cooper et al. 2010 for case studies).

Another factor that will affect the level of genetic diversity within a population is reproductive behaviour (Paxton 2005; Fredsted et al. 2007; Dechmann et al. 2007; McEachern et al. 2009). Every individual struggles to maximise its reproductive success and may use any of various strategies for mating (Waterman 2007), in which differences in the pattern of mating between populations can reflect differential evolution in response to local conditions. For example, random mating may occur between non-divergent populations, while directional or assortative mating may be expected between populations after they have experienced a period of independent evolution (Ganem et al. 2005). It has been suggested that animals may employ mixed strategies in mating as ways of dealing with uncertainty and response to changes in social and ecological characteristics of the environment that vary in space and time (Randall et al. 2002).

A species' mating system is a key component of its life history that can vary between monogamy, where each sex mates with just a single partner, to polygamous and promiscuous mating systems, where individuals mate with multiple partners but either within a social system or with no social bonding respectively (Waterman, 2007). Multiple mating (*i.e.* mating with more than one member of the opposite sex) has been observed as a common system among males of small mammal species (Barash 1981; MeEachern et al. 2009; Perez-Gonzalez et al. 2009). Recently, there is increasing evidence that multiple mating is a commonly-used tactic by female animals (Klemme et al. 2007, 2008; Moran et al. 2009), particularly species of small mammal (see *e.g.* Waser and De Woody 2006; Firman and Simmon 2008; Chapter 3). Several studies have investigated the direct (material) and indirect (genetic) benefit of multiple mating (see *e.g.* Wolff and MacDonald 2004; Jennions and Petrie 2000). For example, multiple mating by females will be adaptive when they gain direct benefits from males such as parental care, increased access to food resource and/or ownership of territory (Yasui 1998; reviewed by Wolff and Macdonald 2004). Indirect genetic benefits (such as greater genetic compatibility between parents, 'good genes', or increased genetic variability of offspring) are hypothesised to generate a higher genetic quality of offspring, thus increasing their survivorship (reviewed by Zeh and Zeh 1996, 2001). The mechanisms by which these benefits are accrued are not fully understood, however, After mating, females could obtain good genes from the superior male either through sperm competition (where more vigorous sperm have greater fertilization success) or via sperm selection (Fisher et al. 2006; reviewed by Simmons 2005; Garcia-Gonzalez 2008; Lupold et al. 2009).

Phylogeography is an integrative field of study that combines information from several disciplines including molecular and population genetics, ethology, demography, phylogenetics, and historical geography to explain the genetic structure of modern populations (Avise 1998). Particularly, it focuses on how historical factors have influenced the gene lineages across its geographical distribution (Avise 1998, 2000). The knowledge of the evolutionary history and genetic status of species is critical for the success of conservation programmes because it allows the definition of management units and the design of appropriate management strategies aimed at minimising genetic erosion while preserving subspecific distinctiveness (Godoy et al. 2004) and natural evolutionary processes (Crandall et al. 2000). Since historical, as well as contemporary factors,

determine the current distribution of a species' genetic diversity, understanding the phylogeographic history of the species is a necessary component of a species' genetic management. Patterns of genetic variability of a species have been studied extensively within the discipline of phylogeography during the past three decades, providing tremendous contributions to fundamental evolutionary issues (Avise 2000, see *e.g.* Jaarola and Searle 2004; Piertney et al. 2005; White and Searle 2008; Lanier and Olson 2009; Searle et al. 2009 for case studies and also Chapter 6). Both speciation (a historical, population-genetic event) and phylogeographic patterns studies are useful to explain ecological and genetic discontinuities observed across the extant range of a species (Brown et al. 1996; Hewitt 2000).

1.2 Conservation biology and role of genetics

Conservation biology is a 'crisis discipline', aiming to provide principles and tools for preserving biodiversity (Soulé 1985) and its emergence was driven primarily by the accelerating and global loss of species (Soulé 1986). Bowen (1999) proposed that conservation biology requires three complementary fields: (1) systematics, identification of organismal lineages, (2) ecology, life-support system protection for the lineages, and (3) evolutionary biology, maintenance of the conditions that produce new lineages. The most significant result of debate on these three challenges was to define the roles of conservation genetics in understanding genetic and evolutionary processes and in delineating the patterns that are relevant to managing endangered populations (Frankham 1995; Kelt and Brown 2000; Loew 2000; DeSalle and Amato 2004).

The most important applications of conservation genetics derive from its ability to help to create a more accurate picture of pattern and process in endangered species (DeSalle and Amato 2004). Specifically, it determines a more precise description on the processes that gave rise to the current endangered state of a population or species. For example, the quantification of inbreeding depression, effective population size and levels of genetic variation and gene flow in natural populations provides specific and comparable measurements of processes that could affect endangered populations (Ralls et al. 1988; Frankham 1995; Crnokrak and Roff 1999; Frankham 2005; Charlesworth 2009; Frankham 2010).

Proponents of systematics believe that not all species should be treated the same in the context of conservation, with priority given to phylogenetic distinctiveness (Bowen 1999; Pérez-Losada and Crandall 2003). Several unit definitions have been coined, the most prominent of which are the Evolutionary Significant Unit (ESU) and the Management Unit (MU), both of which have been very useful in deciding on conservation priorities (Moritz 1994, 1999; Fraser and Bernatchez 2001). The definition and study of different conservation units is also important for *ex situ* (*i.e.* reintroduction) conservation of a species, particularly in identifying the demography and genetics source of reintroduced and resident population in the reintroduction area (reviewed by DeSalle and Amato 2004; Green 2005; de Guia and Saitoh 2007).

1.3 The use of different molecular markers for analysing population genetic structure.

A molecular marker is a DNA sequence used to mark a particular location on a particular chromosome (*e.g.* marker gene) which could be detected by analytical methods or an identifiable DNA sequence that facilitates the study of inheritance of a trait or a gene (Avise 1989; Blanchette et al. 1999; Sunnucks, 2000; Frankham et al., 2002; Vignal et al. 2002; Avise 2004; Okumuş and Ciftci 2004; Excoffier and Heckel, 2006; Selkoe and Toonen, 2006; Allendorf and Luikart, 2007; Galtier et al. 2009). Current advances in molecular-genetic techniques, most notably the development of polymerase chain reaction (PCR) and the discovery of hypervariable microsatellite loci, coupled with the recent explosion of powerful computer programs, offers a wide range of possibilities to study the evolutionary biology and behaviour of organisms that were once thought impossible (Mitra et al. 1999; Sunnucks 2000; Domingo- Roura et al. 2001; Schlötterer, 2004; Galtier et al. 2009).

There are considerable differences in the characteristics of different types of molecular-genetic markers and it is crucial that the choice of marker is appropriate to the problem being tackled (Sunnucks 2000; Schlötterer 2004). Many different competing factors should be given attention before selecting a molecular marker, for example, the sensitivity (*i.e.* level of polymorphism) of loci, their practical pitfalls, their functionality or neutrality and the time and expense associated with a particular technique (Whitlock and McCauley 1999; Beebee and Rowe 2004). In particular, a critical assumption of studies that aim to resolve

evolutionary processes such as patterns and rates of dispersal and genetic drift is that the genetic loci used are selectively neutral (Whitlock and McCauley 1999; Avise 2004; Beebee and Rowe 2004). The most popular genetic markers have been allozymes, mitochondrial DNA (mtDNA), Randomly Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs) and microsatellites (reviewed by Frankham et al. 2002; Beebee and Rowe 2004; Schlötterer 2004). Due to differences in mutation rate (*i.e.* observed level of polymorphism) and modes of inheritance, approaches to genetic equilibrium vary among classes of genetic loci, resulting in differences in the pattern of population genetic structure that can be detected (Wade and Beeman 1994). Among others, genes from the mitochondrial DNA (mtDNA) and microsatellite loci have been the most widely used (Queller et al. 1993; Jarne and Lagoda 1996; Sunnucks 2000; Neff and Gross 2001; Prugnolle and deMeeus 2002; White et al. 2008; Galtier et al. 2009).

1.3.1 Microsatellites

Microsatellites, also known as Short Tandem Repeats (STRs) or Simple Sequence Repeats (SSRs) are highly polymorphic and have been found to be typically abundant in the genomes of all prokaryotic and eukaryotic organisms (Jarne and Lagoda 1996). Unlike genes, microsatellite do not contain a genetic code hat leads to the production of RNA and/or proteins (Queller et al. 1993; Estoup et al. 1998), thus it is thought that most microsatellite loci have no (known) function (Estoup et al. 1998; DeWoody and Avise 2000). Specifically, microsatellites are co-dominant nuclear markers and have very short repeating motifs (Goldstein et al. 1995; Jarne and Lagoda 1996; Beebee and Rowe 2004). An example of microsatellite repeat motifs would be $(TG)_n$ or $(CACG)_n$ where n represents the number of repeats, which typically varies from two to tens of repeats, but dinucleotide (2 bp motifs), trinucleotide (3 by motifs) and tetranucleotide (4 bp motifs) repeats are the most frequently employed categories of repeat unit for molecular genetic studies. Dinucleotide repeats account for the majority of microsatellites isolated for many species (Schlötterer and Wiehe1999; Li et al. 2002), probably because they are the most abundant class of marker in the genome, although they can often suffer from problems when resolving alleles because of slippage during PCR that causes stutter banding. The most likely repeat classes to appear in coding regions are trinucleotide and hexanucleotide (6 bp motifs) because they do not cause a frameshift when alleles expand or contract (Toth et al. 2000).

Microsatellites are inherited in Mendelian fashion and because most microsatellite loci are situated in non-coding genomic regions, they can tolerate high rates of mutation (*e.g.* between 10^{-2} and 10^{-6} mutations per locus per generation, and on average $5x10^{-4}$), which generates the high levels of polymorphism (Schlötterer and Harr 2000). Owing to these characteristics of high variability and ease of amplification, microsatellites have already proven useful in monitoring gene diversity and population genetic structure in a variety of mammalian species designated for conservation and management, particularly those listed as threatened or vulnerable species (*e.g.* Castleberry et al. 2000; Roach et al. 2001; Castleberry et al. 2002; Larson et al. 2002; Haynie et al. 2007; and Chapters 3, 4 and 5).

1.3.2 Mitochondrial DNA (mtDNA)

The mammalian mitochondrial DNA (mtDNA) is a relatively small (typically about 15-18 Kb) circular, double-stranded DNA molecule that occurs as multiple copies in cell mitochondria. The mtDNA genome typically encodes for 37 genes; 22 tRNAs, two rRNAs and 13 mRNAs (da Fonseca et al. 2008).

Some of the principal beneficial characteristics of mtDNA as a genetic marker include ease of isolation, high copy number, primarily selectively neutral and relatively high mutation rates in different regions of the molecule (William et al. 1995; Lunt et al. 1996; Ballard and Rand 2005). mtDNA also provides a type of information concerning population structure that is generally unavailable from nuclear markers (*e.g.* microsatellites). Due to its maternal mode of inheritance and mainly non-recombining nature, the pattern of mtDNA haplotypes among individuals of a population reflects not just allelic diversity but also the genealogical relationship of maternal lineages within a population. Thus, due to this facility in inferring relationships of descent, mtDNA provides a powerful means in evolutionary biology for both phylogenetic (*i.e.* phylogeny reconstruction) and population genetic studies (Avise 1994; William et al. 1995; Ståhls and Nyblom 2000; Beheregaray and Sunnucks 2001; Féral 2002; Ballard and Whitlock 2004 and see Chapter 6). Furthermore, the routine use of PCR, largely because of the availability of 'universal primers' (Kocher et al. 1989), for amplification of regions of the mtDNA have made this class of genetic marker popular for studies of intraspecific genetic diversity. Two regions of the mtDNA commonly used for such purposes are: (1) mtDNA d-loop and (2) mtDNA Cytochrome Oxidase Subunit I (COI).

1.3.2.1 *mtDNA D-Loop*

The d-loop (displacement or control region) comprises a central conserved region and is the only major non-coding segment of animal mitochondrial DNA (Brown et al. 1986), but which contains information essential for the initiation of transcription and DNA replication (Beebee and Rowe 2004). The d-loop lies between the phenylalanine tRNA ($tRNA^{Phe}$) and the praline tRNA ($tRNA^{Pro}$) (Wilkinson and Chapman 1991; Wilkinson et al. 1997) in which the flanking regions vary in sequence and length. Because of its typically high level of sequence variation (see *e.g.* Brown et al. 1982; Yu et al. 2008), d-loop of mtDNA is frequently very informative for the studies of inter- and intra-specific diversity (see *e.g.* Lee et al. 1995; Pesole and Saccone 2001; Aubry et al. 2009; Forster et al. 2009).

1.3.2.2 *mtDNA Cytochrome Oxidase Subunit I (COI)*

Cytochrome Oxidase Subunit I (COI) has been used as a target gene for a number of molecular phylogenetic and identification studies (Lunt et al. 1996; Howland and Hewitt 1995). The size of COI genes can vary between different species of mammal, but tends to be invariable within the same species, ranging from 1,537 to 1,557 bp (see Tobe et al. 2009). The COI gene is the largest of the three mitochondria-encoded Cytochrome Oxidase subunits, and is one of the largest protein-coding genes in the metazoan mitochondrial genome which has led to its use in DNA barcoding (Hebert et al. 2003; Blaxter 2004). DNA barcoding entails genetically characterizing species using a short (*i.e.* 600 bp) fraction of the COI (the DNA barcode), whose sequence could potentially be used as a reliable diagnostic taxonomic character (Hebert et al. 2003; Blaxter 2004; Moritz and Cicero 2004; Frezal and Leblois 2008; Shneyer 2009; Valentini et al. 2009). DNA barcoding has proved feasible for species identification in many groups of organisms as well as revealing cryptic diversity (see *e.g.* Hebert et al. 2003; Armstrong and Ball 2005; Smith et al. 2006; Hajibabaei et al. 2007; Borisenko et al. 2008) and determining the intraspecific diversity (see Rasmussen et al. 2009

1.4 Biology of the common dormouse, *Muscardinus avellanarius*

1.4.1 *Physical characteristics*

The common dormouse, *Muscardinus avellanarius* (see Figure 1.0), which is the smallest of the European dormice, is a habitat specialist and inhabiting deciduous forests that maintain a thick layer of scrub plants and underbrush. *M. avellanarius* is a nocturnal and

arboreal animal, which has a head to tail length of between 115 and 164 mm and weight around 15-30 grams (Morris 2004). The common dormouse has golden fur on the back and a pale, cream-colored underside (Bright et al. 2006). Its feet are very flexible and adapted for climbing and thus dormice are wholly arboreal animals, spending more than 90 percent of their time at two metres or more above the ground in spring and summer. Despite this, dormice may descend to lower areas during the autumn, although they still avoid activity on the ground (Bright and Morris 1994).

1.4.2 Hibernation

In Britain, *M. avellanarius* spend about half the year in hibernation, from October until May, during which time they are inactive and on the ground, seemingly vulnerable to disturbance and predation, in contrast to the active season (Bright and Morris 1996; Morris 2004). Dormice will select a cool damp place where the temperature will remain constant throughout the winter to avoid them drying out. Hibernation is a strategy to overcome the problem posed by lack of food in the winter, but it subjects dormice to significant physiological challenges (Vogel 1997; Morris 2004). Dormice survival during hibernation period mostly depends on the duration of winter and temperatures. Longer winter, higher and variable temperatures can negatively affecting common dormice, especially small individuals (Vogel and Frey 1995; Vogel 1997; Morris 2004; Juškaitis 2005). Hibernation in common dormice through investigations of nests, frequency of arousal and body temperature was first studied in nature by Vogel and Frey (1995). Under natural conditions, warming and arousal are normal in all hibernators, where hibernating dormice wake during the day suggesting that arousal is prompted by daytime temperatures (Morris 2004).

1.4.3 Feeding habits

M. avellanarius feeds on tree flowers (pollen and nectars) during the spring, fruits in the summer and nuts in autumn (Bright and Morris 1996; Bright et al. 2006); insects (including aphids and lepidopteran larvae) are also taken, opportunistically, in the dormouse diet (Bright and Morris 1995). Dormice will also eat buds and young leaves, but only small amounts of mature leaves as they lack a caecum in their digestive system (Bright et al. 2006). Nuts of the hazel, *Corylus avellana*, are regarded as a favourite food of dormice, with nuts eaten by dormice having distinctive tooth marks; the inner rim of hazel nuts nibbled by dormice is carved virtually smooth and the tooth marks are at an angle to the

hole (Bright et al. 2006). Since the association between dormouse and hazel is particularly strong, this animal's Latin name derived from 'avellanarius' which means 'hazel' (Bright et al. 2006). The characteristic way that dormice open hazel nuts provides a highly reliable survey method to determine their presence in woodland, for example the Great Nut hunt 2001-2002 event (PTES 2008; also available at http://www.ptes.org/ moremammals/gnh/).

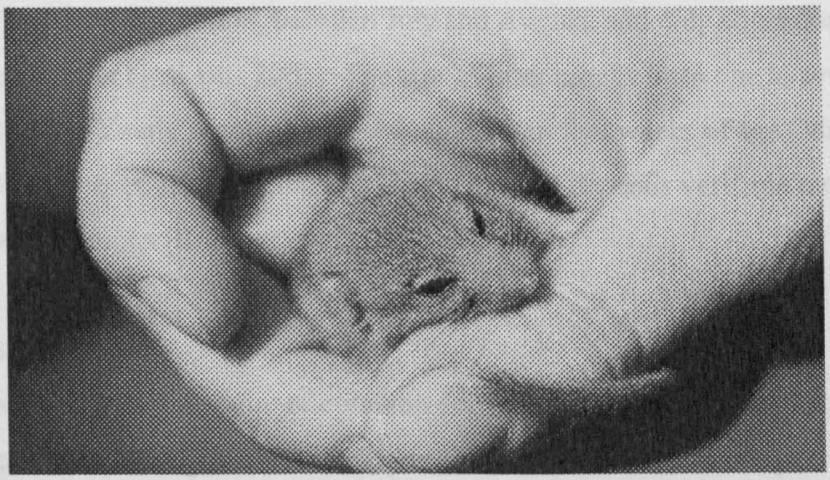

Figure 1.0 A common dormouse *Muscardinus avellanarius* caught during routine monitoring at Bontuchel (see Chapters 3 and 4).

1.4.4 Habitat requirements

Good quality dormouse habitat should comprise a variety of plant species that will ensure availability of food resources throughout the period of dormouse activity (Morris 2004). In Britain, their primary habitat appears to be ancient semi-natural woodland in which there is a high diversity of tree and shrub species (Bright and Morris 1996). Besides ancient woodland, dormice also inhabit deciduous forests, mature scrub, coppice and overgrown hedgerow. These habitats provide abundant food because such places receive plenty of sunlight, which ensures the bushes have abundant flowers, fruits and insects (Morris 2003). However, habitat requirements of dormice may be more flexible in southern European countries (*i.e.* Italy, Ukraine) (Bright and Morris 1994; Capizzi et al. 2002; Morris 2004; Zaytseva 2006). Bright and Morris (1996) found that dormice thrive best in diverse and low growing woodland, especially hazel coppice that is about 10-20 years old, but not in older hazel overgrown by taller trees as this can diminish nut production. During their active period, dormice build spherical nests situated a few feet from the ground in which they

spend most of the day, before emerging after dark to forage in understory (Bright and Morris 1996; Morris 2004).

1.4.5 Reproduction

Dormice have low reproductive potential, and shows large inter annual variation in reproductive success (Bright et al. 1994; Büchner et al. 2003). In most cases, dormice produce only one litter per season, between July and August, and only some dormice will produce a second litter (Juškaitis 2003). The size of litter ranges from one to seven young, but mostly is 3 or 4. Young become independent at about five weeks of age (Miller and Yahnke 2004). However, the breeding season and success depends very much on environmental conditions (Büchner et al. 2003).

1.4.6 Life span

Little is known about the longevity of common dormouse in the wild, however, Juškaitis (1999) reported that the longest known lifespan of a wild individual was 4 years for both sexes. In captivity, individuals have lived for up to 5.3 years (Morris 2004; Bright et al. 2006).

1.4.7 Behaviour

In almost all their distribution range, dormice will descend to the ground during winter to save their energy by hibernation (Juškaitis 2001; Morris 2004). They choose a site that is cool and damp and will remain stable, as these conditions are vital to save fat longer (Bright et al. 2006). Fat accumulates during the summer and the weight of a dormouse changes during the year (Juškaitis 2001). Hibernation begins around the first frosts in autumn, usually in October or November (Bright and Morris 1996), when there is little food available in the trees.

1.4.8 Distribution

Muscardinus avellanarius is distributed across Europe, from the Mediterranean to southern Sweden, eastward to Russia excluding Iberia and extends its range into parts of northern Asia Minor (Figure 1.1) (Juškaitis 2007; IUCN 2009). Island populations occur in southern Britain and also on Corfu and Sicily (Morris 2003; Rossolimo et al. 2001). In many parts of this species' northern range (*i.e.* in the UK, Netherlands, Sweden, Germany and Denmark)

its populations are declining and becoming increasingly fragmented (IUCN 2009). In the UK, for example, common dormice are found in particularly isolated populations in northern Wales (Bright et al. 2006).

Originally, common dormice were widespread over much of southern England and in the Welsh border region (Figure 1.2a) and thus was considered as 'common'; known localities were recorded also in many of midland and northern counties and Wales (Figure 1.2a, shaded pink areas with specific locations shown as yellow dots) (available at http://greenboot.co.uk/dormice/dormouse-distribution/). In 1993 and 2001, public surveys were organised to find and identify hazelnuts eaten by dormice as there was concern for this species. The results demonstrated that in less than a century, common dormice had disappeared from many counties in the UK, being lost from about half of its original range in Britain predominantly from southern England and the Welsh borders (Figure 1.2b). The most recent distributional map, using data from the Great Nut Hunts, sites from the National Dormouse Monitoring Programme, Dormouse Reintroduction Sites and other survey data is shown in Figure 1.2c and indicates that due to the reductions in number in *M. avellanarius*, several reintroductions and National Dormouse Monitoring Programme (NDMP) sites were established. Data from NDMP shows that the average dormice recorded per visit in 2007 was 4.5, 1.2 greater than dormice recorded in 2001 (NDMP, unpublished data). Despite the increased captures, these data include recaptured dormice, and are therefore with respect to the actual number of individuals in all NDMP sites.

1.4.9 Conservation status

M. avellanarius is a charismatic mammal and an important example of a flagship species for conservation (Morris 2004; Bright et al. 2006). The common dormouse is listed on Schedule 6 of the Wildlife and Countryside Act in 1981 (as amended 1986), and was granted full protection as a Schedule 5 species in 1986 (Morris 2004). This species is categorised as "least concern" on the Red List (IUCN 2009) and listed in the Habitat Directive (1994). The dormouse has been on English Nature's species recovery programme since 1992 (MacDonald and Tattersal 2003).

With increased land use and destruction of native habitats by humans, habitat fragmentation is common worldwide and as a result, native habitats have become more fragmented

(Gaines et al. 1997), posing an ever increasing threat to the existence of many plant and animal species (Meffe and Carroll 1994). Habitat loss and fragmentation were identified as a major threat to the population decline of the common dormouse (IUCN 2009) due to isolation of woodland and inappropriate woodland management (Bright et al. 1994; Bright and Morris 1996). In the case of common dormice, it is expected that the population will exhibit decline population trend lead to extinction since this species tends to occur at low population densities, where even in the best habitat, they do not exceed 10 adults per hectare (Morris 2004).

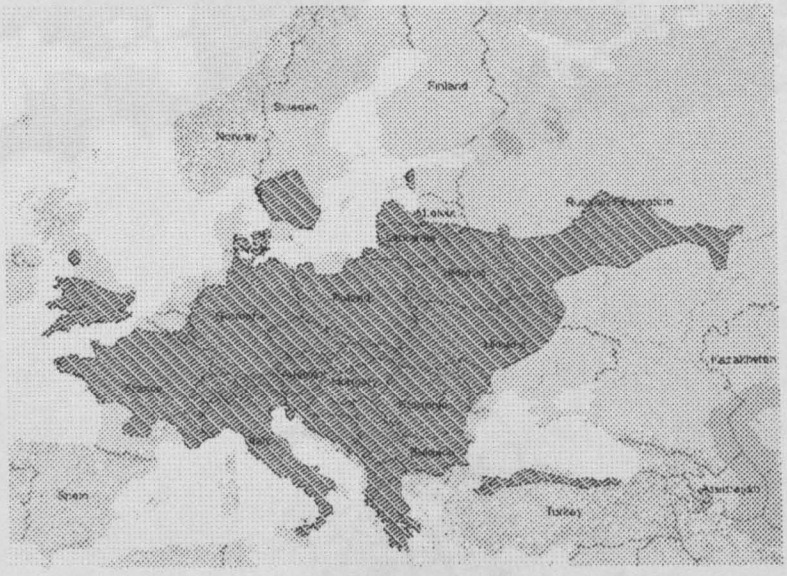

Figure 1.1 Distribution of the common dormouse *Muscardinus avellanarius* (red shaded areas) throughout Europe and Asia Minor (Turkey). *Source*: IUCN (2009) available at http://www.iucnredlist.org/apps/redlist/details/13992/0.

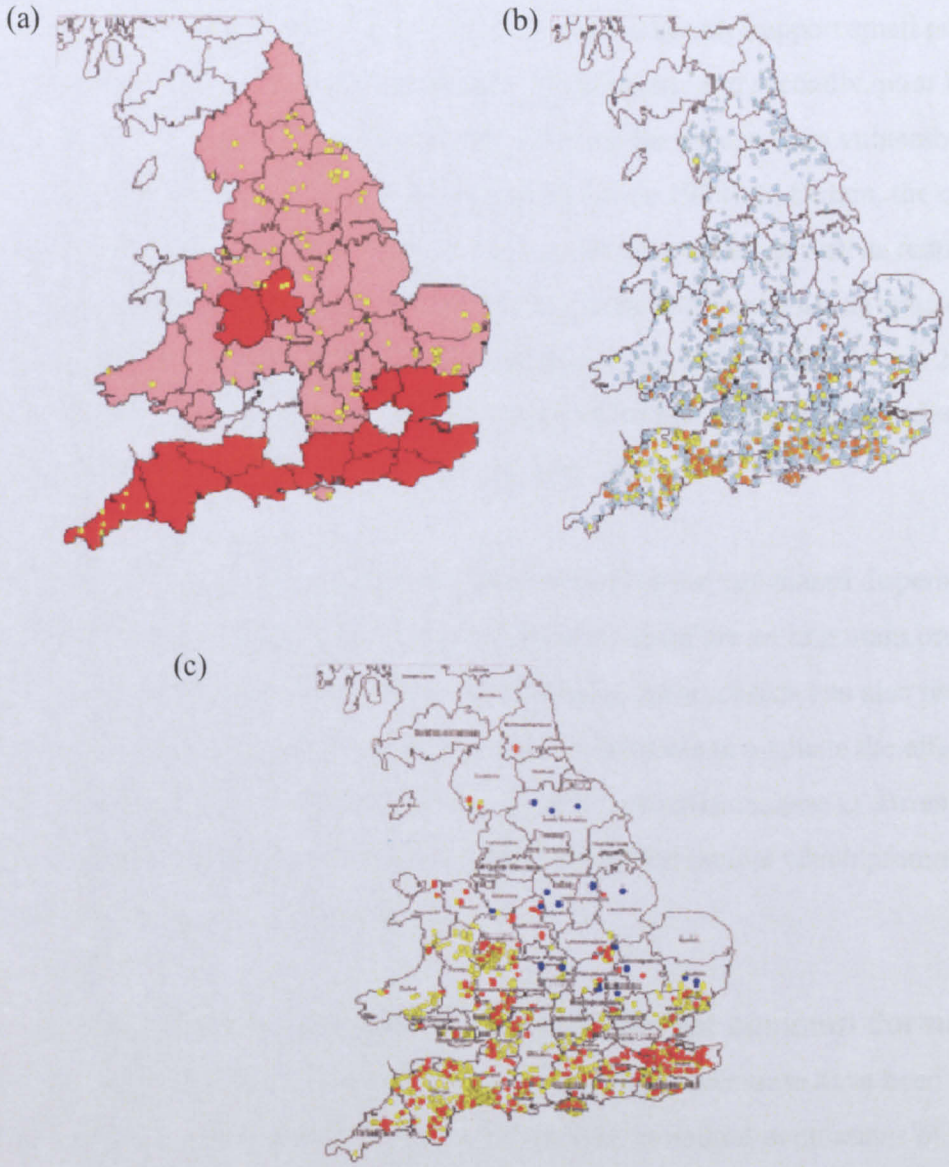

Figure 1.2 Summary of historical and present distributional records of the common dormouse in Great Britain. (a) General dormouse distribution in the early 1900s, with common and known locations of dormice (shaded red and pink respectively), yellow dots shows the specific localities of dormice; (b) results from public survey of dormice (1993-2001) indicating positive (yellow and orange) and negative (blue) records; (c) distribution of common dormice in 2007 - reintroduction sites (blue), National Dormouse Monitoring Programme sites (red) and Great Nut Hunts survey sites (yellow). *Source*: http://greenboot.co.uk/dormice/dormouse-distribution/

There are two ways where extinction risk may be influenced by poor habitat quality as noted by Pimm et al. (1988) and Hakoyama et al. (2000) (reviewed in Griffen and Drake 2008); firstly, poor quality habitats as in small habitats can only support small populations that are prone to extinction from demographic stochasticity, and secondly, poor habitat quality diminishes a population's growth rate delaying the escape from vulnerability when populations are small (*cf.* arguments developed by Lande 1988). In Britain, the clearance of the native woodland has increased the number of habitat patches, as well as reduced habitat richness and quality, and this has affected the distributions of many native species (Peterken 1986, 1997; Harmer et al. 2001; Verheyen et al. 2004; Vellend et al. 2006; Peterken 2009), including the common dormouse which has suffered a 64 % decline in number in Britain since the late 1970s (Bright et al. 2006).

Understanding the genetic and social structure, as well as the sex-biased dispersal and parentage of broods of *M. avellanarius* populations is therefore an important prerequisite for effective conservation of the species. Additionally, genetics data can also provide information for management planning by making it possible to evaluate the effect of genetic changes in the survival and persistence of the populations (see O' Brien 1994), and enable biologists to better identify management needs and factors which promote population expansion or contraction.

1.5 Conservation and current recommendation for common dormouse

Numerous studies of the biology and ecology of common dormouse have been carried out in Europe, for example, studies on dormancy patterns in natural populations of *M. avellanarius* (Juškaitis 2005), assessment of quantitative ultrastructural changes of hepatocyte constituents in euthermic, hibernating and arousing dormice (Malatesta et al. 2002), investigation of seasonal activity patterns (Panchetti et al. 2004) and also reproductive and breeding activities (Büchner et al. 2003). Generally, dormouse studies are based on nestbox assessments of population structure and activity (see *e.g.* Gaisler et al. 1977; Morris et al. 1990).

The common dormouse is a species of high conservation prioroty in the United Kingdom Biodiversity Action Plan (UKBAP) and also under the Species Action Plans, in which the main objectives are to: (1) maintain and enhance dormouse populations in all the counties

in the UK where they still occur and (2) re-establish self-sustaining populations in at least 5 counties where they have been lost. To achieve these objectives, several actions with lead agencies were proposed in all aspects, including site safeguard and management, which focused on mitigation and compensation method – in particular ameliorating the effects of habitat fragmentation by encouraging dispersal. In response to habitat fragmentation, for species that is apparently reluctant to cross open ground, such as the common dormouse, building 'green bridges' that allow some degree of habitat continuity represents a strategy that is hoped to encourage natural dispersal movements. This method was used in Japan, where a dormouse bridge was built and used by a Japanese dormouse (*Glirulus japonicus*) within a year. In Britain, a similar structure is currently being trialed, though its success has yet to be demonstrated (Bright et al. 2006).

In addition there has been an extensive captive-breeding programme, with the view to reintroducing these animals to previously inhabited sites. A specialist consortium of zoos and volunteers (known as Common Dormouse Captive Breeders Group, CDCBG) maintains a captive population of dormice that rescued from dead and with cooperation from English Nature (EN) and Mammals Trust UK, this group maintains the animals in sufficiently large numbers for reintroduction projects (Bright et al. 2006). Some detailed research was required for the success of the reintroduction programs, including determination of the best time of year for reintroductions takes place (Morris 2004), selecting suitable sites for releases and a disease risk analysis (Bright et al. 2006). Starting in 1992, the common dormouse has been reintroduced to a number of sites in England (Joint Nature Conservation Committee, JNCC 2007), with the first reintroduction site in Cambridgeshire; subsequent monitoring has indicated that dormice have spread throughout the habitat site and are beginning to disperse into neighboring hedgerows (Morris 2004). To date, more than a dozen successful reintroductions have taken place (Figure 1.2c; Bright and Morris 2002; see also Chapters 2, 3 and 4 for analysis of one reintroduced population at Wych, England) and most of the new populations show every sign of being successfully established with steadily increasing numbers (Morris 2004; Mitchell-Jones and White 2009).

Most studies that have compared behaviour of captive breeding and wild populations of a species found that captivity has compromised the behaviour of the captive bred individuals

which failed to retain their natural behaviour when released into wild (McPhee 2003; Hellstedt and Kallio 2005; Kelley et al. 2006; Fischer and Lindenmayer 2000, for review). Thus, such information is very crucial as it has impact on common dormice conservation (see Chapters 3 and 4).

In spite of its threatened status and the large amount of work put into captive breeding and reintroductions, no reports or research has been done to analyse the patterns of gene flow and concomitant spatial genetic and social structure of *M. avellanarius* using molecular genetic markers. Moreover, none of the breeding and dispersal characteristics have been explored in any reintroduced site.

1.6 Aims of this thesis

The aims of my thesis are: (**1**) to develop a panel of microsatellite DNA markers that can be utilised to analyse levels of genetic variation and patterns of gene flow among dormice populations (Chapter 2), (**2**) to obtain information about the social structure, particularly the mating pattern, in natural and reintroduced common dormice to determine whether there are any differences in reproductive behaviour between wild and captive-bred individuals (Chapter 3), (**3**) to quantify the natural dispersal patterns of the individuals in large habitats through a combination of field-surveys and molecular-genetic techniques, to determine levels of genetic diversity in natural and reintroduced populations, and to quantify whether the dispersal behaviour differs between wild and captive-bred individuals (Chapter 4), (**4**) to determine the pattern of population genetic structure among discrete habitat patches and contrast this with patterns of spatial structure in a large continuous habitat (Chapter 5), and (**5**) to determine the phylogeographical pattern of genetic variation among UK dormice populations (using sequence variation at mtDNA loci) to better understand genetic differences among regions and the effect of dormouse reintroductions (Chapter 6).

Chapter 2: General Materials and Methods

2.0 Study site descriptions

Common dormice, *Muscardinus avellanarius*, were caught during routine sampling predominantly (but see also Chapters 5 and 6) at two sites at the northern edge of this species' UK distribution: (1) Bontuchel (Denbighshire, Wales; Latitude 53.109364 N; Longitude: -3.370318 W; OS National Grid Reference, SJ082571), and (2) Wych Valley (Cheshire, England; Latitude 52.994994 N; Longitude -2.7745169 W; OS National Grid Reference, SJ4811244 (Figure 2.1). Samples were held at the University of Liverpool under license from Natural England (NE) and the Countryside Council for Wales (CCW).

2.0.1 Bontuchel, Denbighshire, Wales

The 73 ha woodland at Bontuchel, near Ruthin in Denbighshire is a Planted Ancient Woodland Site (PAW), owned by Forestry Commission Wales. This site is a mixed broadleaf and conifer woodland with many of the hard woods being coppice. Conifers in this woodland have now mostly been removed and replaced by natural regeneration of native tree species managed in a variety of ways. In some parts of the site, broadleaf regeneration was allowed to persist due to extent and density of hazel and oak under-storey, which had persisted through the mature larch stands. In 1997 and 2001, other parts of the woodland which had developed mixture of broadleaf and conifer were selected for conifer removal, and this has resulted in a cleaner stand of predominantly oak and other, mixed broadleaf trees.

Today management is focused on PAWs restoration processes where the remaining conifer will be gradually thinned to allow broadleaf regeneration to develop, initially as an under storey and finally producing a mixed conifer and broadleaf high forest with a dense native shrub layer. A large part of the woodland is being managed as a 15-year coppice rotation where a panelling regime and a planned working programme has been produced to retain connectivity. Generally, plantations in Bontuchel consist of blocks of western hemlock, Japanese and European larch, Scots pine, Norway spruce, and Noble and Douglas firs.

Common dormice were discovered at Bontuchel in the early 1990s, occupying bird nest boxes, and the site has been monitored intermittently between 1992 and 2004, and intensively since 2005 by the Northwest Dormouse Partnership (available at http://www.cheshirewildlifetrust.co.uk/proj_dormouse_partnership.htm).

2.0.2 Wych Valley, Cheshire, England

Wych Valley in south Cheshire is one of a number of small enclosed predominantly wooded valleys within the rural Eastern Lowlands of Wrexham. Wych Valley surrounded by unintensive farming, hedgerows and various species of tree, and remnants of lowland pasture, rush pasture and upland mixed ash woodlands. This site is also semi-natural ancient woodland with some areas of planted conifers and other non-native deciduous trees. The main study site in this area is a narrow, steep-sided valley, where the wood is dominated by hazel, *Corylus avellana.* Other plants present of value to dormice include bramble (*Rubus fruticosis*), honeysuckle (*Lonicera periclymenum*) and sycamore *(Acer pseudoplatanus)*. Generally, the wood is not uniform in composition where there are a couple of small stands of poorly grown conifers, which have proved very suitable for dormice.

The Wych Valley was the site of a dormouse reintroduction in 1996 and 1997. These dormice were bred in captivity by the Common Dormouse Captive Breeders Group and given a full bill of health before release by Paington Zoo and the Zoological Society of London (Bright et al. 2006). There were 29 and 24 captive bred dormice that were released in 1996 and 1997 respectively into this site. However, data on the numbers of males and females released is not available. This reintroduction was the third to take place in England, under the guidance of English Nature (now Natural England). The populations have been carefully monitored ever since using nest box surveys. Four years following these reintroductions, the common dormice appeared to be spreading through the small wooded areas available within the site, as evidenced by breeding and population growth data (Morris 2004; Bright et al. 2006).

2.1 Sampling

Monitoring of common dormouse in the Bontuchel and Wych Valley has been the primary focus of a long-term study by North West Dormouse Partnership Project (also available at

http://www.cheshirewildlifetrust.co.uk/proj_dormouse_partnership.htm), started in 2005. Together with the trapping undertaken during the present study (2006-2008), this resulted in a total of 5 years of data available from the trapping sites described above. These data have been utilised in the later chapters (Chapters 3, 4 and 5).

Within each sampling site, a fixed-position of wooden nest boxes have been set up to trap a dormice, laid out in irregular transects. The nest boxes are all positioned on fence posts using wires, mostly very close to existing trees or shrubs at 1.5 m above ground level. The fence posts are used so the boxes can remain in place during management work on different parts of the site. All nest boxes were spaced at 15–26 metre intervals. Nest boxes coordinates were recorded by using a hand-held GPS. There were 230 nest boxes installed in Wych Valley and 250 nest boxes in Bontuchel (Figure 2.1).

All nest boxes at Wych Valley were monitored in May, June, September and October and nest boxes in Bontuchel were inspected in May and June and September and October. During monitoring session, dormice found were scanned for microchips and biological data such as sex, weight, age estimate and breeding status were recorded. Dormice without microchips were taken to the veterinarian in the field and 8 mm microchips were inserted under general anaesthetic, using isoflourane and oxygen for resuscitation. For genetic analysis, hair and buccal swab samples were collected from both sites from 2006 to 2008.

2.2 Sample collection

To collect the saliva samples of common dormice, swabs were scraped with a cotton-stick against the inside of the cheek of each dormouse. Because microsatellite typing becomes more reliable if more than 10 hairs are analysed (Goossens et al. 1998; Md. Naim pers. obs.), bundles of approximately 50-100 hairs with roots were plucked from each dormouse by using a vein clip or forceps. DNA for the initial library construction (see section 2.4.1 below) was extracted from tail tips taken from dead dormice (ones that were found dead during survey work or died during the captive breeding programme). Each sample was placed into a sterile, numbered plastic bag, and stored at -20^{0}C before further analysis.

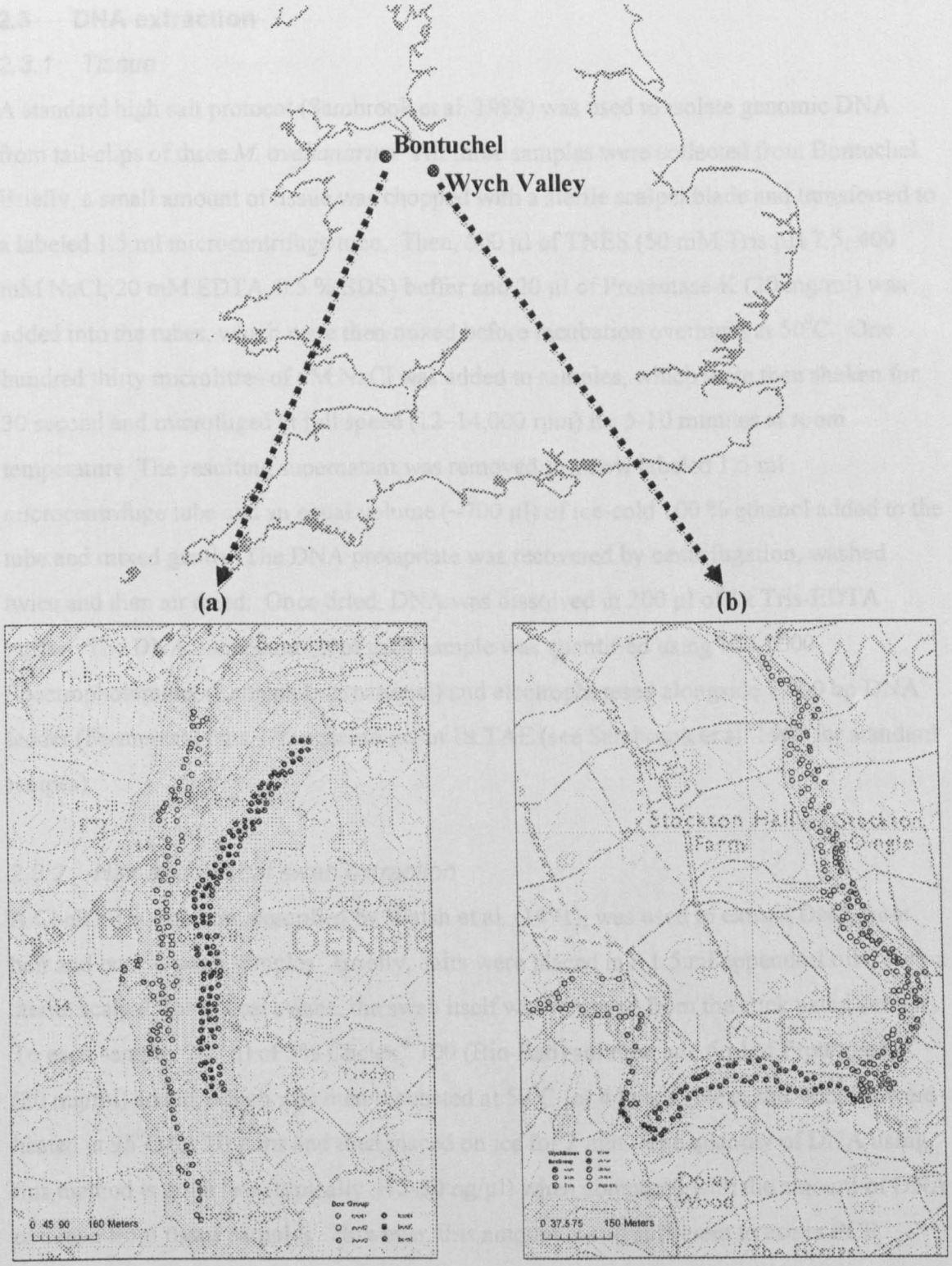

Figure 2.1 The locations of the two main dormouse monitoring sites in this study: (a) Bontuchel and (b) Wych Valley, and the positions of nestboxes within each site (coloured circles).

2.3 DNA extraction

2.3.1 Tissue

A standard high salt protocol (Sambrook et al. 1989) was used to isolate genomic DNA from tail-clips of three *M. avellanarius*. All three samples were collected from Bontuchel. Briefly, a small amount of tissue was chopped with a sterile scalpel blade and transferred to a labeled 1.5 ml microcentrifuge tube. Then, 500 µl of TNES (50 mM Tris pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5 % SDS) buffer and 20 µl of Proteinase-K (20 mg/ml) was added into the tubes, which were then mixed before incubation overnight at 50^{0}C. One hundred thirty microlitres of 6M NaCl was added to samples, which were then shaken for 30 second and microfuged at full speed (12–14,000 rpm) for 5-10 minutes at room temperature. The resulting supernatant was removed to a new labeled 1.5 ml microcentrifuge tube and an equal volume (~700 µl) of ice-cold 100 % ethanol added to the tube and mixed gently. The DNA precipitate was recovered by centrifugation, washed twice and then air dried. Once dried, DNA was dissolved in 200 µl of 1x Tris-EDTA buffer. The DNA concentration of each sample was quantified using ND-1000 Spectrophotometer (Labtech International) and electrophoresed alongside 1,000 bp DNA ladder (Fermentas) on a 1 % agarose gel in 1x TAE (see Sambrook et al. 1989 for standard recipes).

2.3.2 Hair and buccal swab extraction

A Chelex-100 method, described by Walsh et al. (1991), was used to extract DNA from hair and buccal swab samples. Briefly, hairs were placed in a 1.5 ml eppendorf tube with a sterile scalpel; for buccal swabs, the swab itself was removed from the stick using scissors. To each sample, 200 µl of 5% Chelex® 100 (Bio-Rad) solution and 5 µl of Proteinase-K (20 mg/ml) added, which was then incubated at 50^{0}C for 4 hours. Next, the samples were heated at 95^{0}C for 10 mins and then placed on ice for 2 min. The quantity of DNA using this method is quite low (typically ~15-30 ng/µl) when compared with the amount of DNA obtained from tissue samples. However, this amount is still sufficient to use in PCR amplification.

2.4 Molecular markers

2.4.1 Isolation of microsatellite libraries by enrichment

Microsatellites, also known as Short Tandem Repeats (STRs) or Simple Sequence Repeats (SSRs), are loci that vary in the number of repeats of a simple DNA sequence (Slatkin 1995; Goldstein et al. 1995; Jarne and Lagoda 1996; Polziehn et al. 2000; see Chapter 1). This type of genetic marker is highly polymorphic and possesses a number of important features that make them useful for population studies (Jarne and Lagoda 1996). There are several ways to obtain microsatellite markers from a target species, for example by the traditional way of hybridising microsatellite probes against partial genomic libraries and then sequencing the positive clones (Zhou et al. 2007). However, for this study, I used a modified microsatellite enrichment technique, which significantly reduces time and cost required to develop microsatellite loci *de novo*, since greater numbers of positive clones are identified during screening (see Billotte et al. 2001; Zane et al. 2002; Selkoe and Toonen 2006).

2.4.1.1 Digestion of DNA and adapter ligation

The enrichment procedure followed the protocol of Bloor et al. (2001). Approximately 30 ng of genomic DNA was partially digested at 37°C for 2 hours using 40 u *Sau3A* restriction enzyme (Boehringer-Mannheim) in a final volume of 90 µl. Next, the DNA fragments were ligated to 50 pmol of phosphorylated linkers (SauLA -> 5'-GGC CAG AGA CCC CAA GCT TCG -3' annealed to SauLB -> 5'-PO_4-GAT CCG AAG CTT GGG GTC TCT GGC C-3'; Refseth et al.1997) using 40 u T4 DNA ligase (Promega) and incubation at 4°C overnight; the enzymes were inactivated by heating to 65°C for 10 mins.

2.4.1.2 Size selection and PCR-amplification of adaptor-ligated DNA

All digested DNA was electrophoresed for 20 mins at 100 V on a 1.8 % agarose gel containing 0.5 $\mu g ml^{-1}$ ethidium bromide and then run alongside a 100 bp PCR ladder (Promega). Under ultraviolet light the fraction of digested DNA between 500 and 1,100 bp was excised using a sterile scalpel and placed into a pre-weighed 1.5 ml microfuge tube. DNA was then purified using QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. The size-selected DNA was concentrated to a final volume of 15 µl using a Microcon YM-100 spin column (Millipore). Confirmation of successful ligation

was achieved by 10 µl PCR that contained: 1 µl DNA, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20®, 0.2mM each dNTP, 1.5 mM $MgCl_2$, 250 pmol primer SauLA and 0.625 u *Taq* polymerase (ABgene). Thermal cycling conditions were: 95°C for 5 min, followed by 30 cycles of 95°C for 50 s, 56°C for 1 min, and 72°C for 2 min, and finally 72°C for 10 min. PCR success was determined by running 5 µl of PCR product on a 2% agarose gel alongside a 100 bp DNA ladder (Promega) with success indicated by a smear between 400 and 1,100 bp.

2.4.1.3 Capture of microsatellite DNA-containing fragments

This DNA fraction was then enriched for combination of $(CA)_{12}$ and $(TCAC)_6$ repeats by using the following protocol. First, 100 µl of streptavadin-coated magnetic beads (10 $mgml^{-1}$) (M-280 Dynabeads, Dynal) were washed twice with 100 µl of 1X Washing/Binding (W/B) buffer (1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) in a 0.5 ml microcentrifuge tube. Magnetic beads were then resuspended in 200 µl of 2X W/B buffer to which 100 pmol of each of the 3'-biotinylated oligonucleotides (100 mM) $(CA)_{12}$ and $(TCAC)_6$ was added and then the mixture made up to a final volume of 400 µl. The sample was incubated at room temperature for 30 min with gentle agitation by pipetting every 5-10 min. Next, the beads were washed once in 400µl 1X W/B, twice in 400 µl of 6X SSC, and then re-suspended in 50 µl 6X SSC and incubated at 60°C. In a separate 0.5 ml microcentrifuge tube 10 µl of the ligated DNA, 20 pmol of SauLA and 20X SSC (*i.e.* a final concentration 6X) were made up to 50 µl final volume and gently mixed with a pipette and denatured by incubation at 95°C for 5 min. After this, the temperature was ramped down to 60°C and the contents of the re-suspended bead mixture added to the single-stranded DNA sample and gently mixed. The adaptor-ligated / bead-probe mixture was then incubated at 60°C for 30 min, with gentle agitation every 5 min. Next, the magnetic beads were separated from the supernatant using a magnet and the supernatant removed. Magnetic beads were re-suspended in 100 µl of 2X SSC and washed a further four times with 1 ml 2X SSC, incubating the sample for 5 min at room temperature between each wash. Following this, the bead mixture was washed an additional four times in 1X SSC, with incubation for 5 min at room temperature. After the final wash, the bead mixture was re-suspended in 100 µl of 1X SSC and aliquotted into four 25 µl samples. 250 µl of 1X SSC was added to each aliquot which was then incubated at 60°C for 10 min. The

supernatant was removed and the beads rinsed for 30 s at room temperature in 400 µl of 1X TE buffer. The supernatant was again removed and the beads rinsed for 30 s at room temperature in 400 µl 50 mM NaCl. Finally, aliquots were re-suspended in 50 µl PCR-grade water giving a final bead concentration of 5 $\mu g \mu l^{-1}$.

To check quantity of enriched DNA, a PCR was set up in a 10 µl final reaction volume consisting of 40 µg bead suspension, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20®, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 30 pmol SauLA and 0.625 u *Taq* polymerase (ABgene). PCR conditions were 95°C for 3 mins, 3 cycles of 95°C 30 s, 55°C 30 s, 72°C 45 s, followed by 30 cycles of 92°C 30 s, 55°C 30 s, 72°C 55 s, and finally 72°C for 10 min. 5 µl of the PCR product was run on a 2% agarose gel for 20 min at 100 v alongside a 100 bp ladder (Promega), with successful capture and PCR indicated by a smear between approximately 0.4 and 1.1 Kbp. PCR products were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol.

2.4.1.4 Ligation and transformation

Approximately 50 ng of PCR purified products were ligated into 50 ng of pGEM-T vector (Promega) using 3 Weiss units of T4 DNA ligase in a 10 µl final volume following the manufacturer's instructions. The ligation mixture was incubated overnight at 4°C to provide the maximum number of transformants. Several aliquots of 2 µl of the ligation mixture were then each transformed into 100 µl JM109 high efficiency competent *Escherischia coli* cells (Promega), again following the manufacturer's protocol. 50 µl of each transformation reaction was plated onto S-gal agar (Sigma) plates containing 100 µgml-1 ampicillin (Sigma) and incubated overnight at 37°C; bacterial colonies with a vector and an insert were identified because of their white colour.

2.4.1.5 Library construction and microsatellite screening

Using a sterile toothpick, white colonies were picked and swirled in one well (of a 96-well plate) containing 100 µl LB media and ampicillin (final concentration of 100 $\mu g ml^{-1}$). For microsatellite screening, the same toothpick was then swirled into a corresponding well of a 96-well plate containing 20 µl PCR consisting of 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 10 pmol SauLA,

10 pmol CA_{15} oligonucleotide and 0.25 U *Taq* polymerase (ABgene). LB/ampicillin plates were incubated at 37°C for 3-4 hours and then 100 µl of sterile LB/30% glycerol was added to each well; these LB/glycerol plates are then stored indefinitely at -80°C. Thermal cycling conditions for the microsatellite-screening PCR were: 95°C 3 min, 3 cycles of 95°C 30 s, 56°C 30 s, 72°C 45 s, followed by 30 cycles of 92°C 30 s, 56°C 30 s, 72°C for 55 s, and finally 72°C for 10 min. 5 µl of the PCR product was run alongside a 100 bp PCR ladder (Promega) on a 2% agarose gel containing ethidium bromide (at a final concentration of 0.5 μgml^{-1}) at 100 v for 20 min. When visualized under UV light, a double banded PCR product indicated the presence of a microsatellite containing insert (see Figure 2.2).

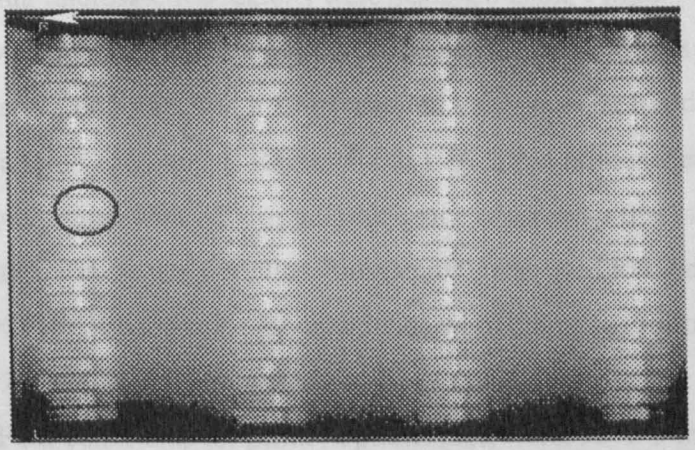

Figure 2.2 PCR screening of enriched clones (on a 2% agarose gel). The circle highlights a double banded PCR product that indicate a plasmid with a microsatellite-containing insert.

2.4.1.6 Sequencing microsatellite containing inserts

From the libraries constructed above, *E. coli* bearing plasmids with microsatellite inserts were streaked onto single LA/ampicillin (100 μgml^{-1}) plates and incubated at 37°C overnight. Single colonies were inoculated into individual 50 ml Falcon tubes containing 10 ml of LB/ampicillin and grown overnight at 37°C with gentle shaking (at 150 rpm). Plasmids were prepared from 2 ml of this culture using a Qiagen plasmid mini kit according the manufacturer's instructions.

I then cycle-sequenced 192 positive clones using standard M13 forward primer (5'- TGT AAA ACG ACG GCC AGT 3') and Big DyeTM chemistry (Applied Biosystems) and electrophoresis on an ABI3100 (Applied Biosystems). Sequences were analysed using ChromasPro ver. 1.5 (Technelysium Pty Ltd.) to identify microsatellite repeat motifs. Samples containing five or more microsatellite repeat units were reverse sequenced using M13 reverse primer (5'- CAG GAA ACA GCT ATG ACC 3') and the reverse and forward sequences aligned with ChromasPro ver. 1.5 (Technelysium Pty. Ltd.) to check for consistency. Microsatellite-containing inserts were then sequenced on an ABI3100 automated DNA sequencer (Applied Biosystems).

2.4.1.7 Primer design and PCR optimisation

Primers flanking microsatellite regions were designed using Primer3 v. 0.4.0 (available at http://fokker.wi.mit.edu/primer3/input.htm). To test for polymorphisms, microsatellite alleles were amplified by PCR in a 10 µl reaction volume using ReddyMix PCR mix (ABgene) on a Dyad Engine (MJ Research Inc.). A tailed primer method, whereby forward primers are synthesised with a 5' (or tail) sequence of a primer that is labeled with 6-FAM, NED, PET or VIC fluorophores (Applied Biosystems), was used to label PCR products (see Schuelke 2000). PCR conditions for each locus were optimised for primer annealing temperature (T_a) and $MgCl_2$ concentration using a gradient PCR that spanned the predicted T_a by ±5°C. PCR conditions for primer optimisation were: 95°C 3 min, 30 cycles of 95°C 30 s, a gradient of T_a°C 30 s, 72°C 45 s, and finally 72°C 10 min. Each PCR contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, either 1.5 or 3.0 mM $MgCl_2$, 5-50 ng template DNA, 10 pmol each primer and 0.25 U *Taq* polymerase (ABgene).

Amplified products with different fluorescent labels or non-overlapping size ranges were pooled with a 500 bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI3100*xl* DNA sequencer and sized using GENEMAPPER software (Applied Biosystems).

2.4.1.8 Characteristics of microsatellite loci

Out of the 192 putative clones sequenced, 83 (43 %) contained a potentially useful (> 6 repeat units) microsatellite motif. Primers could be designed around 28 of these loci

(Appendix 2.1), however eighteen primer pairs were dropped subsequently because they either failed to amplify or produced multiple/spurious bands during PCR: this left just ten microsatellite loci. The characteristics and primer sequences of these loci are shown in Table 2.1, with Appendix 2.2 comprising the "Primer Note" that described these loci in detail (Md. Naim et al. 2009).

2.4.1.9 Analysis of microsatellite polymorphism

Level of polymorphism at 10 microsatellite loci was assessed in139 individuals collected from one site in North Wales, Bontuchel (Denbighshire, Wales; Latitude 53.109364 N; Longitude: -3.370318 W; OS National Grid Reference, SJ082571), UK. The online version of GENEPOP ver. 3.4 (Raymond and Rousset 1995, also available at http://wbiomed.curtin.edu.au /genepop/) was used to calculate basic measures of genetic diversity, the significance of any deviations from expected Hardy-Weinberg equilibrium (HWE) and also for linkage disequilibrium between all pairs of loci. FSTAT ver. 2.9.3 (Goudet 1995) was used to calculate allelic richness (A_R). The software MICROCHECKER (Van Oosterhout et al. 2004) was used to detect the most probable technical cause of HWE departures such as null alleles, mis-scoring due to stuttering and allelic dropout. BOTTLENECK ver.1.2.02 (Piry et al. 1999) software was used to compute an expected distribution of heterozygosities (H_e) under mutation-drift equilibrium from the allelic diversity of each sample for three different models of allelic mutation: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM).

2.4.2 Mitochondrial DNA

2.4.2.1 D-Loop

Mitochondrial (mt) DNA is an organellar genome, has a great variability in structure, gene content, organization and mode of expression in different organisms (Avise 1994; Lunt et al. 1996; Saccone et al. 1999; Ballard and Rand 2005). The primer pair designed by Stacy et al. (1997) (M15997: 5'-TCCCCACCATCAGCACCCAAAGC-3' and H16401: 5'-TGGGCGGGTTGTTGGTTTCACGG-3') were used to amplify the 495 bp target region in the d-loop section. Primer screening was carried out by using a total of 24 samples consisting of swabs and hairs of *M. avellanarius*.

2.4.2.2 Cytochrome Oxidase Subunit I (COI)

A set of primers for the PCR amplification of the Cytochrome Oxidase subunit I (COI) gene in mtDNA was obtained from Pfunder et al. (2004) (RonM: 5'-GGMGCMCCMGATAT RGCATTCCC-3' and NancyM: 5' CCTGGGAGRATAAGAATATAWACTTC-3'). An attempt to gain a preliminary indication of the success of this marker to amplify the 490 bp target region in the COI section gene in *M. avellanarius* has been carried out with a hair samples from 24 individuals.

2.4.2.3 PCR amplifications and sequencing

The PCR technique was used to amplify the target region of the d-loop and COI gene in the mtDNA genome of *M. avellanarius*. Each PCR reaction mixture contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01n % (v/v) Tween 20, 0.2 mM each dNTP, either 1.5 or 3 mM $MgCl_2$, 5-50 ng template DNA, 10 pmol each primer and sterile distilled water to a final volume of 10 µl per reaction. Amplification consisted of an initial 3 min of denaturation (95°C) followed by 6 cycles of (95°C for 30 s, T_a°C for 45 s, 72°C for 45 s), 36 cycles of (92°C for 30 s, T_a°C for 45 s, 72°C for 55 s) with a final extension cycle of 10 min at 72°C, and hold temperature at 4°C, where T_a is the annealing temperature (either 54°C or 58°C depending on the locus). PCR products were then purified with EXOSAP-IT (Amersham) following manufacturer's standard protocol. DNA sequencing was performed using BigDye v1.1, v3.1 terminator (Applied Biosystems) sequencing kit, with approximately 20-30 ng of cleaned PCR products and 1.6 pmol of primer (forward and reverse separately) in each reaction. Sequencing products were cleaned and then electrophoresed on an ABI 3100xl capillary sequencer following standard protocols.

2.4.2.4 Sequence analysis

Sequences were edited and aligned using the Sequencing Analysis ver. 5.2 (Applied Biosystems). The resulting consensus sequences for each individual were then aligned using CLUSTAL W ver. 2.0.12 (Thompson et al. 1994) and were manually checked and trimmed in the BIOEDIT ver. 7.0.4 sequence editing program (Hall 1999); alignments were then subsequently revised by eye in an effort to maximize positional homology.

2.4.2.5 *mtDNA genetic variability*

The frequency of each haplotype, haplotype diversity (i.e. the probability that two randomly selected haplotypes are present in the sample, h) and nucleotide diversity (π) for all 24 samples was estimated using DNASP ver. 4.10 (Rozas et al. 2003).

2.5 Results

2.5.1 *Microsatellite*

The ten microsatellite loci were separated into two genotyping pools so that no two loci with the same fluorescent dye had overlapping allelic size ranges. All loci were highly polymorphic with mean observed heterozygosity of 0.792 (SE±0.077) and mean expected heterozygosity of 0.730 (SE±0.084) with 6-17 alleles per locus (see Table 2.1). There is no evidence for significant (P>0.05) deviation from Hardy-Weinberg equilibrium at any locus, nor did we detect genotypic disequilibrium between any pairs of loci. Similarly, allelic richness (A_R) was high (range: 7.79- 9.1). A few animals (n=3-8) failed to amplify alleles at some loci (see Table 2.1), however there was no significant (P>0.05) evidence for null alleles at any loci. There was no significant heterozygote excess (P>0.05) that is indicative of a population bottleneck for all mutation models and for both statistical tests.

2.5.2 *Mitochondrial DNA (mtDNA)*

Via PCR, both sets of primers consistently amplified approximately 401 bp and 432 bp fragments of the d-loop and COI gene respectively. The 24 individuals analysed generated 17 and 21 haplotypes from d-loop and COI gene respectively. Overall, the values of haplotype and nucleotide diversities were high, and greater in the COI (h=0.989±0.015; π=0.0498±0.0035) than in the d-loop (h=0.960±0.025; π=0.0090±0.0021) (see Chapter 6 for more details).

2.6 Discussion

I developed a panel of ten polymorphic microsatellite loci that were subsequently utilised to determine the breeding structure (see Chapter 3) and spatial genetic structure within and between populations (see Chapters 4 and 5) of *M. avellanarius*. To be useful for population genetic studies, these loci must be unlinked (see e.g. Goldstein et al. 1999; Pritchard and Rosenberg 1999). When tested with 139 individuals of *M. avellanarius*, the genotypes at these loci were independent. The level of genetic variation detected (H_e=0.615-0.889,

Table 2.1) in the study site is comparable to those at microsatellite loci in other mammal species (i.e. Harley et al. 2005; Lecis et al. 2008; Neaves et al. 2009) particularly species of rodents (see e.g. Loew et al. 2005; Brouat et al. 2007; Vega et al. 2007; Gauffre et al. 2008; Abdelkrim et al. 2009). The pattern of high genetic variability in *M. avellanarius* is unlikely to represent a signature of bottleneck and rapid range expansion of population (also discussed in Chapters 4 and 5), although clearly further characterisation of additional *M. avellanarius* populations from its range in Europe is required to confirm this result.

The level of average heterozygosities found in this study was generally higher than those of several other rodents (see Chapter 3, 4 and 5), indicating the higher genetic diversity of dormice over those species. However, it was noted that all the average observed heterozygosity H_o were higher than expected H_e in all loci except at two loci (Mav G6 and Mav H3; Table 2.1). This showing that there was a high degree of genetic variability in the dormice population studied and also might suggest an isolate-breaking effect (*i.e.* the mixing of two previously isolated populations) on the population studied (see Marson et al. 2005). Several other studies also reported the higher H_o values than H_e, *i.e.* Marson et al. 2005; Yue et al. 2008; Jacubczak et al. 2009; Pascoal et al. 2009; Shasavarani and Rahimi-Mianji (2010).

Numerous studies have reported a positive correlation between population size and within-population genetic diversity (see e.g. O'Ryan et al. 1998; Knaepkens et al. 2004; White and Searle 2007). However, any correlation is expected to be more pronounced for numbers of alleles (N_a) rather than expected heterozygosity (H_e) because rare alleles, which are uncovered with increasingly larger sample sizes, contribute little to H_e (see Stow and Briscoe 2005). Sample sizes of studies used to characterise microsatellite variability in this study are (n=192, Bontuchel; n=140, Wych; see Chapter 3), (n=296, Bontuchel; n=135, Wych; see Chapter 4) and (n=70, Bontuchel North; n=236, Bontuchel South; see Chapter 5). Clearly, variation in sample *per se* may explain the high diversity of *M. avellanarius*, thus the signal of generally high genetic diversity of *M. avellanarius* in this study site is certainly real rather than an artefact of poor sampling effort.

Table 2.1 Levels of variability at 10 polymorphic microsatellite loci in the common dormouse (*Muscardinus avellanarius*) from Bontuchel, Wales (n=139). N_a indicates number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; T_a, annealing temperature of primer during PCR. VIC, NED, 6-FAM and PET indicate the fluorophores used for genotyping. n_s=number of animals (out of 139) that produced a genotype.

Locus/ GenBank Accession No.	Primer Sequence (5'-3')	Repeat motif	T_a	Size range (bp)	n_s	N_a	H_o	H_e
MavE3	F: VIC-ATAGCCCAGGTAGAAAGC	$(CA)_{49}$	58	110-170	136	11	0.664	0.615
GF089515	R: TAGCATCCCGTTCTCAAACC							
MavB5	F: NED-GAAGGGCTGGGTATATATCATGG	$(CA)_{40}$	53	110-160	136	8	0.763	0.678
GF089516	R: AGTGCAAGGAGAGAGGGAGAGAGG							
MavG3	F: FAM-CACATGTGTTGACTGATTGAGTGG	$(GT)_{54}$	53	110-180	135	9	0.844	0.821
GF089517	R: AGAGCTGTTGTGGTTCTAAGG							
MavF10	F: PET-GTAGAGCTGAGGGTATAACTTGG	$(CA)_{43}$	53	130-180	131	9	0.846	0.801
GF089509	R: TTTCCCAGCCTCTCTGAACC							
MavG9	F: NED-CCATTGGTCCAAGCCACATCC	$(GT)_{34}$	53	190-260	136	8	0.819	0.652
GF089510	R: GTGCCAGCAAGAGTCACAGC							
MavG6	F: FAM-TCTTGCCTCGAAATGACTGG	$(GT)_{38}$	58	240-300	135	8	0.832	0.856
GF089511	R: AGGTGTAAGGGTATAGCTTGG							
MavA5	F: VIC-AAGTTGCTTGGTCTCTTTGG	$(GT)_{38}$	53	180-280	131	17	0.908	0.889
GF089512	R: CTCAAACCCAAGGCTGTTCC							
MavH3	F: PET-GTGTAGCTTGAAGGTAGAATGC	$(GT)_{29}$	58	230-310	132	6	0.670	0.675
GF089518	R: CAAGAAAGGTGATGGTGGTACTTGG							
MavC4-2	F: FAM-GATCACAGGTTTGACGACAGC	$(CA)_{12}$	60	110-230	134	7	0.786	0.730
GF089513	R: CTCTCTTTGCTTCCTGCATCG							
MavF1-2	F: PET-TTCAGGGAAGGGAGTAAGG	$(CA)_{22}$	56	180-290	135	8	0.788	0.711
GF089514	R: CAGGCATCAGTGAGCCAAGG							

Appendix 2.1 Primers designed from the sequences flanking the microsatellite regions of *M. avellanarius*. T_a (^{0}C) – PCR annealing temperature; size – expected product size in base pairs.

Locus	T_a (^{0}C)	Size	SSR motif	Primer sequence (5'-3')
E1	61.82	216	$(CA)_{28}$	F: TCTAACAGTCCTGCATTGCTAACC
	63.35			R: CAACTGTCCTCTCACCCTCACC
D10	65.26	179	$(CA)_{16}$	F: GCCAGCCTCAGCAATTTAGGG
	62.61			R: AGTGAGTCTGTGTGCGTGTGC
B5	60.74	182	$(CA)_{40}$	F: GAAGGGCTGGGTATATATCATGG
	61.15			R: GCAACATCTCTGATGGAGAAGG
A1	55.42	189	$(CA)_{35}$	F: ATGTAGCTCAGAGGTAGAATGC
	62.10			R: GGTAGAATGCTCCTGGGTTCC
F9	64.25	184	$(CA)_{29}$	F: TACAGGGAAATGGATGGAACTGG
	65.60			R: CCAGTTTGTGGTCATTTGTTGTGG
D9	64.25	186	$(CA)_{30}$	F: TACAGGGAAATGGATGGAACTGG
	65.60			R: CCAGTTTGTGGTCATTTGTTGTGG
F11	64.48	268	$(GT)_{35}$	F: TTTACAATCCGCCTGCTCACC
	57.88			R: TTAGCGAGACCCTGTTTCC
E11	61.21	300	$(CA)_{43}(CT)_{43}$	F: CAAGATGACCCAAGAGATACAAGG
	65.78			R: AGTGCAAGGAGAGAGGGAGAGAGG
C5	57.15	176	$(CA)_{46}$	F: TGAAGGTAGAAAGCCTCTGG
	60.21			R: TTCCAGGCTTCCAAGTATGC
C6	62.25	249	$(GT)_{35}(CTAA)_8$	F: AGTTGGCCATTGTGCTACACC
	63.54			R: CAAGGCTGATTCCTCACCTAAGC
H9	59.76	222	$(CA)_{38}$	F: TTAAGGCAGGTAGCAAGATTCC
	63.43			R: AATGCTCCTGGGTTCCATCC
H5	65.94	209	$(GT)_{46}$	F: CGTTCTTACACACCTTCCCACACC
	65.49			R: CCAAGACAAGGCCAGTGAGACC
A5	57.05	297	$(GT)_{39}$	F: AAGTTGCTTGGTCTCTTTGG
	61.94			R: CTCAAACCCAAGGCTGTTCC
A3	62.61	186	$(CA)_{39}$	F: TTTCCAGTGACTGACAAGTGTGC
	61.71			R: GCACCAAACAAACAGAACAACC
F10	56.36	193	$(CA)_{43}$	F: GTAGAGCTGAGGGTATAACTTGG
	61.67			R: TTTCCCAGCCTCTCTGAACC
E3	55.32	197	$(CA)_{49}$	F: ATAGCCCAGAGGTAGAAAGC
	60.12			R: TAGCATCCCGTTCTCAAACC
B9	62.83	199	$(GT)_{32}$	F: GGTGCTGGTGACACTTGTGC
	60.57			R: AGAGGGATTGGGCATAGAGC
G6	61.32	269	$(GT)_{38}$	F: TCTTGCCTCGAAATGACTGG
	55.15			R: AGGTGTAAGGGTATAGCTTGG
G9	66.88	240	$(GT)_{34}$	F: CCATTGGTCCAAGCCACATCC
	62.20			R: GTGCCAGCAAGAGTCACAGC
E12	64.19	287	$(GT)_{37}$	F: CCATTTGAAGGTTCTTCCTCATCC
	56.22			R: AGAGCTGTTGTGGTTCTAAGG
F8	65.44	246	$(CA)_{31}$	F: AAGAGGAGACGCTTGGGAGAGG
	64.92			R: CAAGAAAGGTGATGGTGGTATCTTGG
H3	55.89	297	$(GT)_{29}$	F: GTGTAGCTTGAAGGTAGAATGC
	55.26			R: AAACTTGGTACTAGTGCAGACC
G3	62.72	198	$(GT)_{54}$	F: CACATGTGTTGACTGATTGAGTGG
	59.00			R: TGAAGATGTAGCTCATAGGTAGCC
E4	61.01	284	$(GT)_{45}$	F: AAAGTAGGGCAGAGGGTGTAGC
	64.64			R: GGGAGTGTAGCCCAGAGATAGAAGG

Appendix 2.2 Description of microsatellite loci for the common dormouse *Muscardinus avellanarius* in *Molecular Ecology Notes*.

PCR products was verified on agarose gels stained with ethidium bromide. The primer pairs designed for each of the 34 microsatellite markers amplified a single DNA fragment from *L. mammosus*

Allelic diversity in the 34 microsatellite markers was assessed across 31 *L. mammosus* sporocarps collected at Voumayaare, Sweden. Appropriate dilutions of the PCR products were analysed on the MegaBACE 1000 automated sequencer (Molecular Dynamics and Amersham Life Science). Band sizes were determined using the MegaBACE Genetic Profiler version 1.5 software (Amersham Biosciences) and GeneMarker Demo version 1.51 (SoftGenetics LLC). Of the 34 microsatellite loci examined with the two methods, 25 were monomorphic and nine were polymorphic, of which eight were isolated from the ecogenics GmbH enriched library and one (LmCAC89 locus) from the enriched library built according to the FIASCO protocol (Table 1). Out of the nine polymorphic microsatellite loci developed in this study for *L. mammosus*, the observed number of alleles per locus ranged from 2 to 5, and the expected heterozygosities ranged from 0 to 0.84 (Table 1). Significant heterozygote deficiency was detected at two loci (Lacmam 03 and Lacmam 08) using the chi-squared goodness-of-fit test of Hardy–Weinberg equilibrium. This heterozygote deficiency may be due to null alleles (Pemberton *et al.* 1995). Genotypic linkage disequilibrium between all pairs of loci was tested by means of a contingency exact test using GenePop version 3.1 (Raymond & Rousset 1995). No significant departure from the null hypothesis of linkage equilibrium was detected. The nine polymorphic microsatellite markers developed in this study should prove useful for studying the distribution of genets as well as genetic structure and gene flow in populations of *L. mammosus*.

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Isolation and characterization of 10 microsatellite loci in the common dormouse *Muscardinus avellanarius*

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Abstract

To assess the level and spatial pattern of genetic diversity of the common dormouse, *Muscardinus avellanarius*, we developed polymorphic microsatellite loci from partial genomic libraries enriched for microsatellite motifs. Ten dinucleotide polymorphic microsatellites were isolated and levels of genetic diversity were assessed in 139 individuals from Bontuchel, (Denbighshire, Wales). We observed high levels of heterozygosity (mean $H_O = 0.792 \pm 0.077$; mean $H_E = 0.730 \pm 0.084$) and a large number of alleles ($N_A = 6–17$). There

Appendix 2.2 *cont.* Description of microsatellite loci for the common dormouse *Muscardinus avellanarius* in *Molecular Ecology Notes*.

was no evidence of deviations from Hardy–Weinberg conditions, or genotypic disequilibrium between any pairs of loci. This is the first description of microsatellite primers from a common dormouse and these loci are currently being used to quantify dormouse spatial genetic structure.

Keywords: conservation, microsatellites, *Muscardinus avellanarius*, population structure

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Anthropogenic land use has impacted on many species by restricting their ability to disperse throughout an increasingly fragmented landscape, often leading to small, isolated populations that, for example, are more susceptible to demographic and environmental stochasticity, edge effects and inbreeding depression (e.g. Bright 1993). In Britain, the clearance of the native woodland has affected the distributions of many native species (Peterken 2002). One such species is the common dormouse, *Muscardinus avellanarius* (Rodentia; Gliridae), that has suffered a 64% decline in number in Britain since the late 1970s (Bright *et al.* 2006). Accordingly, in Europe, common dormice are listed in the Bern Convention (Annex III) and are protected under the Wildlife and Countryside Act in the UK (Bright *et al.* 2006). Common dormice are categorized as 'least concern' on the Red List (IUCN 2008). For better management and conservation of this species, we developed a panel of microsatellite loci that can be used to determine levels of genetic variation and dispersal among habitat patches.

Genomic DNA was extracted using a high salt method (Sambrook *et al.* 1989) from tail clips of three captive-bred dormice that had died of natural causes. The enrichment procedure is described by Bloor *et al.* (2001). Briefly, ~8 μg DNA was digested with 40 U *Sau3A1* (Boehringer–Mannheim), and the 400–1200 bp size fraction was then excised from an agarose gel and purified using QIAquick gel extraction kit (QIAGEN). Next, the fragments were ligated to 50 pM phosphorylated linkers (SauLA: 5′-CGCCAGAGACCCCAA-GCTTCG-3′ annealed with SauLB: 5′-GATCCGAAGCTT-CCCGTCTCTGGCC-3′) (Refseth *et al.* 1997) using 40 U T4 DNA ligase (Promega).

The DNA fragments were then hybridized with M2–80 streptavidin-coated magnetic beads (Dynal) that had been incubated with 3′-end biotin-labelled $(CA)_{12}$ and $(TCAC)_6$ oligonucleotides. After a series of differential stringency washes in 2× SSC and 1× SSC, the enriched DNA was amplified in a 50-μL polymerase chain reaction (PCR) primed with 30 pM SauLA. The enriched fraction was then purified using a QIAquick PCR purification kit (QIAGEN), ligated into a pGEM-T plasmid vector (Promega) and transformed into JM109 competent cells (Promega). The presence of recombinant plasmids was identified with black/white selection on S-gal (Sigma) agar/ampicillin plates. Plasmids containing a microsatellite insert were identified by a double band when screened with PCR. Positive clones were sequenced on an ABI3100xl automated DNA sequencer (Applied Biosystems). Primers flanking microsatellite regions were designed using Primer 3 version 0.4.0 (Rozen & Skaletsky 2000).

To test for polymorphisms, microsatellite alleles were amplified in a 10-μL PCR on a Dyad Engine (MJ Research Inc.). A tailed primer method (Schuelke 2000), whereby forward primers are synthesized with a 5′ (or tail) sequence of a primer that is labelled with either 6-FAM, NED, PET or VIC fluorophores (Applied Biosystems), was used to label PCR products. The PCR consisted of 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 3 mM $MgCl_2$, 5–50 ng template DNA, 3 pM of tailed, reverse and labelling primer, 10 μg BSA, 1.25 U DNA polymerase (ABgene). PCR conditions for all loci were 95 °C for 3 min, *6×* (95 °C for 30 s, T_a °C for 45 s, 72 °C for 45 s), 25× (92 °C for 30 s, T_a °C for 45 s, 72 °C for 55 s), where T_a is the primer annealing temperature (Table 1). PCR products were pooled with a 500-bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI3130xl and sized using GeneMapper software (Applied Biosystems).

We sequenced 192 putative positive clones, of which 83 (43%) contained a reasonable (> 6 repeat units) microsatellite motif. Primers could be developed around 28 of these loci; however, 18 primer pairs were dropped subsequently because they either failed to amplify or they produced multiple/spurious bands during PCR: this left just 10 polymorphic loci (Table 1). We quantified the level of polymorphism in *M. avellanarius* from one site in North Wales, Bontuchel (Ordnance Survey SJ082571; $n = 139$), using DNA extracted from hair samples (see Walsh *et al.* 1995 for protocol). The online version (3.4, http://wbiomed.curtin.edu.au/genepop/) of GenePop (Raymond & Rousset 1995) was used to calculate basic measures of genetic diversity, the significance of any deviations from expected Hardy–Weinberg equilibrium (HWE) conditions and also for linkage disequilibrium between all pairs of loci. The software Micro-Checker (Van Oosterhout *et al.* 2004) was used to detect the most probable technical cause of HWE departures such as null alleles, mis-scoring due to stuttering and allelic dropout.

All loci were highly polymorphic with mean observed heterozygosity at 0.792 (SE ± 0.077) and mean expected

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Appendix 2.2 *cont.* Description of microsatellite loci for the common dormouse *Muscardinus avellanarius* in *Molecular Ecology Notes*.

Table 1 Levels of variability at 10 polymorphic microsatellite loci in the common dormouse (*Muscardinus avellanarius*) from Bontuchel, Wales ($n = 139$)

Locus/GenBank Accession no.	Primer sequence (5′–3′)	Repeat motif	T_a	Size range (bp)	n_a	N_a	H_O	H_E
MavE3	F: VIC-ATAGCCCAGGTAGAAAGC	$(CA)_{16}$	58	110–170	136	11	0.684	0.615
GF089515	R: TAGCATCCCGTTCTCAAACC							
MavB5	F: NED-GAAGGGCTGGGTATATATCATGG	$(CA)_{40}$	53	110–160	136	8	0.765	0.678
GF089516	R: AGTGCAAGGAGAGAGGGAGAGAGG							
MavG3	F: FAM-CACATGTGTTGACTGATTGAGTGG	$(GT)_{34}$	53	110–180	135	9	0.844	0.821
GF089517	R: AGAGCTGTTGTGGTTCTAAGG							
MavF10	F: PET-GTAGAGCTGAGGGTATAACTTGG	$(CA)_{45}$	53	130–180	131	9	0.846	0.801
GF089509	R: TTTCCCAGCCTCTCTGAACC							
MavG8	F: NED-CCATTGGTCCAAGCCACATCC	$(GT)_{34}$	53	180–260	136	8	0.819	0.652
GF089510	R: GTGCCAGCAAGAGTCACAGC							
MavG6	F: FAM-TCTTGCCTCGAAATGACTGG	$(GT)_{58}$	58	240–300	135	8	0.832	0.726
GF089511	R: AGGTGTAAGGGTATAGCTTGG							
MavA5	F: VIC-AAGTTGCTTGGTCTCTTTGG	$(GT)_{35}$	53	180–280	131	17	0.908	0.889
GF089512	R: CTCAAACCCAAGGCTGTTCC							
MavH3	F: PET-GTGTAGCTTGAAGGTAGAATGC	$(GT)_{59}$	58	230–310	132	6	0.670	0.675
GF089518	R: CAAGAAAGGTAATGGTGGTACTTGG							
MavC4-2	F: FAM-GATCACAGGTTTGACGACAGC	$(CA)_{12}$	60	110–230	134	7	0.786	0.730
GF089513	R: CTCTCTTTGCTTCCTGCATCC							
MavF1-2	F: PET-TTCAGGGAAGGGAGTAAGG	$(CA)_{32}$	56	180–290	135	8	0.788	0.711
GF089514	R: CAGGCATCAGTGAGCCAAGG							

N_a, indicates number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; T_a, annealing temperature of primer during PCR. VIC, NED, 6-FAM and PET indicate the fluorophores used for genotyping. n_a, number of animals (out of 139) that produced a genotype.

heterozygosity at 0.730 (SE ± 0.084), and with between 6 and 17 alleles per locus (Table 1). There is no evidence for significant ($P > 0.05$) deviation from Hardy–Weinberg equilibrium at any locus, nor did we detect genotypic disequilibrium between any pairs of loci. A few animals ($n = 3$–8) failed to amplify alleles at some loci (see Table 1); however, there was no significant ($P > 0.05$) evidence for null alleles at any loci. We are presently using these loci to determine reproductive patterns and quantify dispersal among habitat patches in this species.

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Chapter 3: Prevalence of multiple mating by female common dormice, *Muscardinus avellanarius*, in natural and reintroduced populations.

3.0 Introduction

A species' mating system is a key component of its life history and evolution. For example, the efficacy of sexual selection depends largely on the extent of competition between males and female choice. Typically, the pattern of reproductive behaviour exhibited by either sex is driven mainly by the associated investment that is directed towards producing and raising offspring (Clutton-Brock and Vincent 1991). Thus many bird species are monogamous as both sexes must contribute to raise their young (Wachtmeister 2001), while, by contrast, the males of many species of mammal are relieved from parental care duties and accordingly direct their efforts towards attempting to mate with multiple females (Wolff and Sherman 2007). While the putative fitness benefits associated with promiscuous behaviour by males is uncontroversial (McEachern et al. 2009), females, on the other hand, typically are believed to be more selective in their choice of mate – for example, selecting a single, best male (Klemme et al. 2008). However, increasing recognition that multiple mating by females is a commonly-employed tactic (*e.g.* Ribble and Millar 1996; Waser and De Woody 2006; Clutton-Brock and McAuliffe 2009), particularly among species of small rodents (see Waterman 1998; Topping and Millar 1999; Hohoff et al. 2003; Matocq and Lacey 2004), has driven interest into determining the natural patterns of reproductive behaviour by females.

Multiple mating by females will be adaptive when they gain direct, material benefits from males such as parental care, increased access to food resource and/or ownership of territory (Yasui 1998; reviewed by Wolff and Macdonald 2004). Nonetheless, there is emerging evidence for fitness consequences in the absence of direct benefits (see *e.g.* Stockley 2003; Simmons 2005). Indeed, a number of possible indirect, genetic benefits have been proposed to explain the evolution of multiple mating by females, including for example, inbreeding avoidance, genetic compatibility and 'good genes' (Jennions and Petrie 2000; Zeh and Zeh 2001). Typically, these are speculated to generate a higher genetic quality of

offspring (Wolff and Macdonald 2004) and as such one key feature of mating behaviour is the amount of genetic diversity (Moore et al. 2007).

From a more practical perspective it is crucial to understand natural patterns of reproduction to assess anthropogenic impact on species in their environment. Mating behaviour potentially is influenced by the consequences of loss and fragmentation of habitat, such as reduced social neighbourhood sizes and lower rates of dispersal and elevated relatedness among potential mates that could lead to Allee effects (Courchamp et al. 1999) and/or inbreeding (Banks et al. 2005). Moreover, with increasing use of captive breeding programmes for species augmentation and reintroductions, it seems prudent to try to recreate and sustain appropriate natural behaviours *ex situ*. Knowledge of a species' natural mating system clearly permits informed decisions regarding the appropriate demography and management strategy for any managed population.

The common dormouse, *Muscardinus avellanarius*, is a nocturnal and arboreal mammal that inhabits areas of deciduous forest with a thick layer of scrub plants and underbrush (Bright et al. 2006). This species distributed across Europe, from the Mediterranean to southern Sweden, eastward to Russia excluding Iberia and extends its range into parts of northern Asia Minor (Juskaitis 2007; IUCN 2008). It reaches the northwest limit of its European range in the UK, where it can be found over much of southern England and also in isolated populations in northern Wales (Bright et al. 2006). In many parts of this species' northern range (*i.e.* in the UK, Netherlands, Sweden, Germany and Denmark) its populations are declining and becoming increasingly fragmented (IUCN 2008). The detrimental effects of loss and fragmentation of forest habitat on *M. avellanarius* populations have been highlighted in the UK where this species has become extinct in up to seven English counties (about half of its former range) during the past 100 years (Morris 2004; Bright et al. 2006); more recently, this species has suffered by a 64 % decline in number in Britain since the late 1970s (Bright et al. 2006). Several factors are thought to have contributed to the decline of dormice including unfavorable woodland management, a succession of poor breeding years and habitat fragmentation leading to increases in the isolation of populations (Bright and Morris 1996; Bright et al. 1996). Indeed, now *M. avellanarius* is regarded as a "Flagship Species" for nature conservation and as excellent bioindicator of woodland quality (Morris 2004; Bright et al. 2006). Accordingly, *M.*

avellanarius is a conservation priority and is protected throughout its range, categorised as 'least concern' on the Red List (IUCN 2008). In the UK, it is granted full protection as a Schedule 5 species and was placed on the English Nature's Species Recovery Programme in 1992 (Macdonald and Tattersal 2003). Captive breeding and reintroduction of *M. avellanarius* is aimed at conserving and protecting dormice in the UK. This *ex-situ* programme has proved feasible with most reintroduced populations of *M. avellanarius* providing evidence of breeding and spreading into available habitat (Morris 2004; PTES 2009).

Despite a recent slowdown in the rate of population decline, *M. avellanarius* remains a conservation priority (PTES 2009). In particular, there is some concern about the ability of *M. avellanarius* populations to recover as this species has a low reproductive potential, a wide inter-annual variation in reproductive success and a limited breeding period in the northern parts of its range (Bright et al. 1994; Büchner et al. 2003; Juŝkaitis 2003a, b; Morris 2004). In most cases, the majority of dormice produce just one litter per season (between July and August) of between one and seven young, although litters of three to four young are most common (Büchner et al. 2003; Juskaitis 2003). Since *M. avellanarius* are small, cryptic and nocturnal, it is clearly impractical to attempt to make direct observations on mating behaviour. Hence, the only feasible method of obtaining such data is to use molecular-genetic analyses of litters to determine natural patterns of reproductive behaviour (see *e.g.* Slate et al. 2000; Waser and DeWoody 2006; Crawford et al. 2008 for case studies).

The principal aims of this study are (1) to quantify the mating system of *M. avellanarius* at a natural site and in a reintroduced population and (2) to determine whether there are any differences in reproductive behaviour between these two sites. I find that female dormice typically mate more than once at both sites and discuss the possible factors that influence this mating system.

3.1 Materials and methods

3.1.1 Sample collection

Muscardinus avellanarius were sampled at two sites in the UK (1) Bontuchel (Denbighshire, Wales; Latitude 53.109364 N; Longitude: -3.370318 W; OS National Grid

Reference, SJ082571) and (2) Wych Valley (Cheshire, England; Latitude 52.994994 N; Longitude -2.7745169 W; OS National Grid Reference, SJ4811244) as part of a continued monitoring programme by the Northwest Dormouse Partnership (available at http://www.cheshirewildlifetrust.co.uk/proj_dormouse_partnership.htm). The natural population at Bontuchel inhabits an area of mixed broadleaf and conifers, while the animals at the Wych Valley are the descendents of 29 and 24 captive-bred dormice that were released (in 1996 and 1997 respectively) into a habitat consisting of ancient woodland and native broadleaves (see Chapter 1). To monitor *M. avellanarius* at these sites, 250 and 230 nestboxes were installed at Bontuchel and Wych respectively. All nestboxes are situated on tree trunks, with the entrance facing the trunk at 1.5 m above ground level, and are spaced at approximately 20-40 m intervals. Nestboxes at Wych are monitored in May, June, September and October, while nestboxes in Bontuchel are inspected monthly from May until October. Every dormouse is scanned for a microchip and then its sex, weight, estimated age and breeding status is recorded; dormice without microchips are anaesthetised and then microchipped using 8 mm microchips. For genetic analyses, hair and buccal swab samples have been collected from all animals that were encountered during annual monitoring surveys since 2006.

3.1.2 DNA extraction and genotyping

Total genomic DNA was extracted from hair and buccal swab samples using a CHELEX-100 protocol (Walsh et al. 1991). All samples were genotyped at ten polymorphic microsatellite loci (Md. Naim et al. 2009) in separate 10 µl PCR reactions that contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 3 mM $MgCl_2$, 1 µl extracted template DNA, 3 pmol of each primer, 10 µg BSA and 1.25 u DNA polymerase (ABgene). Thermal cycling conditions (on a Dyad Engine; MJ Research Inc) were 95°C for 3 min, 6x [95°C for 30s, T_a°C for 45s, 72°C for 45s], 25x [92°C for 30s, T_a °C for 45s, 72°C for 55s] and a final incubation at 72°C for 5 min, where T_a is the annealing temperature (either 53°C or 58°C depending on the locus). PCR products were then pooled with a 500 bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI3130*xl* and sized using GeneMapper software (Applied Biosystems). All samples were genotyped at least twice to ensure accuracy.

3.1.3 Basic analyses of genetic data

MICROCHECKER ver.2.2.3 (Van Oosterhout et al. 2004) was used to identify any systematic genotyping errors, for example, null alleles, large allele dropout and possible mis-scoring due to stutter. Next, basic estimates of genetic diversity at each locus were made by calculating number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e) and allelic richness (A_R, standardised to the minimum sample size of 105 diploid individuals) using FSTAT ver. 2.9.3.2 (Goudet 2001). Genotype data (for each site and sample period separately) were tested for deviation from Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium using the online version of GENEPOP ver. 3.1 (Raymond and Rousset 1995).

3.1.4 Multiple paternity

Paternity was determined in litters with two or more offspring only as a single-offspring "litter" provides no information about the degree of (attempted or otherwise) multiple mating. Paternities were determined using two methods. First, I manually estimated the minimum number of sires that could account for the spectrum of genotypes in each litter. For this method, the genotypes of offspring and adult female(s) that shared a nestbox were checked to confirm that the adult female present was the mother of the litter (*i.e.* the adult and juveniles shared alleles); knowledge of the maternal alleles permits the range of paternal alleles to be identified – *i.e.* alleles that are present in the offspring but not in the mother (except in homozygous offspring). This technique was also used to directly reconstruct the paternal genotype of offspring in each family. When heterozygous offspring shared their genotype with heterozygous mothers a conservative approach was taken with paternal alleles inferred in a way that minimised the total number of paternal alleles from the litter. Clearly, one or two total paternal alleles in a litter could have originated from just one (heterozygous) father, while three or four paternal alleles indicates that the female has mated with a minimum two fathers, and so on. I considered evidence for multiple paternity when more than two paternal alleles were observed at one or more microsatellite loci.

For the second method of estimating the minimum number of fathers per litter, I used a combination of softwares. First, I used GERUD ver. 2.0 (Jones 2005), which identifies litters with multiple paternity and then estimates the ratio of paternal contribution of males involved. I next used CERVUS ver. 3.0 (Marshall et al. 1998) to identify the most likely

father, from the available pool of fathers that had been sampled during surveys, for each of the offspring. Finally, I attempted to match the genotype of the most likely father identified by CERVUS against the panel of manually reconstructed paternal genotypes, both without any allelic mismatch and also allowing for some genotyping error by permitting one allelic mismatch.

Finally, using the GERUD output of minimum numbers of fathers, I determined whether there was any significant difference between (1) litter size and (2) the minimum number of sires at each study site, and also (3) whether there was any correlation between average body weight of females and the size of their litters (note that these tests do not include pregnant females). Finally, in Bontuchel during 2007, two nestboxes (NB791 and NB916) each contained a pair of adult females and a single litter (one female was identified as the sole mother of all offspring in each nestbox). I determined the relatedness of the other adult female to the mother by calculating Ritland's (1996) estimator of pairwise relatedness (r, which is the proportion of shared alleles between the individuals weighted by the allele frequencies in the whole population) using SPAGeDI ver.1.2 (Hardy and Vekemans 2002).

3.2 Results

3.2.1 Demographic parameters

In total, 192 and 140 dormice were sampled in Bontuchel and 61 and 74 dormice were caught at Wych during 2006 and 2007 respectively. The mean litter size at Bontuchel (3.43±1.27 SD) and Wych (3.80±1.92 SD) did not differ significantly (t-test, t=-0.531, d.f.=26, P>0.05). After removing litters with a single individual and where the mother was not present in the nestboxes, paternity analyses could be conducted on a total of 23 litters (23 adult females and 79 offspring) in Bontuchel and 5 litters (5 adult females and 19 offspring) in Wych.

3.2.2 Genetic diversity

Average genotyping error rates were less than 1%, and none of the loci showed evidence of null alleles (P>0.05). Genetic variability was greater at Bontuchel, where the number of alleles per locus varied from 10 up to 18, than in Wych, with between 5 and 15 alleles per locus (data pooled over both sample periods; Table 3.1). Similarly, allelic richness A_R, was greater at Bontuchel (range=8.2-14.8) than in Wych (range=4.5-12.0), as was the average

expected heterozygosity (Bontuchel mean H_e=0.770±0.064 SD; Wych mean H_e=0.695±0.070) (Table 3.1). There was no significant (P>0.05) deviation from Hardy-Weinberg equilibrium conditions at any locus, nor was there any significant (P>0.05) linkage disequilibrium between any pairs of loci. Thus, this panel of microsatellite loci is sufficiently powerful to uncover patterns of multiple mating in these populations of *M. avellanarius*.

3.2.3 Parentage

All offspring genotypes were compatible (*i.e.* they shared alleles at all loci) with the putative mother (the adult female (s) that occupied the same nestbox as the litter) (Appendix 3.1), and therefore null alleles or mutations are unlikely to represent a significant bias in my results. There were some discrepancies (8.7%) in the estimated minimum number of potential sires between the two methods (manual observations vs. GERUD), however both methods provided unambiguous evidence that multiple mating by female dormice is commonplace (Table 3.2). By allele counting, multiple mating was detected in 16 out of 23 (69.6%) litters and 4 out of 5 litters (80.0%) in Bontuchel and Wych respectively. Using GERUD, I detected multiple paternity in 14 out of the 23 litters (60.9%) in Bontuchel and 4 of the 5 litters at Wych (data pooled for both years; Table 3.2 and Appendix 3.1). Most of the multiply-sired litters were fathered by at least two males (although, depending upon the level of heterozygosity, there could be more than two males) at Bontuchel (56.5%) and at Wych (60.0%), while just three litters in Bontuchel and one litter in Wych were definitely sired by at least three males respectively (Table 3.1; Figure 3.1).

The manually reconstructed paternal genotypes for each offspring in all litters within each site are listed in Appendix 3.2. From this panel, it is estimated that 38 (Bontuchel) and 11 (Wych) different males have sired the multiply-mated offspring in Bontuchel and Wych respectively. Thus, the proportion of fathers apparently present in my sample is 15 out of 38 (39.5%) and 4 out of 11 (36.4%) in the sampled families from Bontuchel and Wych respectively. When I allow a genotyping error at a rate of one allele mismatch, these proportions increase to 47.4% (Bontuchel) and 45.5% (Wych).

CERVUS returned the most likely father for all offspring tested (see Appendix 3.3). When no allelic mismatch was allowed, 18.8% (Bontuchel) and 11.8% (Wych) of the paternal genotypes of the most likely father matched with a males' genotype present in my dataset (*i.e.* 15 out of the 80 and four out of the 34 adult males that had been caught in Bontuchel and Wych respectively); however, when one and two allelic mismatch per genotype was allowed, these percentages increased to 22.5% and 25.0% in Bontuchel and 11.8% and 14.7% in Wych respectively. No evidence of a significant reproductive skew was evident, as the males involved in multiple paternity were generally equally successful in siring offspring in the litter (Table 3.2).

The mean minimum number of fathers per litter did not differ significantly between sites (Bontuchel=1.83±0.65 SD; Wych=2.00±0.71 SD; *t* test, t=-0.523, d.f.=26, P>0.05) (data combined for both years). There was a significant, positive correlation between average body weight of females and the size of the litter in Bontuchel (Spearman's rank correlation, r_s=0.998, P<0.01) and in Wych (r_s=0.943, P<0.01) (Figure 3.2; see also Appendix 3.4). The two pairs of adult females that were found co-habiting were likely to be half-siblings, as they had pairwise relatedness values (r) of approximately 0.25 (r=0.273 at NB791 and r=0.319 at NB916).

3.3 Discussion

This first study of the natural patterns of parentage in the common dormouse, *M. avellanarius*, has identified a prevalence of multiple mating by females, with between 69 and 87 % of the litters sired by more than one male at one or more microsatellite loci (when the litters with just one offspring are included as a single mating by a female). These estimates are still high (between 57 % and 60 %) using the conservative approach of inferring multiple paternity only when three or more paternal alleles were detected at two or more microsatellite loci. Thus one striking feature of my data is the high proportions of multiple paternity observed in both populations that are among the highest reported in such studies in small rodents, for example: 20 % in *Mus musculus* (Dean et al. 2006), 47 % in *Mastomys natalensis* (Kennis et al. 2008) and 38 % in *Microtus oeconomus* (Borkowska et al. 2009).

This raises the issue of what costs or benefits are associated with this reproductive behaviour. Females may gain a clear material/direct benefit from males by multiple mating such as increased protection and/or access to food resources (Yasui 1998; Wolff and Macdonald 2004). With this in mind, it is interesting that male *M. avellanarius* are territorial (Morris 2004) and therefore possible that females can access more resources by mating with several males. Moreover, since up to 65 % of adult male dormice share a nestbox with one or more females outside the natal period (Morris 2004), territorial behaviour may provide lactating females with a secure nest site (see also Solomon and Keane 2007). However, male *M. avellanarius* do not care for juveniles (Morris 2004) and direct benefit models may not fully account for the reasons that female *M. avellanarius* frequently mate multiply.

Numerous indirect genetic benefit hypotheses have been proposed to explain multiple mating in the absence of any perceived direct benefits, including increased heterozygosity, inbreeding avoidance, 'good genes' and genetic incompatibility avoidance (*e.g.* Stockley 2003; Simmons 2005; reviewed by Jennions and Petrie 2000). Broadly, these hypotheses propose that multiple mating by females reduces the chance of reproductive failure and/or generates fitness benefits to the females themselves or to their offspring (Murie 1995; Hoogland 1998; Drickamer et al. 2000). Indeed, numerous investigations have suggested that multiple paternity improves offspring fitness (Tregenza and Wedell 1998; Garcia-Gonzalez and Simmons 2005; Fisher et al. 2006; Klemme et al. 2006, 2007), however with the absence of lifetime reproductive success data no robust conclusions about the fitness of single- versus multiple-paternity dormice litters can be made (data not shown). More recently, Lane et al. (2008) found no support for the prediction that females use multiple mating to enhance genetic diversity of their offspring in North American red squirrels *Tamiasciurus hudsonicus*. Clearly females may be limited in their capability to manipulate the genetic diversity of their offspring. Indeed, it is simply plausible that female dormice mate multiply to avoid some cost of being harassed by males, rather than gaining a tangible benefit from multiple mating (see Lee and Hays 2004). More work is required to ascertain what benefits, if any, are accrued through multiple mating by female dormice.

A second issue raise by multiple mating is the extent of female mate choice, and, if females are selective, then what criteria are desirable? For example, female mammals may select

mates based on maturity, fertility, weaponry, physical conditions, odour and relatedness, (Lehmann et al. 2007; Clutton-Brock and McAuliffe 2009), but the impact of such characters are unknown in dormice. It is interesting that despite extensive surveying of dormice in both study sites, I note that a considerable proportion of the reconstructed paternal genotypes (up to 80% when assuming no allele mismatch) were not present in the samples in both sites. This probably represents individuals that are overlooked during sampling, possibly because they avoid nestboxes and inhabit the tree canopy. The high reproductive success rate of these 'unsampled' males suggests that female dormice preferentially mate with 'unfamiliar' individuals or males that stay away from nestboxes (*i.e.* the unidentified individuals) and/or are not normally encountered during routine foraging. Such behaviour is sometimes employed as a mechanism to avoiding mating with relatives (see *e.g.* Pusey and Wolf 1996; Loew 2000; Linklater and Cameron 2009). Alternatively, this probably reflects larger than anticipated population sizes and the additional available habitat (beyond the area covered by nestboxes) at both study sites. This certainly raises questions about the behavioural characteristics of animals that do and do not use nestboxes; if differences exist, then the information about population characteristics of populations that are monitored using nextboxes (rather than random trapping, for example) should be interpreted with caution.

Thus, in addition to the unsampled males, one of the key findings in this study was the large proportion (70-80%) of males that apparently present in my samples in both sites that did not reproduce. This raises a probability of non-breeding alloparents (conspecifics that participate in offspring care; Solomon and Keane (2007)), which can be common in birds and mammals (Hauber and Lacey 2005). Hauber and Lacey (2005) demonstrated that the presence of a large number of non-breeding males, particularly when coupled with social suppression of reproduction among females, may alter the relative variance in direct fitness between the sexes. For example, Lacey and Sherman (1997) have argued that the reason the breeding female of naked mole rat (*Heterocephalus glaber*) can have four to five very large litters per year is that the alloparents do almost everything (*i.e.* foraging, tunnel building, colony defense) but Jarvis (1991) has shown that alloparents are not essential for successful rearing of offspring which suggest that one or both of the breeders may benefit in terms of time or energy saved from the assistence provided by non-breeding colony members. This implies that the presence of non-breeding males is an important determinant of the

variability in reproductive success. Alternatively, the proportion of non-breeding males in this study could also represent individuals that did breed but were missed in paternity analyses probably due to limited nestbox sampling or non-random sampling, or simply die after reproduction. In reality, all of these processes are likely to have occurred to some degree, suggesting that the effects of non-breeding males and unsampled nestboxes (see paragraph above) may to some extent offset each other (*i.e.* Krakauer 2008).

A positive correlation between body weight and litter size in dormice (Figure 3.2) is consistent with the reproductive biology of other small mammals, particularly rodents, where larger individuals usually have greater reproductive success (Myers and Master 1983; Wauters and Dhondt 1989; Holt et al. 2004; Schulte-Hostedde et al. 2002). Since weight typically correlates with age in small rodents, this effect may reflect age-dependent fecundity similar to that exhibited by many mammals (*e.g.* Clutton-Brock et al. 1987; Broussard et al. 2003; Radespiel and Zimmermann 2003; Havelka and Millar 2004). Dormice typically live up to five years (Bright and Morris 1996) but it was not possible to determine the ages of sufficiently-many animals to explore this potential phenomenon with any statistical rigour. Equally, litter size may correlate with the number of mates (see *e.g.* Schilling et al. 1968; Keil and Sacher 1998; Schulte-Hostedde et al. 2004). My data also display this trend (unpublished data) but it likely reflects the increased probability of detecting multiple mating in larger litters. Nonetheless, an outcome of these effects is that multiply-mated female dormice are, on average, heavier than singly mated females (this effect was significant only at Bontuchel because of the greater sample size), implying that female reproductive behaviour is linked to condition and possibly age. On the one hand, female dominance may affect reproductive success, with dominant females gaining priority access to better quality of food resources and are therefore able to sustain larger litters (Holand et al. 2004; Kinahan and Pillay 2008). This phenomenon has been observed in other rodent species (*e.g. Clethrionomys glareolus*, Jonsson et al. 2002; *Rhabdomys pumilio*, Kinahan and Pillay 2008), including the garden dormouse *Eliomys quercinus* (Bertolino et al. 2001) and edible dormouse *Glis glis* (Pilastro et al. 2003). Alternatively, as discussed above, female dormice may gain more access to resources by mating multiply.

Certain mammals communally nest or form crèches and this may provide benefits as a result of cooperative foraging and feeding, allogrooming, group defence and assistance in

thermoregulation (Garza et al. 1997; Lacey et al. 1997; reviewed in Hayes 2000), and this raises questions about the extent of kin recognition (Holmes and Mateo 2007). That the adult females who shared a nestbox when offspring were present were half-sibs suggest that female dormice who exhibit communal nesting behaviour, possibly in the form of a créche, recognise and tolerate related individuals in their breeding grounds (see *e.g.* Sato et al. 1987; Packer and Pusey 1995). Additionally, I found large litter consist of 11 offspring in Wych (2007) with two different ages (juveniles and brown young), further supporting the idea of a créche in dormice. Similarly, kinship between communally breeding females has been reported in *G. glis* (Marin and Pilastro 1994) and red-backed voles *Clethrionomys rufocanus* (Kawata 1987) where sibling females recognised and associated with each other, and maintained nearby home ranges.

Given the extent of multiple mating and the lack of an obvious paternity skew (*i.e.* a particular male does not sire most of the offspring) (Table 3.2), male *M. avellanarius* apparently do not dominate access to females. This is someone surprising given reports of aggressive behaviour by male dormice (Morris 2004) and the expectation that dominant individuals will gain more copulations than subordinates (Spritzer et al. 2005, 2006). Exceptions to this occur, of course, such as when females escape from such male precopulatory tactic (Koprowski 1993; Waterman 2007) and/or subordinate males are able to engage in "sneaky-matings" (*e.g.* Koford 1982; Ohsawa et al. 1993; reviewed by Reichard et al. 2007). In *M. avellanarius*, strongest indications that female mating behaviour actively counteract attempted monopolisation by males are (1) the high proportions of multiple paternity (in both populations) and (2) the fact that most litters are not sired by the apparently nearest (*i.e.* male that was present in a nestbox, see discussion above) male neighbour. Thus, the absence of paternity skew indicates that dormice reproductive behaviour contrasts with other small mammals where, for example, neighbouring males tend to monopolise access to females (Waser and DeWoody 2006) or the first male to mate dominates the litter (Firman and Simmons 2008).

3.4 Implications for conservation

An analysis of a species' mating system provides crucial behavioural insights relevant to conservation efforts. *M. avellanarius* is a species of international conservation concern and considerable effort has been directed towards habitat management, captive breeding and

reintroductions (Bright et al. 2006). Generally, despite some investigations into the reproductive biology in various dormice species (*e.g.* Nevo and Amir 1964; Pilastro 1992; Marin and Pilastro 1994), including some work on *M. avellanarius* (Büchner et al. 2003; Juškaitis 2003), molecular genetic techniques have not yet been applied to determine mating behaviour of dormice. I provide the first direct evidence that female *M. avellanarius* typically mate with multiple males, and that this behaviour occurs in a reintroduced population that was founded from a small number of captive-bred individuals. Thus, even though the *ex situ* programme maintains dormice either singly or as a breeding pairs (*i.e.* monogamy) (J. Chapman, Paignton Zoo, *pers. comm.*), when returned to the wild dormice appear to resume their natural reproductive behaviour. Moreover, despite some differences in genetic diversity between sites that may be linked to the relatively small founding population, the lack of harem structure/male dominance and concomitant prevalence of multiple mating would appear to have limited the extent of genetic erosion at Wych. In future, it would be useful for managers of captive-breeding efforts to consider the genetic benefit of multiple mating by rotating resident females with a group of males. Another crucial aspect of this study is the larger litter size associated with larger females, and the associated greater level of genetic diversity. Releasing larger females, therefore, should provide more rapid population growth and limit the extent of genetic erosion.

3.5 Conclusions

To conclude, multiple mating by females is a significant characteristic of the breeding strategy of *M. avellanarius*, both in natural and reintroduced populations. The extent of female choice in driving this mating behaviour remains unknown, though it is possible that females apparently show preference towards unsampled or unidentified males in this study. The large proportion of non-breeding males that present in both study sites may be a consequence of limited nestbox sampling or non-random sampling, thus raises a probability of non-breeding alloparents. I provide evidence for possible crèche behaviour in dormice that is linked to kin recognition. Further studies on additional populations are required to explore the consequence of these findings in more detail.

Table 3.1 Summary statistics for the panel of 10 microsatellite loci used to study paternity in the common dormouse *Muscardinus avellanarius* sampled from two sites in the UK during 2006 and 2007 (data combined over both years). N_a=numbers of alleles; H_o=observed heterozygosity; H_e=expected heterozygosity; A_R=allelic richness.

Locus	Bontuchel				Wych			
	N_a	H_o	H_e	A_R	N_a	H_o	H_e	A_R
MavE3	14	0.601	0.576	11.63	9	0.700	0.600	7.44
MavB5	13	0.658	0.633	10.97	8	0.683	0.638	6.77
MavG3	10	0.733	0.759	9.40	10	0.847	0.755	7.38
MavF10	11	0.740	0.745	10.95	11	0.825	0.654	7.68
MavG9	10	0.807	0.740	8.19	8	0.567	0.600	7.17
MavG6	15	0.747	0.771	10.79	7	0.745	0.718	6.06
MavA5	13	0.702	0.673	10.38	13	0.750	0.685	9.10
MavH3	11	0.560	0.606	9.29	5	0.694	0.740	4.47
MavC4-2	18	0.766	0.789	14.77	11	0.951	0.781	8.56
MavF1-2	12	0.600	0.708	10.72	15	0.885	0.781	12.01
Mean	12.7	0.763	0.770	10.71	9.7	0.765	0.695	7.66

Table 3.2 Paternity analyses of litters of the common dormouse *M. avellanarius* using direct allele counting (DAC) and GERUD ver. 2.0 methods. *N*=number of individuals in the litter; M_s=minimum number of sires; PC=paternal contribution ratio.

			DAC	GERUD	
Site (Year)	Family	*N*	M_s	M_s	PC
Bontuchel	F1	3	2	2	2:1
(2006)	F2	3	2	2	2:1
	F3	3	2	1	2:1
	F4	2	1	1	-
	F5	4	2	2	2:2
	F6	2	1	1	-
	F7	5	2	2	2:3
	F8	3	2	2	2:1
	F9	5	2	2	2:3
	F10	2	1	1	-
	F11	2	1	1	-
	F12	5	2	2	2:3
	F13	2	1	1	-
	F14	3	2	2	2:1
	F15	4	2	2	2:2
	F16	4	2	2	2:2
Bontuchel	F17	5	3	3	2:2:1
(2007)	F18	3	2	1	2:1
	F19	2	1	1	-
	F20	4	2	2	2:2
	F21	5	3	3	2:2:1
	F22	2	1	1	-
	F23	6	3	3	2:2:2
Wych	FW1	4	2	2	2:2
(2006)	FW2	3	2	2	2:1
	FW3	3	2	2	2:1
Wych	FW4	2	1	1	-
(2007)	FW5	7	3	3	3:2:2

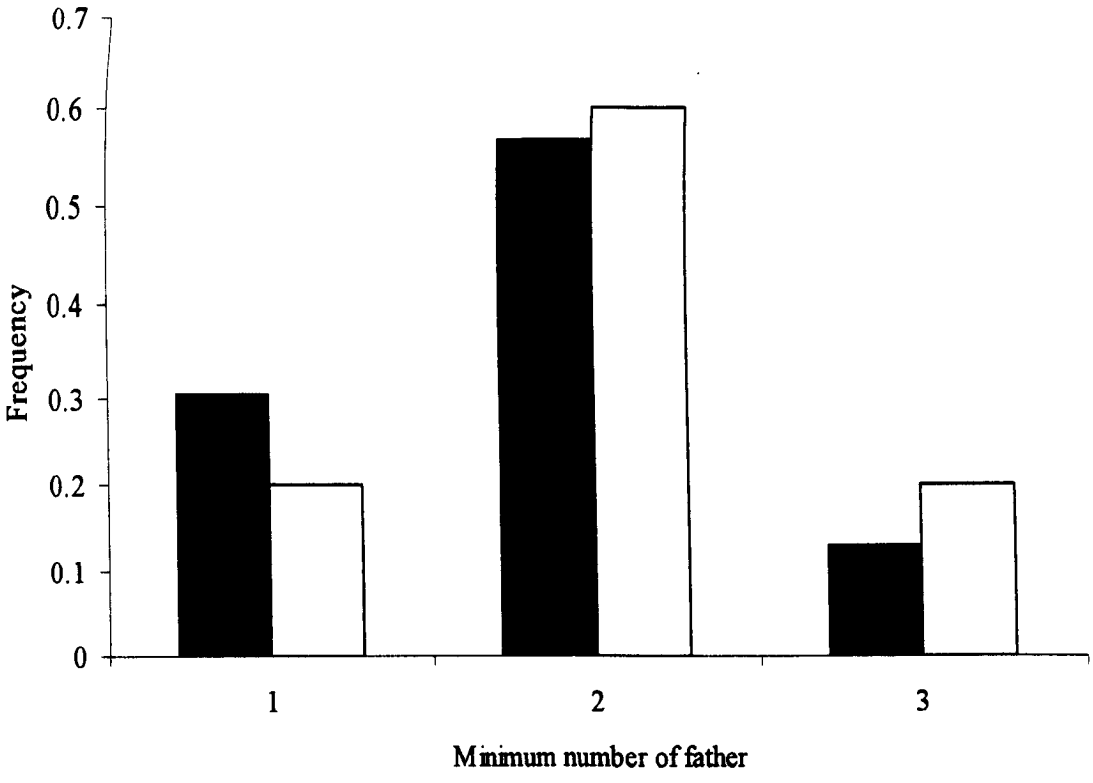


Figure 3.1 Frequency of multiple mating in relation to the number of minimum number of fathers detected combined for both years in Bontuchel (filled bars, n=79) and Wych (open bars, n=19).

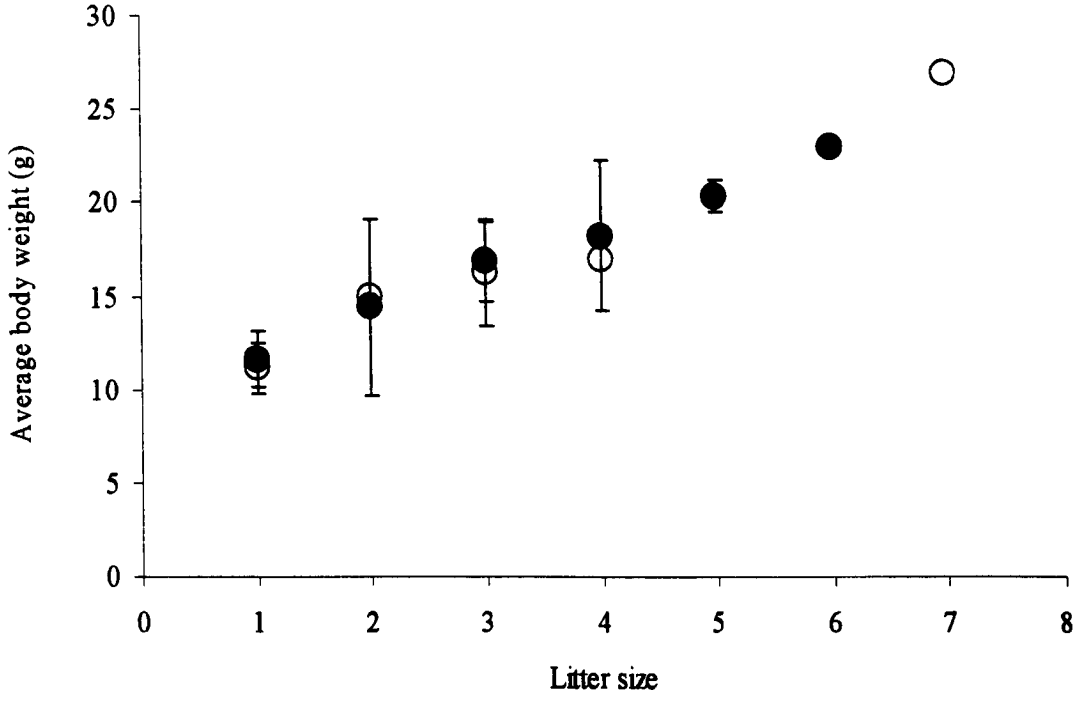


Figure 3.2 Relationship between adult female average body weight (mean±SD) and litter size in common dormice *M. avellanarius* found at two UK populations: Bontuchel (black) and Wych (white).

Appendix 3.1 Evidence of multiple paternity at a panel of ten microsatellite loci for families of the common dormouse *M. avellanarius* in Bontuchel and Wych during 2006 and 2007. *NPA*= number of paternal alleles.

Family/Locus		MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	MavC4-2	MavF1-2
F1	Mother	137/139	135/135	155/161	139/148	251/255	261/277	231/235	260/262	127/139	232/238
	Offspring 1	139/145	135/139	155/157	139/143	251/253	261/279	231/235	260/260	127/135	232/232
	Offspring 2	139/145	135/135	155/157	146/148	245/255	277/279	229/231	256/262	127/127	232/232
	Offspring 3	139/143	135/143	143/155	143/148	251/253	261/267	227/235	260/260	139/141	224/232
	NPA	2	3	2	2	2	2	3	2	3	2
F2	Mother	139/151	141/143	157/161	143/143	223/253	261/277	229/235	260/262	139/139	224/224
	Offspring 1	139/147	139/141	157/161	143/148	223/253	261/279	227/229	260/266	139/141	224/224
	Offspring 2	139/141	141/143	143/157	143/146	223/245	277/279	229/255	256/260	139/143	224/232
	Offspring 3	139/139	139/143	157/159	139/143	223/235	261/261	229/231	260/260	139/143	224/224
	NPA	3	2	3	3	3	2	3	3	2	2
F3	Mother	143/145	143/143	143/143	143/143	253/257	277/279	235/235	256/260	141/143	224/232
	Offspring 1	143/145	139/143	143/157	143/148	223/253	277/281	231/235	256/256	135/143	232/232
	Offspring 2	139/143	143/143	143/161	143/148	223/253	279/281	231/235	256/256	139/141	232/234
	Offspring 3	137/145	139/143	143/157	139/143	223/253	261/277	227/235	256/262	139/143	224/232
	NPA	3	2	2	2	1	2	2	2	2	3
F4	Mother	139/143	141/143	143/161	143/148	223/257	279/281	233/235	260/266	143/143	234/234
	Offspring 1	139/151	143/163	157/161	143/146	223/243	279/281	233/235	262/266	143/163	224/234
	Offspring 2	139/145	139/143	143/161	143/143	223/223	277/279	231/235	260/260	135/143	232/234
	NPA	2	2	2	2	2	2	2	2	2	2
F5	Mother	139/151	139/141	143/157	143/146	223/253	261/277	229/231	262/264	139/143	224/232
	Offspring 1	139/145	139/143	143/163	143/148	223/253	277/277	229/229	262/266	135/143	224/224
	Offspring 2	143/151	139/143	143/157	143/148	223/253	277/279	231/235	262/262	135/139	228/232
	Offspring 3	139/145	139/157	157/163	143/148	223/235	261/277	231/235	260/262	143/143	224/232
	Offspring 4	139/143	139/139	157/159	143/143	223/253	277/279	229/231	262/266	143/163	224/224
	NPA	3	3	3	2	2	2	3	3	3	2
F6	Mother	137/139	139/143	157/161	137/143	223/255	261/277	231/235	260/262	139/143	224/232
	Offspring 1	139/145	143/163	155/157	143/148	223/253	257/261	229/235	252/260	141/143	224/234
	Offspring 2	139/143	141/143	143/157	143/257	223/243	261/277	233/235	252/262	143/163	224/224
	NPA	2	2	2	2	2	2	2	1	2	2
F7	Mother	139/143	141/143	157/161	143/148	249/253	261/277	231/235	262/262	143/163	234/238
	Offspring 1	143/143	139/143	157/163	143/146	249/235	261/277	229/235	262/262	135/163	224/238
	Offspring 2	135/143	143/143	157/157	143/143	253/253	257/261	227/231	262/268	141/143	224/234
	Offspring 3	139/141	139/143	161/165	143/257	223/253	277/277	231/235	262/262	143/143	234/234

Appendix 3.1 *cont.*

Family/Locus		MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	MavC4-2	MavF1-2
	Offspring 4	135/139	135/143	159/161	143/146	253/253	277/285	235/235	262/262	135/143	232/234
	Offspring 5	139/139	141/143	157/159	139/143	253/253	261/279	235/246	252/262	143/145	232/234
	NPA	3	4	4	4	3	4	5	3	4	3
F8	Mother	139/143	139/143	143/161	143/146	223/257	277/279	229/231	262/266	139/141	224/232
	Offspring 1	141/143	141/143	157/161	139/146	223/253	277/277	229/235	262/262	139/143	232/232
	Offspring 2	139/141	141/143	143/147	146/148	257/253	279/279	231/235	262/266	141/143	224/224
	Offspring 3	143/143	143/143	143/157	143/143	253/257	277/277	231/239	266/266	135/139	224/228
	NPA	2	2	2	3	1	2	2	2	2	3
F9	Mother	139/151	143/143	143/157	143/143	235/253	267/277	229/235	260/262	139/143	224/232
	Offspring 1	137/139	143/143	157/161	143/143	223/235	277/279	229/235	260/262	135/139	232/234
	Offspring 2	137/139	143/148	157/161	143/148	223/235	267/279	227/229	260/262	135/139	224/224
	Offspring 3	139/141	141/143	155/157	143/257	253/253	277/281	231/235	262/262	135/143	232/234
	Offspring 4	139/143	139/143	143/161	143/146	223/253	261/277	235/248	260/266	139/143	232/232
	Offspring 5	139/139	143/143	141/157	143/143	223/253	267/279	229/235	260/262	139/141	224/234
	NPA	4	4	3	4	2	2	4	2	3	3
F10	Mother	139/141	127/143	141/143	143/146	223/253	261/279	235/239	260/262	139/139	232/234
	Offspring 1	139/141	143/143	143/143	141/143	223/253	279/283	229/235	262/262	139/143	232/234
	Offspring 2	141/145	127/143	143/157	143/146	223/253	279/279	231/235	260/260	139/141	234/234
	NPA	2	2	2	2	1	2	2	2	2	1
F11	Mother	143/151	143/148	141/157	143/146	253/255	261/261	233/235	260/260	139/143	224/234
	Offspring 1	143/151	143/143	157/161	143/143	223/253	261/277	229/235	260/260	139/141	224/224
	Offspring 2	143/149	141/143	141/143	143/148	235/253	261/277	233/235	260/268	141/143	224/232
	NPA	2	2	2	2	2	1	2	2	1	2
F12	Mother	139/145	143/145	143/145	146/148	223/249	277/279	229/235	262/264	135/145	224/232
	Offspring 1	139/143	143/143	143/143	146/146	223/223	263/279	229/235	262/262	139/145	232/232
	Offspring 2	143/145	143/143	143/143	143/146	223/235	277/279	235/235	262/266	135/135	224/234
	Offspring 3	141/145	145/145	145/147	146/148	223/223	263/277	229/235	264/266	135/139	232/232
	Offspring 4	143/145	141/143	143/161	143/148	223/257	261/277	231/235	264/264	135/145	224/228
	Offspring 5	139/139	143/143	145/161	143/148	223/223	279/279	229/235	262/266	135/139	224/234
	NPA	3	3	3	3	3	4	2	3	3	3
F13	Mother	139/145	143/157	143/145	141/148	223/253	277/279	229/231	260/264	137/143	224/234
	Offspring 1	139/143	143/155	143/157	143/148	223/257	261/279	231/231	260/266	135/137	224/224
	Offspring 2	141/145	143/143	145/161	141/143	253/257	279/279	229/235	264/266	135/143	232/234
	NPA	2	2	2	1	1	2	2	2	1	2
F14	Mother	141/143	135/143	157/161	146/148	251/253	261/279	231/235	262/264	143/163	228/232

Appendix 3.1 *cont.*

Family/Locus		MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	MavC4-2	MavF1-2
	Offspring 1	139/143	143/143	143/157	143/146	223/253	261/277	229/235	262/266	139/143	224/232
	Offspring 2	141/143	135/143	161/161	146/148	223/251	261/261	229/231	260/262	141/143	228/242
	Offspring 3	143/145	143/143	143/161	143/148	253/253	267/279	235/235	260/264	143/143	232/232
	NPA	3	2	2	2	2	3	2	2	3	3
F15	Mother	139/143	143/143	157/161	137/143	253/257	261/261	231/235	260/268	143/143	232/232
	Offspring 1	143/145	141/143	161/165	137/143	223/253	261/277	229/235	260/262	143/177	224/232
	Offspring 2	141/143	135/143	161/161	143/148	223/253	261/279	229/231	262/268	143/163	232/232
	Offspring 3	139/139	137/143	157/161	129/137	235/253	261/277	229/231	260/260	139/143	224/232
	Offspring 4	143/145	139/143	139/161	143/148	253/253	261/279	233/235	260/260	141/143	232/242
	NPA	3	4	3	3	3	2	3	2	4	3
F16	Mother	139/151	141/143	143/157	143/146	223/257	277/279	231/235	260/262	139/143	224/232
	Offspring 1	139/143	141/143	143/161	143/148	223/235	277/279	233/235	262/262	141/143	224/224
	Offspring 2	139/151	143/143	143/161	143/148	223/235	277/285	231/235	262/266	143/143	224/230
	Offspring 3	149/151	143/163	143/165	143/146	235/257	279/281	229/235	262/268	139/177	232/232
	Offspring 4	139/147	137/143	157/159	141/146	257/257	261/277	233/235	260/262	135/139	224/232
	NPA	4	3	3	3	2	4	3	4	4	3
F17	Mother	139/143	139/143	157/163	143/146	253/257	277/279	231/235	262/264	149/177	224/232
	Offspring1	137/143	135/139	157/161	143/148	223/253	277/281	229/235	260/262	143/149	224/224
	Offspring2	139/143	139/143	143/157	146/148	249/253	277/279	229/231	260/262	141/149	224/232
	Offspring3	139/143	141/143	155/157	143/143	253/253	261/279	231/233	264/264	143/149	232/234
	Offspring4	143/143	141/143	157/161	143/143	223/253	277/279	235/235	260/264	139/149	224/232
	Offspring5	137/143	141/143	161/163	141/146	249/257	263/277	235/237	258/262	139/149	224/232
	NPA	3	3	3	3	4	5	5	3	3	3
F18	Mother	139/141	143/143	143/159	143/146	223/253	261/263	235/235	260/262	135/141	228/232
	Offspring1	139/147	143/143	143/161	143/143	253/253	261/277	229/235	260/260	135/143	228/232
	Offspring2	141/143	143/143	143/157	143/146	253/253	263/277	229/235	262/264	141/143	228/232
	Offspring3	135/141	143/143	157/159	143/143	223/253	263/279	229/235	260/260	141/143	228/232
	NPA	3	1	2	2	2	2	1	2	1	1
F19	Mother	139/139	139/143	157/159	139/143	223/235	261/261	229/231	260/260	139/143	224/224
	Offspring1	139/143	139/143	157/161	139/143	235/257	261/261	231/235	260/262	135/143	224/238
	Offspring2	139/143	143/143	157/165	143/143	223/257	261/277	229/231	260/262	141/143	224/234
	NPA	1	1	2	1	1	2	2	1	2	2
F20	Mother	143/145	127/143	157/159	143/143	223/253	261/279	235/235	262/262	139/143	232/232
	Offspring1	135/143	141/143	139/157	135/143	223/223	277/279	235/235	260/262	139/139	232/232
	Offspring2	139/143	143/143	157/161	143/143	253/257	261/277	229/235	260/262	139/155	224/232

Appendix 3.1 *cont.*

Family/Locus		MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	MavC4-2	MavF1-2
	Offspring3	133/143	127/141	159/161	143/146	253/257	261/277	231/235	260/262	143/145	224/232
	Offspring4	141/143	127/143	157/161	143/143	223/253	261/261	235/235	262/264	143/143	224/232
	NPA	4	2	2	3	3	2	3	2	4	2
F21	Mother	139/143	139/143	157/163	143/151	253/257	277/277	229/235	260/264	139/145	224/232
	Offspring1	135/143	141/143	157/161	139/143	249/257	277/279	229/235	260/260	141/145	234/232
	Offspring2	143/143	141/143	157/157	143/146	253/257	277/277	229/235	262/264	139/145	224/224
	Offspring3	133/139	143/143	157/165	143/151	223/253	261/277	231/235	260/260	139/139	232/232
	Offspring4	143/143	135/143	143/157	143/146	253/253	261/277	229/235	260/262	139/143	228/232
	Offspring5	135/139	135/139	163/163	151/151	257/257	277/277	229/231	264/264	135/139	228/232
	NPA	3	3	5	5	4	3	3	3	4	3
F22	Mother	135/139	135/141	157/161	135/143	253/253	263/279	229/235	262/268	135/145	228/234
	Offspring1	139/139	135/143	161/165	143/146	223/253	263/277	229/235	262/262	135/141	224/228
	Offspring2	139/147	139/141	157/161	143/143	253/257	261/263	231/235	260/262	135/143	232/234
	NPA	2	2	2	2	2	2	2	2	2	2
F23	Mother	141/143	135/143	157/159	143/146	253/257	261/279	229/235	262/262	143/143	224/232
	Offspring1	139/143	135/141	159/163	143/148	253/261	279/279	235/235	262/262	133/143	224/232
	Offspring2	139/143	139/143	143/157	143/143	253/253	261/279	235/235	262/262	141/143	224/234
	Offspring3	139/143	141/143	143/157	143/148	223/257	277/279	231/235	262/264	141/143	224/224
	Offspring4	143/149	143/163	157/165	143/146	223/253	279/281	229/231	262/264	141/143	232/232
	Offspring5	139/143	135/143	157/157	143/146	253/253	261/261	235/235	260/262	143/145	228/232
	Offspring6	141/143	135/139	157/161	146/146	253/253	261/277	235/235	260/262	139/143	228/224
	NPA	3	5	5	3	3	4	2	3	4	4
FW1	Mother	147/151	139/143	161/163	143/146	247/249	267/277	227/227	260/262	141/143	224/230
	Offspring1	147/151	139/143	161/165	146/148	249/253	267/277	227/248	256/262	141/143	228/230
	Offspring2	147/151	143/143	161/163	146/146	245/247	277/277	227/235	260/262	137/141	224/232
	Offspring3	149/151	143/163	161/165	143/146	249/249	267/277	227/248	256/262	141/159	222/224
	Offspring4	143/147	135/143	161/171	141/143	245/249	267/277	227/235	260/260	141/145	228/230
	NPA	3	3	3	4	3	1	2	2	4	3
FW2	Mother	147/149	143/145	161/165	146/148	245/247	267/267	235/246	260/262	141/143	232/234
	Offspring1	149/151	139/143	161/165	146/148	245/247	267/285	235/248	260/260	139/141	232/238
	Offspring2	149/151	143/145	145/161	146/148	245/247	267/277	235/246	260/262	141/143	234/238
	Offspring3	143/147	143/145	161/163	143/146	245/247	267/271	231/235	256/260	143/143	232/238
	NPA	2	2	3	2	1	3	2	3	3	1
FW2	Mother	143/147	143/145	143/161	143/146	223/245	261/285	231/235	256/260	139/141	230/238
	Offspring1	143/147	141/143	157/161	143/146	245/247	261/277	235/235	256/256	139/163	236/238

Appendix 3.1 *cont.*

Family/Locus		MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	MavC4-2	MavF1-2
	Offspring2	147/151	143/145	161/165	141/143	245/245	277/285	227/235	260/260	141/143	220/230
	Offspring3	147/147	143/143	161/163	137/143	223/247	261/267	227/231	260/260	139/143	230/230
	NPA	2	3	3	3	2	2	2	2	2	3
FW4	Mother	147/147	143/145	161/163	143/146	243/245	267/277	227/248	256/260	143/145	224/250
	Offspring1	147/149	139/145	161/161	143/143	245/255	267/267	246/248	260/262	143/143	224/238
	Offspring2	147/149	139/145	161/163	146/146	245/255	277/285	227/235	256/256	139/145	224/238
	NPA	1	1	2	2	1	2	2	2	2	1
FW5	Mother	145/145	141/143	157/161	143/146	243/255	267/277	246/248	260/262	143/143	224/230
	Offspring1	145/145	139/143	161/161	143/146	243/249	277/285	235/248	260/262	141/143	230/232
	Offspring2	145/145	143/145	161/171	143/151	243/243	277/285	246/248	262/262	143/145	230/230
	Offspring3	145/147	141/145	161/161	143/146	255/255	277/279	227/248	260/260	141/143	230/250
	Offspring4	145/145	141/145	157/163	143/146	243/245	267/285	227/246	262/262	143/143	230/250
	Offspring5	145/149	141/143	161/163	141/143	245/255	277/285	246/246	262/262	143/145	224/238
	Offspring6	145/147	143/143	161/165	137/143	243/247	277/279	231/248	256/262	143/143	230/250
	Offspring7	145/145	143/143	157/163	143/143	243/245	261/277	233/248	260/260	143/145	230/250
	NPA	3	3	4	5	5	4	5	3	2	4

Appendix 3.2 Paternal genotypes at 10 microsatellite loci reconstructed manually for all offspring tested.

Site/ Year	Family		Locus MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	mavC4-2	mavF1-2
Bontuchel/ 2006	F1	Mother	137/139	135/135	155/161	139/148	251/255	261/277	231/235	260/262	127/139	232/238
		Father1	143/145	135/139	157/157	143/143	245/245	279/279	235/235	260/260	135/135	232/232
		Father2	145/145	139/143	157/143	143/146	245/253	279/267	229/227	256/260	127/141	232/224
	F2	Mother	139/151	141/143	157/161	143/143	223/253	261/277	229/235	261/262	139/139	224/224
		Father1	141/147	139/143	161/143	146/148	245/253	279/261	227/231	266/260	141/143	224/224
		Father2	139/139	139/139	159/161	139/146	235/253	279/279	255/231	256/260	143/143	224/232
	F3	Mother	143/145	143/143	143/143	143/143	253/257	277/279	235/235	256/260	141/143	224/232
		Father1	139/145	139/139	157/157	148/148	223/223	261/281	231/231	256/256	135/139	232/232
		Father2	137/139	139/143	157/161	139/148	223/223	281/281	227/231	256/262	139/139	232/234
	F4	Mother	139/143	141/143	143/161	143/148	223/257	279/281	233/235	260/266	143/143	234/234
		Father1	151/145	163/139	143/157	146/143	223/243	281/277	235/231	262/260	163/135	224/232
	F5	Mother	139/151	139/141	143/157	143/146	223/253	261/277	229/231	262/264	139/143	224/232
		Father1	145/145	143/143	163/163	148/148	235/253	277/277	235/235	260/266	135/163	224/224
		Father2	143/143	139/137	157/159	143/148	253/253	277/279	229/231	266/262	135/143	224/228
	F6	Mother	137/139	139/143	157/161	137/143	223/255	261/277	231/235	260/262	139/143	224/232
		Father1	145/143	163/141	155/143	148/257	253/243	257/261	229/233	252/252	141/163	234/224
	F7	Mother	139/143	141/143	157/161	143/148	249/253	261/277	231/235	262/262	143/163	234/238
		Father1	143/139	135/141	163/157	146/139	235/253	277/277	229/231	262/262	143/143	232/232
		Father2	135/141	139/143	165/159	146/257	253/223	279/285	229/246	262/252	135/145	224/224
		Father3	135/139	139/139	159/159	143/146	253/253	257/277	227/235	262/268	135/141	224/232
	F8	Mother	139/143	139/143	143/161	143/146	223/257	277/279	223/231	262/266	139/141	224/232
		Father1	141/141	141/143	157/157	139/143	253/253	277/277	235/235	262/266	135/143	224/232
		Father2	141/143	141/141	157/147	148/143	253/253	277/279	235/239	266/266	143/143	232/228
	F9	Mother	139/151	143/143	143/157	143/143	235/253	267/277	229/235	260/262	139/143	224/232
		Father1	137/137	141/139	161/161	143/148	223/253	261/279	227/231	262/262	135/135	234/234
		Father2	139/141	143/148	155/161	146/257	223/223	281/279	235/235	262/266	135/139	224/234

Appendix 3.2 *cont.*

Site/ Year	Family		Locus MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	mavC4-2	mavF1-2
	F10	Mother	139/141	127/143	141/143	143/143	223/253	261/279	235/239	260/262	139/139	232/234
		Father1	141/145	143/123	143/157	141/146	253/253	283/279	229/231	262/260	143/141	234/234
	F11	Mother	143/151	143/148	141/157	143/146	253/255	261/261	233/235	260/260	139/143	224/234
		Father1	149/151	143/141	161/143	143/148	223/235	277/277	229/233	260/268	141/141	224/232
	F12	Mother	139/145	143/145	143/145	146/148	223/249	277/279	229/235	262/264	135/145	224/232
		Father1	143/141	143/145	143/147	146/143	223/235	261/263	235/235	266/266	139/139	232/232
		Father2	145/139	143/141	161/161	148/143	223/257	263/279	235/231	266/264	139/145	234/228
	F13	Mother	139/145	143/157	143/145	141/148	223/253	277/279	229/231	260/264	137/143	224/234
		Father1	143/141	143/145	157/161	143/143	257/257	279/279	231/235	266/266	135/135	224/232
	F14	Mother	141/143	135/143	157/161	146/148	251/253	261/279	231/235	262/264	143/163	228/232
		Father1	139/141	143/143	143/143	143/143	223/223	277/277	229/229	260/266	139/141	224/232
		Father2	141/145	135/135	143/161	143/146	223/253	261/267	229/235	260/260	141/143	242/232
	F15	Mother	139/143	143/143	157/161	137/143	253/257	261/261	231/235	260/268	143/143	232/232
		Father1	145/145	139/141	161/165	143/148	223/223	277/277	229/231	260/260	141/177	224/232
		Father2	141/139	135/137	139/157	148/129	235/253	279/279	231/233	262/262	163/139	224/242
	F16	Mother	139/151	141/143	143/157	143/146	223/257	277/279	231/235	260/262	139/143	224/232
		Father1	143/139	143/143	161/161	148/148	235/235	277/285	233/231	262/266	141/143	224/230
		Father2	147/151	163/137	165/159	141/146	235/257	281/261	233/229	262/268	177/135	232/232
Bontuchel/ 2007	F17	Mother	139/143	139/143	157/163	143/146	253/257	277/279	235/231	260/264	143/149	224/232
		Father1	137/143	135/141	161/155	148/143	223/253	281/279	229/229	260/260	141/143	232/232
		Father2	139/139	143/141	161/143	148/141	249/249	277/261	229/233	258/264	143/143	232/234
		Father3	139/143	141/141	143/155	148/148	223/253	277/263	235/237	260/258	139/139	224/224
	F18	Mother	139/141	143/143	143/157	143/146	223/253	261/263	235/235	260/262	135/143	228/232
		Father1	135/147	143/143	157/157	143/143	253/253	277/277	229/229	260/260	143/143	232/232
		Father2	143/135	143/143	157/161	146/146	253/223	277/279	229/229	260/264	143/143	232/232
	F19	Mother	139/139	139/143	157/159	139/143	223/235	261/277	231/229	260/260	139/143	224/224

Appendix 3.2 *cont.*

Site/ Year	Family		Locus MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	mavC4-2	mavF1-2
		Father1	143/143	143/143	161/165	143/143	257/257	261/277	235/231	262/262	135/141	234/238
	F20	Mother	143/145	127/143	157/159	143/143	223/253	261/279	235/235	262/262	139/143	232/232
		Father1	141/139	141/141	161/161	143/143	223/257	261/277	231/235	260/260	143/145	224/224
		Father2	135/133	143/143	139/161	135/146	223/257	277/277	235/229	264/260	139/155	232/224
	F21	Mother	143/139	139/145	157/163	143/151	253/257	277/277	229/235	260/264	139/145	224/232
		Father1	135/143	141/141	161/157	139/146	249/257	279/277	229/231	262/264	139/135	224/228
		Father2	135/133	135/135	165/163	151/151	253/223	261/261	235/231	260/262	139/143	232/228
		Father3	143/133	141/143	165/143	146/151	249/253	279/279	231/231	260/260	141/135	234/228
	F22	Mother	135/139	135/143	157/161	135/143	253/253	263/279	229/235	262/268	135/145	228/234
		Father1	139/147	139/143	161/165	146/143	223/257	261/277	231/235	260/262	141/143	224/232
	F23	Mother	141/143	135/143	157/159	143/146	253/257	261/279	229/235	262/262	143/143	224/232
		Father1	139/139	141/143	143/143	143/148	223/253	261/281	235/235	262/262	133/141	232/234
		Father2	139/139	163/139	165/157	146/148	253/261	281/277	235/231	262/260	141/145	224/228
		Father3	139/141	139/139	161/163	146/146	223/223	261/277	235/248	260/260	141/139	232/228
Wych/ 2006	FW1	Mother	147/151	139/143	161/163	143/146	247/249	267/277	227/227	260/262	141/143	224/230
		Father1	151/149	143/163	165/163	143/148	245/249	277/277	248/235	256/260	159/145	228/222
		Father2	151/143	143/135	165/171	141/146	253/245	277/277	248/235	256/260	141/137	228/232
	FW2	Mother	147/149	143/145	161/165	146/148	245/247	267/267	235/246	260/262	141/143	232/234
		Father1	151/143	139/143	165/163	146/143	245/245	285/277	231/246	256/262	139/141	238/238
		Father2	151/151	143/143	145/163	146/143	245/245	285/271	246/248	260/256	139/143	238/238
	FW3	Mother	143/147	143/145	143/161	143/146	223/245	261/285	231/235	256/260	139/141	230/238
		Father1	147/151	141/145	157/165	146/141	247/245	277/277	235/227	260/260	141/163	220/236
		Father2	147/147	141/143	163/157	146/137	247/247	277/267	235/227	256/260	139/141	236/230
Wych/ 2007	FW4	Mother	147/147	143/145	161/163	143/146	243/245	267/277	227/248	256/260	143/145	224/250
		Father1	147/145	139/145	161/161	143/151	245/243	267/277	246/227	260/256	143/139	238/232
		Father2	149/149	139/139	161/165	143/148	255/255	267/285	235/246	256/262	139/143	238/238

Appendix 3.2 *cont.*

Site/ Year	Family		Locus MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	mavC4-2	mavF1-2
	FW5	Mother	145/145	141/143	157/161	143/146	243/255	267/277	246/248	260/262	143/143	224/230
		Father1	147/149	141/143	161/165	141/146	255/247	279/261	227/233	260/260	141/145	230/232
		Father2	145/145	141/143	163/171	137/151	243/245	285/285	231/246	260/256	143/143	250/238
		Father3	145/147	139/145	163/163	146/146	245/249	279/285	227/246	262/262	141/143	230/250

Appendix 3.3 The most likely father for offspring in each family generated by CERVUS with 80% confidence level.

Site	Family	No. of offspring	Offspring ID	Mother ID	The most likely father ID	Trio LOD score
Bontuchel	F1	3	BON.002	BON.004	BON.079	1.08E+00
			BON.007	BON.004	BON.029	6.97E+00
			BON.009	BON.004	BON.029	9.98E+00
	F2	3	BON.010	BON.011	BON.035	1.07E+01
			BON.073	BON.011	BON.141	8.60E+00
			BON.012	BON.011	BON.035	7.92E+00
	F3	3	BON.026	BON.027	BON.100	4.29E+00
			BON.038	BON.027	BON.056	2.61E+00
			BON.034	BON.027	BON.056	4.98E+00
	F4	2	BON.031	BON.032	BON.079	6.88E+00
			BON.033	BON.032	BON.079	2.93E+00
	F5	4	BON.053	BON.055	BON.132	1.32E+01
			BON.054	BON.055	BON.035	1.73E+01
			BON.060	BON.055	BON.132	1.28E+01
			BON.066	BON.055	BON.035	1.78E+01
	F6	2	BON.042	BON.043	BON.071	2.46E+01
			BON.044	BON.043	BON.071	1.42E+01
	F7	5	BON.072	BON.076	BON.021	6.01E+00
			BON.075	BON.076	BON.113	8.60E+00
			BON.074	BON.076	BON.102	1.62E+01
			BON.081	BON.076	BON.102	1.27E+01
			BON.077	BON.076	BON.113	3.61E+00
	F8	3	BON.128	BON.132	BON.064	1.51E+01
			BON.129	BON.132	BON.125	4.85E+00
			BON.130	BON.132	BON.064	1.40E+01
	F9	5	BON.021	BON.020	BON.133	8.31E+00
			BON.023	BON.020	BON.128	6.02E-01
			BON.024	BON.020	BON.047	1.37E+00
			BON.028	BON.020	BON.133	3.88E+00
			BON.138	BON.020	BON.047	1.20E+01
	F10	2	BON.094	BON.095	BON.035	1.61E+01
			BON.096	BON.095	BON.035	1.28E+01
	F11	2	BON.107	BON.109	BON.064	4.82E+00
			BON.108	BON.109	BON.064	1.34E+01
	F12	5	BON.080	BON.071	BON.065	7.29E+00
			BON.073	BON.071	BON.089	4.21E+00
			BON.089	BON.071	BON.003	3.66E+00
			BON.109	BON.071	BON.065	4.29E+00
			BON.137	BON.071	BON.003	1.75E+01
	F13	2	BON.111	BON.132	BON.128	2.43E+00
			BON.114	BON.132	BON.128	7.64E+00
	F14	3	BON.103	BON.042	BON.018	3.75E-02
			BON.104	BON.042	BON.131	2.59E+00
			BON.112	BON.042	BON.018	1.40E+00
	F15	4	BON.061	BON.118	BON.088	5.93E+00
			BON.067	BON.118	BON.010	2.81E+00
			BON.069	BON.118	BON.010	7.83E+00

			BON.070	BON.118	BON.088	2.73E-01
	F16	4	BON.057	BON.055	BON.079	1.32E+01
			BON.058	BON.055	BON.035	1.73E+01
			BON.062	BON.055	BON.079	1.28E+01
			BON.068	BON.055	BON.035	1.78E+01
	F17	5	BON.082	BON.086	BON.053	6.07E+00
			BON.083	BON.086	BON.047	2.93E+00
			BON.084	BON.086	BON.008	4.90E+00
			BON.085	BON.086	BON.008	4.47E+00
			BON.099	BON.086	BON.047	1.97E+00
	F18	3	BON.006	BON.004	BON.079	1.08E+00
			BON.008	BON.004	BON.029	6.97E+00
			BON.005	BON.004	BON.029	9.98E+00
	F19	2	BON.013	BON.011	BON.035	1.07E+01
			BON.012	BON.011	BON.035	7.92E+00
	F20	4	BON.025	BON.027	BON.035	4.29E+00
			BON.030	BON.027	BON.056	2.61E+00
	F21	5	BON.136	BON.137	BON.131	1.00E+01
			BON.138	BON.137	BON.035	1.66E+01
			BON.139	BON.137	BON.035	2.91E+01
			BON.140	BON.137	BON.102	2.00E+01
			BON.135	BON.137	BON.131	1.00E+01
	F22	2	BON.101	BON.125	BON.039	5.75E+00
			BON.115	BON.125	BON.053	1.28E-01
			BON.036	BON.027	BON.056	4.98E+00
			BON.037	BON.027	BON.035	6.88E+00
	F23	6	BON.116	BON.056	BON.035	2.95E+00
			BON.117	BON.056	BON.129	3.91E+00
			BON.118	BON.056	BON.035	2.58E+00
			BON.119	BON.056	BON.131	1.41E+00
			BON.059	BON.056	BON.129	6.12E+00
			BON.063	BON.056	BON.131	8.83E+00
Wych	FW1	4	Wych.055	Wych.048	Wych.044	6.12E+00
			Wych.045	Wych.048	Wych.044	5.93E+00
			Wych.073	Wych.048	Wych.090	7.74E-01
			Wych.049	Wych.048	Wych.090	8.83E+00
	FW2	3	Wych.052	Wych.057	Wych.061	3.91E+00
			Wych.053	Wych.057	Wych.089	2.58E+00
			Wych.054	Wych.057	Wych.061	1.41E+00
	FW3	3	Wych.049	Wych.056	Wych.038	8.83E+00
			Wych.050	Wych.056	Wych.035	6.01E+00
			Wych.051	Wych.056	Wych.038	2.95E+00
	FW4	2	Wych.047	Wych.046	Wych.044	5.93E+00
			Wych.075	Wych.046	Wych.044	7.74E-01
	FW5	7	Wych.060	Wych.059	Wych.035	6.01E+00
			Wych.062	Wych.059	Wych.061	2.95E+00
			Wych.063	Wych.059	Wych.061	3.91E+00
			Wych.064	Wych.059	Wych.071	2.58E+00
			Wych.065	Wych.059	Wych.061	1.41E+00
			Wych.066	Wych.059	Wych.071	6.12E+00
			Wych.067	Wych.059	Wych.071	6.12E+00

Appendix 3.4 Number of offspring and body weight for females included in analysis in both study sites. IB1-IB8 and IW1-IW7 are individual females randomly selected and that only have one offspring in nestbox.

Site	Female ID	Number of offspring	Date obtained	Weight (g)
Bontuchel	F1	3	20/06/2006	16
	F2	3	18/09/2006	19
	F3	3	18/09/2006	13
	F4	2	18/09/2006	15
	F5	4	18/09/2006	19
	F6	2	18/09/2006	15
	F7	5	18/09/2006	19
	F8	3	18/09/2006	16
	F9	5	18/09/2006	20
	F10	2	18/09/2006	16
	F11	2	18/10/2006	14
	F12	5	18/10/2006	21
	F13	2	18/10/2006	13
	F14	3	18/10/2006	20
	F15	4	18/10/2006	22
	F16	4	18/10/2006	20
	F17	5	18/05/2007	19
	F18	3	18/05/2007	17
	F19	2	18/05/2007	12
	F20	4	18/05/2007	15
	F21	5	17/09/2007	20
	F22	2	17/09/2007	14
	F23	6	17/09/2007	23
	IB1	1	17/09/2007	12
	IB2	1	17/09/2007	9
	IB3	1	27/06/2007	13
	IB4	1	27/06/2007	14
	IB5	1	18/05/2007	11
	IB6	1	18/05/2007	12
	IB7	1	18/05/2007	11
	IB8	1	18/05/2007	11
Wych	FW1	4	16/10/2006	18
	FW2	3	16/10/2006	16
	FW3	3	16/10/2006	16
	FW4	2	16/10/2006	15
	FW5	7	22/05/2007	27

IW1	1	16/10/2006	10
IW2	1	16/10/2006	9
IW3	1	16/10/2006	11
IW4	1	16/10/2006	11
IW5	1	16/10/2006	12
IW6	1	16/10/2006	12
IW7	1	16/10/2006	13

Chapter 4: Dispersal characteristics of natural and reintroduced populations of the common dormouse, *Muscardinus avellanarius*.

4.0 Introduction

Species' dispersal characteristics influence a range of fundamental demographic and evolutionary processes (Boudjemadi et al. 1999; Hanski 1999; Clobert et al. 2001). Of particular relevance for conservation are studies that have inextricably linked dispersal with population persistence. For example, dispersal and subsequent gene flow maintains intra-population genetic diversity (*e.g.* Dossantos et al. 1995; Sommer et al. 2002; Nunes 2007; Gauffre et al. 2008) and thus limits the rate of genetic erosion; the corollary, particularly for small populations, is that reduced dispersal can increase the probability of extinction, either through inbreeding effects or loss of evolutionary potential (see *e.g.* Soulé 1988; Saccheri et al. 1998; Reed 2004; Ewing et al. 2008). Accordingly much research effort has been directed towards quantifying the putative roles of various life-history traits and ecological factors that influence dispersal, such as landscape heterogeneity, matrix quality and resource distribution (e.g. Manel et al. 2003; Berthier et al. 2005; Aars et al. 2006; Scribner et al. 2006; Russell et al. 2007). In this context, establishing species' natural patterns of dispersal and subsequent gene flow will provide insights into some of the key factors that are critical for the maintenance of viable populations – information that should underpin decisions about *in situ* management of species (Lidicker et al. 1987).

Reintroduction of captive-bred animals is an appealing concept that represents a viable solution to restore or augment populations of endangered or locally-extinct species without affecting the demography of other native populations (Gippoliti and Amori 2007; Armstrong and Seddon 2008; Bowkett 2009). However, there is increasing recognition that captive-bred animals can lack certain behaviours necessary for survival (*e.g.* Csermely 2000; Hellstedt and Kallio 2005; Mathews et al. 2005; Peignot et al. 2008; Maran et al. 2009). For example, rather than being innate, certain behaviours may have to be learnt from adults and an unnatural setting may limit or even prevent this from taking place. Alternatively, dispersal, like some other complex behavioural traits, can be, at least partly, a

heritable trait (Ferriere et al. 2000; see also Hansson et al. 2003; Krackow 2003 for case studies). If this were the case, a species' dispersal behaviour may change depending upon the variation present in the pool of breeding individuals and the specific breeding programme that is implemented. Most studies have focus on viability of reintroduced animals and whether they are capable of some dispersal (Tweed et al. 2003; Dzialak et al. 2005; Diefenbach et al. 2006; Ausband and Moehrenschlager 2009; Whitifield et al. 2009) and, to my knowledge, no studies have considered whether the reintroduced populations retain their natural dispersal tendencies.

Dispersal is a difficult behaviour to study directly in wild populations, with logistical constraints on the size of study areas typically leading to underestimates in both the scale and frequency of dispersal (Koenig et al. 1996; Broquet and Petit 2009). Particular problems may be encountered when there is natal dispersal, movement of juveniles from their place of birth prior to breeding (which occurs in many species of mammal; Nunes 2007) as individuals may disperse prior to being observed in the population (see *e.g.* Telfer et al. 2003b). Over many decades, therefore, a burgeoning literature has centered on the use of molecular-genetic markers to quantify patterns of gene flow (*i.e.* dispersal behaviour) among natural populations, with a recent emphasis on the effect of landscape (see *e.g.* Antolin et al. 2006; Gauffre et al. 2008; Perez-Espona et al. 2008; Neaves et al. 2009). Such approaches have proved invaluable at documenting species' response to changing environments. For example, increasing habitat fragmentation can lead to reduced dispersal through an inhospitable matrix (Banks et al. 2005; Redeker et al. 2006) and therefore potentially affect natural patterns of dispersal.

The common dormouse *Muscardinus avellanarius* is a cryptic mammal that inhabits areas of deciduous forest with a thick layer of scrub plants and underbrush (Bright et al. 2006). This species is distributed from the Mediterranean to southern Sweden, eastward to Russia (excluding Iberia) and into parts of northern Asia Minor (Juskaitis 2007; IUCN 2009) and reaches the northwest limit of its European range in the UK, where it can be found over much of southern England and in isolated patches in northern Wales (Bright et al. 2006). The detrimental effects of loss and fragmentation of forest habitat on *M. avellanarius* populations have been highlighted in the UK, where this species has suffered by a 64 % decline in numbers since the late 1970s (Bright et al. 2006). *M. avellanarius* now is

regarded as a "Flagship Species" for nature conservation and a bioindicator of woodland quality (Morris 2004; Bright et al. 2006). The status of *M. avellanarius* in Europe varies, depending on the country (Vilhelmsen 2003; Hofmann 2004; Morris 2004; Foppen et al. 2002) but generally this species categorised as 'least concern' on the Red List (IUCN 2009). In the UK it is listed as a Schedule 5 species and was placed on the English Nature's Species Recovery Programme in 1992 (Macdonald and Tattersal 2003).

Owing to its conservation priority, captive breeding and reintroduction programmes have been developed, which are aimed at conserving and protecting common dormice in the UK. By 2008, a total of 635 captive-bred dormice had been released in 16 reintroduction sites across the UK (PTES 2009). Generally, these *ex-situ* programmes have proved feasible with most reintroduced populations of *M. avellanarius* breeding and even spreading into adjacent available habitat (Morris 2004; PTES 2009). Effective planning of future reintroduction programs, as well as a more general evaluation of the viability of persisting natural populations, is limited by a lack of knowledge about the dispersal patterns exhibited by both natural and reintroduced populations of *M. avellanarius*. Prior studies using direct observations of tagged animals have indicated that *M. avellanarius* do not move far - typically less than 500 m in a lifetime (Morris 2004; Büchner 2008). However these data may suffer from the well-documented limitations associated with direct tracking of dispersal in animals (see Koenig et al. 1996; Broquet and Petit 2009). The natural dispersal pattern of this species has not yet been assessed using genetic methods. Moreover, a molecular genetic approach may also provide important information for assessing the success of reintroduction programmes (see Grenier et al. 2007).

The main aim of this study is, through a combination of field-surveys and molecular-genetic techniques, to quantify the natural dispersal pattern within a large natural population of *M. avellanarius* as this circumvents potential problems associated with habitat loss and fragmentation. I then contrast these data with the dispersal behaviour exhibited by individuals at a reintroduced population that were sourced from a captive-bred population. Specifically, I (1) quantify the level of genetic diversity and pattern of spatial genetic structure of *M. avellanarius* in natural and reintroduced populations, (2) quantify sex-biased dispersal characteristics and (3) determine whether the dispersal behaviour differs between wild and captive-bred individuals. The study therefore represents a first

step to understanding dispersal in this protected species, by focusing on dispersal patterns and genetic structure within large populations.

4.1 Materials and methods

4.1.1 Sample collection

Muscardinus avellanarius were sampled at two sites in the UK (1) Bontuchel (Denbighshire, Wales; Latitude 53.109364 N; Longitude: -3.370318 W; OS National Grid Reference, SJ082571) and (2) Wych Valley (Cheshire, England; Latitude 52.994994 N; Longitude -2.7745169 W; OS National Grid Reference, SJ481124) for three consecutive years (2006-2008) as part of a continued monitoring programme by the Northwest Dormouse Partnership (further information available at http://www.cheshirewildlifetrust.co.uk/proj_ dormouse_ partnership.htm). The natural population at Bontuchel inhabits an area of mixed broadleaf and conifers, while animals at the Wych Valley are the descendents of 29 and 24 captive-bred dormice that were released (in 1996 and 1997 respectively) into a habitat consisting of ancient woodland and native broadleaves habitat. To monitor *M. avellanarius*, 250 and 230 nestboxes, spaced at approximately 20-40 m intervals, were installed within 0.55 km^2 in Bontuchel and 0.19 km^2 in Wych respectively and were geolocated using a Global Positioning System (GPS). Nestboxes at Wych are consistently monitored for one day in May, June, September and October from 2006 to 2008. In Bontuchel, nestboxes are inspected in May, June and July and in September and October in year 2006. From 2007 to 2008, nestboxes consistently inspected in May, June, September and October. Every captured dormouse was scanned for a microchip and then its sex, weight, approximate age and breeding status recorded before being returned to its nestbox; dormice without microchips are anaesthetised and then chipped using 8 mm microchips. Age classes were determined as follows: brown young (brown fur, eyes open, weight approximately 4 g), juvenile (with non-orange fur, individual weight <12 g) and adult (orange fur, weight >12 g) (Juškaitis 2001; Bright et al. 2006). For genetic analyses, hair and buccal swab samples were collected during nestbox sampling.

To evaluate dispersal tendencies of dormice from field data, I calculated the average distance moved per month between capture (*i.e.* nestbox) locations for each sex and age separately. For animals first caught as juveniles, the cumulative distances may combine both natal dispersal and subsequent movements within home ranges, whilst for animals first

caught as adults the cumulative distances may only represent home range movements. Here the principal aim was to determine whether there were differences in movement patterns between the sexes and between populations. Therefore I checked whether there was a difference between these groups in (a) the proportion of animals first caught when young and (b) the length of time between first and last capture.

4.1.2 DNA extraction and genotyping

Total genomic DNA was extracted from hair and buccal swab samples using CHELEX-100 (Walsh et al. 1991). All samples were genotyped at 10 polymorphic microsatellite loci (Md. Naim et al. 2009). Each 10 µl PCR contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 3 mM $MgCl_2$, 1 µl extracted template DNA, 3 pmol of each primer, 10 µg BSA and 1.25 u DNA polymerase (ABgene). Thermal cycling conditions were 95°C 3 min, 6x [95°C 30s, T_a°C 45s, 72°C 45s], 25x [92°C 30s, T_a °C 45s, 72°C 55s], where T_a is the locus-specific annealing temperature, on a Dyad Engine (MJ Research Inc). PCR products were pooled with a 500 bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI3130*xl* and sized using GeneMapper software (Applied Biosystems).

4.1.3 Genetic diversity

MICROCHECKER ver. 2.2.3 (Van Oosterhout et al. 2004) was used to check for systematic genotyping errors (*i.e.* null alleles, large allele dropout and any mis-scoring of stutter peaks). Tests for linkage disequilibrium between all locus-pair combinations were carried out using GENEPOP ver.3.1d (Raymond and Rousset 1995) (Markov chain parameters were 1,000 dememorisation, 100 batches and 1,000 iterations per batch). Deviations from Hardy–Weinberg Equilibrium (HWE) conditions were quantified using FSTAT ver.2.9.3 (Goudet 1995) by making 2,000 permutations of alleles among individuals within samples. FSTAT was used to calculate allelic richness (A_R) standardised to 75 individuals, expected heterozygosity (H_e) and Wright's (1951) inbreeding coefficient (f) Sequential Bonferroni corrections for k multiple tests were applied where appropriate (Rice 1989).

4.1.4 Detection of recent population bottleneck

Evidence of a recent population bottleneck may be taken from the characteristic signature of significant excess of heterozygotes than expected under genetic equilibrium conditions

(Cornuet and Luikart 1996, Luikart and Cornuet 1998). I used BOTTLENECK ver.1.2.02 (Piry et al. 1999) software to compute an expected distribution of heterozygosities (H_e) under mutation-drift equilibrium from the allelic diversity of each sample for three different models of allelic mutation: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM). Both the Wilcoxon signed-rank test and a sign test were used to assess significance of whether the observed H_e is greater than that expected at equilibrium, although the latter is only robust when more than 20 loci are used (see Cornuet and Luikart 1996 for details).

4.1.5 Effective population size

A point estimate of short-term effective population size (N_e) was calculated from the strength of linkage disequilibrium (LD) (Hill 1981) using Waples' (2006) correction for a downward bias that occurs when the sample size is small relative to N_e. Briefly, the LD method of estimating N_e is based on the premise that if there is no immigration, population substructure or selection and the genetic sample is representative, then genetic drift in a finite population generates a measurable correlation between alleles among different loci that informs on the N_e. Estimates of N_e were computed using LDNe ver. 1.31 (Waples and Do 2008) for each sample and year separately. For this analysis, I assumed random mating populations and excluded alleles with frequencies less than 0.05, as inclusion of rare alleles causes an upward bias in N_e estimates (Waples and Do 2008); parametric 95 % confidence intervals were calculated using Equation 12 in Waples (2006).

4.1.6 Temporal genetic variation

Hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was used to partition the contribution to genetic diversity arising from spatial variation with that occurring among successive sampling periods.

4.1.7 Sex-biased dispersal

I determined whether there was sex-biased dispersal by estimating F_{is} (Weir and Cockerham's (1984) estimator of inbreeding within a population) and r (average relatedness of individuals within a population, where $r=2F_{st}/(1+F_{it})$) for both juvenile and adult categories (based on age at first capture) of both sexes using FSTAT ver. 2.9.3.2 (Goudet 2001), and where F_{st} and F_{it} are measures of inbreeding due to differences among

subpopulations and of individuals relative to the total population respectively. Because populations can contain different levels of gene diversity, the probability of occurrence of multi-locus genotypes of individuals in different populations are not directly comparable (Goudet 2001); to remove this problem, the data are log-transformed and the average probability of the sample is subtracted from the individual multi-locus probabilities to calculate the "corrected assignment index" *AIc* (Favre et al. 1997; Mossman and Waser 1999). If there is sex-biased dispersal, *r* and *mAIc* (the mean corrected assignment index) are lower in the dispersive sex, whereas F_{is} and *vAIc* (the variance of corrected assignment index) will be higher (see Goudet et al. 2002). The significances of the calculated values were estimated using 10,000 randomisations. I predicted that juvenile dormice would not show evidence of sex-biased dispersal as they will have not yet exhibited differences in movement patterns (*i.e.* differences in natal dispersal propensity or home range movements).

4.1.8 Spatial genetic structure

Spatial genetic structure (SGS) was examined by spatial autocorrelation (Hardy and Vekemans 1999). SPAGeDi ver.1.2 (Hardy and Vekemans 2002) was used to calculate the correlation in average kinship (F_{ij}, Loiselle et al. 1995) relative to the whole data set between pairs of *M. avellanarius* separated by a range of increasing spatial scales. This approach measures the genetic similarity between individuals *i* and *j* relative to the mean genetic similarity between random individuals in the sample (Hardy and Vekemans 2002). To visualize SGS, kinship coefficients values were averaged over a set of distance intervals and then plotted against geographical distance. To test for significant SGS, spatial group locations were permuted 1,000 times to generate 95 % confidence intervals (CI) for multilocus kinship coefficients at each distance class. For all spatial statistics, Euclidian geographical distances between individuals were calculated from the GPS *x* and *y* coordinates and analyses were conducted separately based on sex, age and year.

Finally, to determine whether there was immigration into the study sites, I conducted assignment tests to evaluate the proportion of first-generation immigrants among adults within each locality and for each sex separately using the program GENECLASS2 ver. 1.0 (Piry et al. 2004). Likelihood computation was performed using the frequency method (Paetkau et al. 1995) and the statistic L_h (*i.e.* likelihood computed from the population

where the individual was sampled) as recommended when all source populations for immigrants have not been sampled (Paetkau et al. 2004). The probability of an individual being resident was then assessed using a Monte Carlo resampling procedure of Paetkau et al. (2004). Individuals with a probability of less than 0.05 were excluded as resident and assigned as unknown population.

4.2 Results

4.2.1 Demographic parameters

The capture-mark-recapture data included a total of 508 and 212 captures in Bontuchel and Wych respectively, that corresponded to 174 (2006), 93 (2007) and 54 (2008) new dormice caught in Bontuchel and 51 (2006), 60 (2007), and 44 (2008) new dormice caught in Wych over the three sampling years (Table 4.1). From these samples, a total of 296 and 135 dormice were genotyped successfully in Bontuchel and Wych respectively, of which 108, 138 and 50 (Bontuchel) and 53, 67 and 15 (Wych) were new males, females and juveniles respectively. The adult sex ratio did not differ significantly from unity at either site (Bontuchel, χ^2=2.22, d.f.=1, P>0.05; Wych, χ^2=0.39, d.f.=1, P>0.05). While the proportion of juveniles in the samples was higher in Bontuchel (0.36, n=296) than in Wych (0.26, n=135), this difference was not significant (χ^2=0.03, d.f.=1, P>0.05). There is no significant difference between sites in the proportion of males first caught as juvenile (χ^2=0.80, d.f.=1, P>0.05) or the proportion of females first caught as juvenile (χ^2=1.62, d.f.=1, P>0.05). Similarly, there is no significant difference between the sites in the length of time between first and last capture in males first caught as juveniles (Kruskal-Wallis test value H=0.18, d.f.=1, NS) or the length of time between first and last capture in females first caught as juvenile (Kruskal-Wallis test value H=0.09, d.f.=1, NS). Only 10 juveniles from Bontuchel (4 F, 6 M) and 9 juveniles from Wych (3F, 6M) were caught as adults subsequently. Note that the frequency of sampling in Bontuchel in year 2006 (5 times) was greater than in 2007 and 2008 (4 times per year), whereas the frequency of sampling in Wych is consistent across years (4 times).

4.2.2 Dispersal

Based on the field data, a similar sex-biased pattern of movement was evident at both the natural and the reintroduced site, with male *M. avellanarius* moving further than the females (Figures 4.1a, b). At Bontuchel, the average distance moved per month by

(recaptured) adult females and males was 53.62±4.21 (SD) m and 64.17±6.13 (SD) m respectively, and at Wych the average distances moved by adults were 51.57±3.28 (SD) m (females) and 66.60±4.21 (SD) m (male). The difference in average distance moved between sexes was significant at both sites (Bontuchel; Mann-Whitney *U* test: n_1n_2=40, 47, *U*=773, *P*<0.05, Wych; Mann-Whitney *U* test: n_1n_2=24, 25, *U*=383, *P*<0.05), however, there was no significant difference in the average distance moved by dormice between the two sites for either sex (males, Mann-Whitney *U* test: n_1n_2=40, 24, *U*=538, *P*>0.05; females, Mann-Whitney *U* test: n_1n_2=47, 25, *U*=459, *P*>0.05).

The average period between the first and last capture for adult female dormice was not significantly different between sites (Bontuchel: 7.63±3.23 SD months; Wych: 7.92±0.33 SD months; Kruskal-Wallis test value H=0.05, d.f.=1, NS). Likewise, there was no significant difference between the first and last capture dates of male dormice (Bontuchel: 10.01±1.87 (SD) months; Wych: 9.23±2.54 (SD) months; Kruskal-Wallis test value H=1.62, d.f.=1, NS). However, the average period between the first and last capture was significantly different between sexes in both sites (Bontuchel: Kruskal-Wallis test value H=4.20, d.f.=1, *P*<0.05; Wych: Kruskal-Wallis test value H=6.57, d.f.=1, *P*<0.05). There was no significant difference in the distance moved between first and last capture of males and females that were first caught as juveniles and later caught as adults in Bontuchel (Mann-Whitney *U* test: n_1n_2=6, 4, *U*=3, *P*>0.05) and in Wych (Mann-Whitney *U* test: n_1n_2=6, 3, *U*=6.5, *P*>0.05).

4.2.3 Genetic diversity

None of the loci suffered from errors due to stuttering, large allele dropout or null alleles, and all ten microsatellite loci were polymorphic (Table 4.2). After sequential Bonferroni correction, no locus deviated significantly (*P*>0.05) from expected HWE conditions and significant linkage disequilibrium was not found between any pair of loci. Genetic variability was greater in Bontuchel with number of alleles (N_a) per locus ranging between 6 and 14 compared with 5 to 11 alleles in the Wych samples. Similarly, mean allelic richness A_R, was greater in Bontuchel (range: 8.60- 9.30) than in Wych (range: 5.20 - 6.80) (Table 4.2). However, mean expected (H_e) heterozygosity in Bontuchel (H_e=0.691±0.01 SD) and Wych (H_e=0.667±0.03 SD) did not differ significantly between sites (*t*-test, *t*=1.46, d.f.=4, *P*>0.05) and was relatively constant over the three years of study.

4.2.4 Bottleneck

At both sites there was no evidence for a significant heterozygote excess, after Bonferroni correction (k=3), that is indicative of a population bottleneck, for all mutation models and for both statistical tests (Appendix 4.1).

4.2.5 Effective population size and temporal genetic differences

The estimated effective population size varied somewhat across years but was consistently over 100 animals - N_e at the natural population in Bontuchel ranged from 138.2 (2007) to 230.8 (2006) whilst N_e at the reintroduced site in Wych ranged from 109.9 (2007) up to 149.1 (2006). Thus, at both sites the estimates of N_e were greater than the numbers of animals found during surveys and, in particular, in Wych the estimates of N_e were higher than the number of founder individuals. While N_e estimates were generally greater at Bontuchel, this difference was not significant (overlapping 95 % CI) (Table 4.3).

At both sites, no significant genetic differences were attributed to variation among temporal groups of samples (P>0.05), which accounted for between 2 to 3 % of the total genetic variance (Table 4.4).

4.2.6 Sex-biased dispersal

There was genetic evidence for male-biased dispersal (MBD) in adults at both sites that is consistent with the dispersal tendencies described above (from nestbox surveys of chipped animals). Thus, average relatedness, r, was significantly (P<0.05) lower in adult males than in adult females at both populations, and significantly negative *mAIc* and significantly greater values of *vAIc* and F_{is} were observed in males than in females. I also found qualitative evidence of MBD in juveniles both at Bontuchel (n=50) and at Wych (n=15), whereby the values of *mAIc* and *vAIc* were lower and higher respectively in males, but differences between male and female juveniles were not significant probably because of the small sample size (Table 4.5).

4.2.7 Spatial genetic structure

Individual correlogram profiles of the relationship between average kinship (F_{ij}) and spatial separation varied slightly between sampling years, but any differences were not significant as the 95 % confidence intervals for the average value of F_{ij} at each distance class

overlapped (Appendices 4.2a, b). Therefore the data were pooled over all years to demonstrate the broad pattern of dispersal behaviour (Appendix 4.2c). Again, when combining data over all sample periods, a significant pattern of spatial autocorrelation was observed in adult females in both sites, with pairs of individuals up to 200 m apart having significant F_{ij} values and then significantly negative F_{ij} values from 300 m and beyond (Figures 4.2a, b). Consistent with the analyses described above that indicate male-biased dispersal, a contrast in the pattern of spatial genetic structuring between adult males and females was evident in both populations. Adult males at both sites displayed lower levels of relatedness at short distance classes (Figures 4.2a, b) and only average F_{ij} at 500 m distance classes were significantly different from zero. Overall, juvenile male and female dormice showed no apparent relationship between relatedness and distance even though there is a qualitative pattern of declining average pairwise kinship in both sexes with increasing distance (Appendix 4.3a, b); however none of the F_{ij} values significantly different from zero. Generally, the F_{ij} values for adult dormice in Bontuchel were consistently higher than in Wych but the difference was not significant (t-test, t=1.32, d.f.=16, P>0.05); this pattern was observed also in juveniles in both sites (t-test, t=1.06, d.f.=16, P>0.05).

Assignment tests revealed a relatively low rate of immigration and supported the idea of male-biased dispersal, with a high proportion of individuals (96.3 % of the 188 adults at Bontuchel and 91.0 % of the 100 adults in Wych) likely to be residents (at >95 % likelihood). There were 11 individuals (9 M and 2 F) and nine individuals (6 M and 3 F) in Bontuchel and Wych respectively assigned as potential immigrants (P<0.05; data pooled over years), although none of the sampled individuals were identified as suspected immigrants at a more stringent probability (*i.e.* P<0.01).

4.3 Discussion

The main outcomes of this study are that (1) a natural pattern of dispersal is maintained in the reintroduced population founded with individuals from a captive breeding programme, and (2) common dormice are relatively sedentary but exhibit sex-biased dispersal, with adult males dispersing further than females. I present evidence for reduced genetic diversity in the reintroduced population, but no significant bottleneck. Moreover, while there is no evidence for strong spatial structure that is manifest as discrete subpopulations

within large (i.e. 2.5 km) habitat patches, dispersal is sufficiently limited to allow isolation by distance genetic structure to develop.

Both the scale of dispersal and a pattern of male-biased dispersal were evident at both sites (these topics are discussed in more detail below), indicating that a period of captive breeding has not affected dispersal behavior. This is somewhat surprising as my result is contrary to the study conducted by McPhee (2003) in which captivity had compromised the behaviour of oldfield mice *Peromyscus polionotus* that had been in captivity for multiple generations. Other studies (Hellstedt and Kallio 2005; Kelley et al. 2006; Fischer and Lindenmayer 2000, for review) also have demonstrated the failure of captive bred animals in retaining its natural behaviour when release into wild. This study also implies that dispersal behaviour, and the differential response of the males and females, is innate. Most vertebrates are predisposed at birth to make innate dispersal movements (Howard 1960) and have been well documented in literatures (see e.g. Krackow 2003; Hansson et al. 2003; Weimerskirch et al. 2006; Kynard et al. 2007).

4.3.1 Dispersal of marked individuals

The propensity of tagged adult males to move further than a female (Figures 4.1a, b) is consistent with the genetic data (Figures 4.2a, b), suggesting that the greater movements made by males (combining both natal dispersal and subsequent movements as breeding adults) apparently translates into movement of genes. The agreement between direct (*i.e.* tagging) and indirect (*i.e.* genetic) methods of dispersal has been demonstrated in some other studies, particularly when dispersal has been studied within continuous habitat patches (Watts et al. 2007a; Selonen et al. 2009). However, studies at larger spatial scales often detect more frequent and longer dispersal events using indirect than by direct approaches (*e.g.* see Telfer et al. 2003a). The maximum movement distance of tagged animals detected in this study (600 m) is comparable to that recorded at other study sites; for example, Büchner (2008) reported that dormice in Germany typically travel less than 500 m during their lifetime. Although, data on the patterns of dispersal by dormice between discrete habitat patches are lacking, the restricted movement patterns observed here indicate that common dormice are expected to move more frequently within sites and to neighbouring areas, thus isolation by distance (IBD) genetic structure (see Rousset 2001, 2004; Watts et al. 2004a) should be typical.

4.3.2 Patterns of genetic diversity

Almost certainly because of the relatively small number of founders, the reintroduced population at Wych harbours less genetic diversity than the population at Bontuchel (Table 4.2). Indeed, many populations established from a limited number of founders typically show a reduction in genetic variability compared with more-established, natural populations (*e.g.* Fitzsimmons et al. 1997; Hedrick et al. 2004; Sigg 2006). Nonetheless, that there was no significant difference in gene diversity (H_e) between sites points towards a relatively limited extent of genetic erosion at Wych. One likely reason for this is the prevalence of multiple mating by female dormice (see Chapter 3) that prevents one or few males from dominating the genetic make-up of the population. In addition, a high survival rate of the reintroduced animals will limit any loss of diversity. Certainly, the majority of common dormice reintroductions have been apparently successful as indicated by high survival rates after the first hibernation and subsequent establishment of breeding populations (PTES 2009). My genetic data support this – the Wych population is genetically diverse and there is no evidence that it has passed through a significant population bottleneck (Appendix 4.1). Likewise, the population at Bontuchel has not undergone an apparent significant reduction in size recently, although it has to be noted that any heterozygote excess is maintained for just $2N_e$-$4N_e$ generations after the bottleneck event (Piry et al. 1999).

A somewhat surprising outcome however is the similar estimates of N_e at both sites, albeit slightly higher at Bontuchel (Table 4.3), that supports the idea that the Wych population has sustained a relatively large/expanding population since its re-establishment. Since the estimates of N_e are consistently higher than the numbers of adults caught, this raises the practical issue for dormice monitoring that many reproductively active animals are not caught in nestboxes. It is important to note also that this result does not indicate that the sites contain similar numbers of individuals (*i.e.* the population census, N) as the ratio N_e/N is not likely to be constant. For example, several studies have uncovered some type of "genetic compensation" whereby smaller populations have larger N_e/N ratios (see Ardren and Kapuscinski 2003, Rowe and Beebee 2004, Jehle et al. 2005; Watts et al. 2007b). The mechanism(s) behind this are not fully-understood although it is likely to be driven by a reduction in variance in reproductive success in smaller populations (Ardren and Kapuscinski 2003). Moreover, the single generation method of estimating N_e may lack

precision (see Frankham 1995; Wang 2005; Waples 2006) so these data must be interpreted cautiously. Further work using more precise estimators of N_e (*i.e.* from the temporal variation in allele frequencies) along with more accurate surveys that would allow determination of the adult census sizes would permit an evaluation of the level of variation in the ratio N_e/N and concomitant insights into the relative influences of demographic factors that determine the successful breeding population (see Frankham 1995; Frankham et al. 2002 for reviews).

4.3.3 Sex-biased dispersal

Partitioning the patterns of genetic differentiation for each sex separately is a crucial part of understating a species' ecology. I provide clear genetic evidence for female philopatry and male-biased dispersal (MBD) in common dormice populations (Figures 4.2a, b; Table 4.5). Such behaviour is a common feature of many species of mammal that have polygynous mating systems (Greenwood 1980; Clutton-Brock 1989; Devillard et al. 2004; Nunes 2007), particularly rodents (Lacey and Sherman 2007), although the degree and the direction of biased dispersal can vary among species (Greenwood 1980; Clutton-Brock 1989; Favre et al. 1997).

For females, philopatry is generally argued to circumvent any cost of dispersal and can provide a range of benefits associated with increased reproductive success, such as co-operation between kin, and familiarity with food resources and the breeding site (O'Riain and Braude 2001; Lacey and Sherman 2007). Intriguingly, there is some evidence to suggest that communally-nesting female dormice are related, and that they may use a créche to support their young (see Chapter 3). Such behaviour occurs in many social animals (Jennisons and MacDonalds 1994; Emlen 1995) particularly rodents (see Hayes 2000, for review). However, other factors such as resource abundance and distribution are also expected to influence philopatry (Jones 1984, Ratnayeke et al. 2002).

In contrast to females, male dormice are mobile at a local scale, and, accordingly, gene flow is mediated largely by male dispersal. Male-biased dispersal (gene flow) is a typical feature of many mammal species (*e.g.* Hazlitt et al. 2004; Janečka et al. 2007; Gauffre et al. 2009) and is expected when there is strong male-male competition for resources (Dobson 1982), or as a response to limit inbreeding and kin competition (Gauffre et al. 2009). By

dispersing to areas with less competition for mates, this may be an important factor of the reproductive ecology of common dormice. That juvenile dispersal was not significant in both populations is consistent with my predictions that juveniles were not expected to exhibit a pattern of movement (Appendix 4.3a, b).

4.3.4 Spatial genetic structure

Spatially limited dispersal causes an increase in genetic differentiation among individuals separated by increasing geographic distances (Wright 1943; Rousset 1997, 2000). I find isolation-by-distance (IBD) (Figures 4.2a, b) developing within 1 km, similar to that recorded in other mammals (Hazlitt et al. 2004; Schweizer et al. 2007; Busch et al. 2009), that is driven by a combination of broadly limited dispersal distances and female philopatry. However, no significant sub-populations were detected by AMOVA (Table 4.4) or Bayesian clustering techniques (data not shown) suggesting that rate and pattern of dispersal is sufficient to prevent the formation of discrete subpopulations in continuous habitat patches of more than 0.5 km^2. Crucially, however, I demonstrate that this dispersal behaviour is similar in both the wild and the reintroduced population (Figures 4.2a, b).

That fewer immigrant females than males were identified in both populations lend further support to the patterns of male-biased dispersal discussed above. Moreover, the possibility of immigrant dormice into both sites is intriguing given this species' conservation status. Certainly at Bontuchel there is evidence of dormice inhabiting parts of the surrounding area (Rhian Hughes, North Wales Wildlife Trust, unpubl. data) that could serve as potential source patches. If this is confirmed, then dormice occasionally disperse further than 1 km, and across inhospitable agricultural habitat (though likely using hedgerows as corridors). Potential immigrants at Wych raise the possibility of neighbouring dormice populations, and indeed, these immigrants may have augmented the genetic diversity of the re-introduced Wych population. Alternatively, the suspected immigrants may reflect the genetic signature of the mixed population origins of the individuals used for captive breeding. Unfortunately, there are no records of the origin of the founding populations in Wych, but founders in this site were mostly from several regions in southern England (Sue Tatman, Cheshire Wildlife Trust, CWT; *pers. comm.*).

4.4 Implications for conservation

Despite its high conservation profile it is surprising that to date no study has determined the pattern and extent of spatial genetic structure in common dormice, despite the considerable effort that has been directed towards habitat management, captive breeding and reintroductions. By comparing between the wild and reintroduced dormice population that was established from captive bred population, this study demonstrates how aspects of a species' behaviour (particularly dispersal) can generate a population genetic structure over a small geographical scale (less than 1 km) in continuous habitat patches with gene flow largely mediated by dispersing males. This short movement behaviour has important implications for the conservation of the species as it is a feature that considerably increase the vulnerability of populations to extinction (Lawes et al. 2000). However, the detection of some immigrants in both population sites would appear to have increased the genetic diversity and as such genetic monitoring may be used to identify new dormice populations.

4.5 Conclusions

To conclude, a reintroduced population of common dormice founded from captive-bred populations shows similar dispersal behaviour as a natural population, particularly in exhibiting the same pattern of isolation by distance at local scales with a large habitat patch. As expected for a typical mammalian system, dormice show male-biased dispersal and female philopatry and therefore the apparent IBD spatial structuring is driven by the pattern of male dispersal. The relatively high and temporally stable estimate of N_e in the population at Wych is encouraging as it points towards widespread and successful breeding by the reintroduced individuals.

Table 4.1 Summary of dormice captured in Bontuchel and Wych from 2006 to 2008.

	Bontuchel				Wych			
	2006	2007	2008	Total	2006	2007	2008	Total
Mark	174	93	54	321	51	60	44	155
Recaptured	-	113	74	187	-	38	20	58
Total	174	206	128	508	51	98	64	213

Table 4.2. Summary genetic diversity statistics of two common dormouse (*M. avellanarius*) populations: numbers of allele (N_a), observed (H_o) and expected (H_e) heterozygosity, allelic richness (A_R) and inbreeding coefficient (f). Bold values indicate a significant ($P<0.05$) deviation from Hardy-Weinberg Equilibrium (HWE), however none of the tests remain significant ($P<0.05$) after a sequential Bonferroni correction for $k=3$.

Site	Year		Locus MavG3	MavE3	MavF10	MavB5	MavA5	MavG6	MavG9	MavH3	mavC4-2	mavF1-2	Average
Bontuchel	2006	N_a	6.00	10.00	11.00	10.00	10.00	10.00	8.00	8.00	14.00	10.00	9.70
		H_o	0.717	0.588	0.695	0.616	0.657	0.7551	0.798	0.581	0.717	0.657	0.678
		H_e	0.739	0.579	0.737	0.653	0.669	0.759	0.739	0.598	0.782	0.732	0.699
		A_R	6.00	9.88	11.00	9.82	9.87	9.74	7.88	8.00	12.63	7.88	9.27
		f	0.030	-0.015	0.058	0.057	0.018	0.005	-0.080	0.029	0.084	0.103	0.029
	2007	N_a	9.00	11.00	11.00	9.00	10.00	7.00	6.00	6.00	12.00	11.00	9.20
		H_o	0.573	0.738	0.772	0.536	0.646	0.769	0.744	0.522	0.707	0.706	0.671
		H_e	0.682	0.685	0.770	0.576	0.685	0.744	0.685	0.565	0.764	0.779	0.694
		A_R	8.52	10.43	10.94	8.72	9.57	6.87	5.84	6.00	11.47	10.59	8.89
		f	0.161	-0.078	-0.002	0.070	0.057	-0.034	-0.087	0.077	0.075	0.094	0.032
	2008	N_a	9.00	10.00	11.00	7.00	8.00	8.00	8.00	7.00	12.00	12.00	9.20
		H_o	0.711	0.644	0.800	0.413	0.732	0.837	0.675	0.564	0.727	0.622	0.673
		H_e	0.721	0.780	0.741	0.447	0.642	0.740	0.685	0.543	0.753	0.810	0.680
		A_R	8.70	9.45	10.18	5.67	7.90	7.81	7.95	5.00	11.51	11.42	8.56
		f	0.014	0.093	-0.080	0.077	-0.142	-0.133	0.015	-0.039	0.034	0.234	0.007
Wych	2006	N_a	5.00	7.00	7.00	7.00	8.00	7.00	7.00	5.00	7.00	9.00	6.90
		H_o	0.806	0.694	0.906	0.714	0.771	0.706	0.571	0.821	0.944	0.861	0.780
		H_e	0.741	0.606	0.686	0.648	0.673	0.723	0.547	0.760	0.773	0.744	0.693
		A_R	5.00	6.54	6.50	6.36	7.19	6.61	6.16	5.00	6.77	8.44	6.46
		f	-0.088	-0.148	-0.329	-0.104	-0.148	0.024	-0.045	-0.082	-0.226	-0.160	-0.132
	2007	N_a	8.00	7.00	6.00	7.00	8.00	5.00	7.00	6.00	8.00	11.00	7.30
		H_o	0.758	0.769	0.750	0.800	0.811	0.710	0.750	0.567	0.750	0.800	0.746
		H_e	0.689	0.666	0.633	0.643	0.593	0.679	0.781	0.650	0.738	0.602	0.667
		A_R	7.73	6.71	5.83	6.43	7.62	4.97	6.81	4.00	7.25	10.18	6.75
		f	0.040	-0.158	-0.188	-0.248	-0.023	-0.046	0.040	0.131	-0.016	0.039	-0.037
	2008	N_a	6.00	5.00	6.00	6.00	6.00	5.00	6.00	7.00	8.00	8.00	6.30
		H_o	0.524	0.700	**0.032**	0.591	0.667	0.700	0.571	**0.041**	0.455	0.842	0.512
		H_e	0.777	0.533	0.528	0.569	0.779	0.613	0.751	0.542	0.659	0.658	0.641
		A_R	5.99	4.70	2.81	3.76	5.94	4.70	5.81	3.00	7.04	7.89	5.16
		f	0.331	-0.323	0.284	-0.040	0.148	-0.147	0.244	0.135	0.315	0.019	0.097

Table 4.3 Effective population size estimates (N_e) for *M. avellanarius* in Bontuchel and Wych in each year with 95 % confidence intervals (CI).

Population	Year	Estimated N_e	95 % CI (Jackknife)
Bontuchel	2006	230.8	22.4-270.0
	2007	132.8	19.4-741.1
	2008	150.2	32.5-300.0
Wych	2006	149.1	23.5-290.0
	2007	109.9	13.5-250.6
	2008	119.5	20.9-210.3

Table 4.4 Analyses of molecular variance (AMOVA) for populations of the common dormouse *M. avellanarius* in Bontuchel and Wych. d.f., degrees of freedom; SS, sum of squares; MS, mean squares; Est. var., estimated variance, %D, distribution of total variance.

Source	d.f	SS	MS	Est. var	%D	*P*
Bontuchel						
Among temporal groups	2	38.234	19.117	0.153	2%	>0.050
Within populations	227	1794.118	7.904	7.904	98%	0.000
Wych						
Among temporal groups	2	32.320	16.160	0.254	3%	>0.050
Within populations	95	767.497	8.079	8.079	97%	0.000

Table 4.5 Juvenile and adult sex-biased dispersal analysis. *n*, sample size; see Materials and Methods section for definitions of F_{is}, H_s, *r*, *mAIc*, *vAIc*; *P* values were based on 10 000 randomization using FSTAT ver. 2.9.3.2 (Goudet 2001).

	Juvenile						Adult					
	Bontuchel			Wych			Bontuchel			Wych		
	Female	Male	*P*	Female	Male	*P*	Female	Male	*P*	Female	Male	*P*
	n=31	*n*=19	value	*n*=9	*n*=6	value	*n*=138	*n*=108	value	*n*=67	*n*=53	value
F_{is}	-0.015	0.025	>0.05	-0.037	0.054	>0.05	0.018	0.064	<0.05	-0.012	0.055	<0.05
mAIc	0.606	-0.581	>0.05	0.529	-0.37	>0.05	0.192	-0.155	<0.05	0.048	-0.029	<0.05
vAIc	15.468	17.007	>0.05	11.121	12.339	>0.05	16.434	21.006	<0.05	6.364	13.46	<0.05
r	0.052	0.037	>0.05	0.207	0.158	>0.05	0.334	0.327	<0.05	0.254	0.054	<0.05
H_s	0.614	0.593		0.681	0.611		0.694	0.649		0.714	0.732	

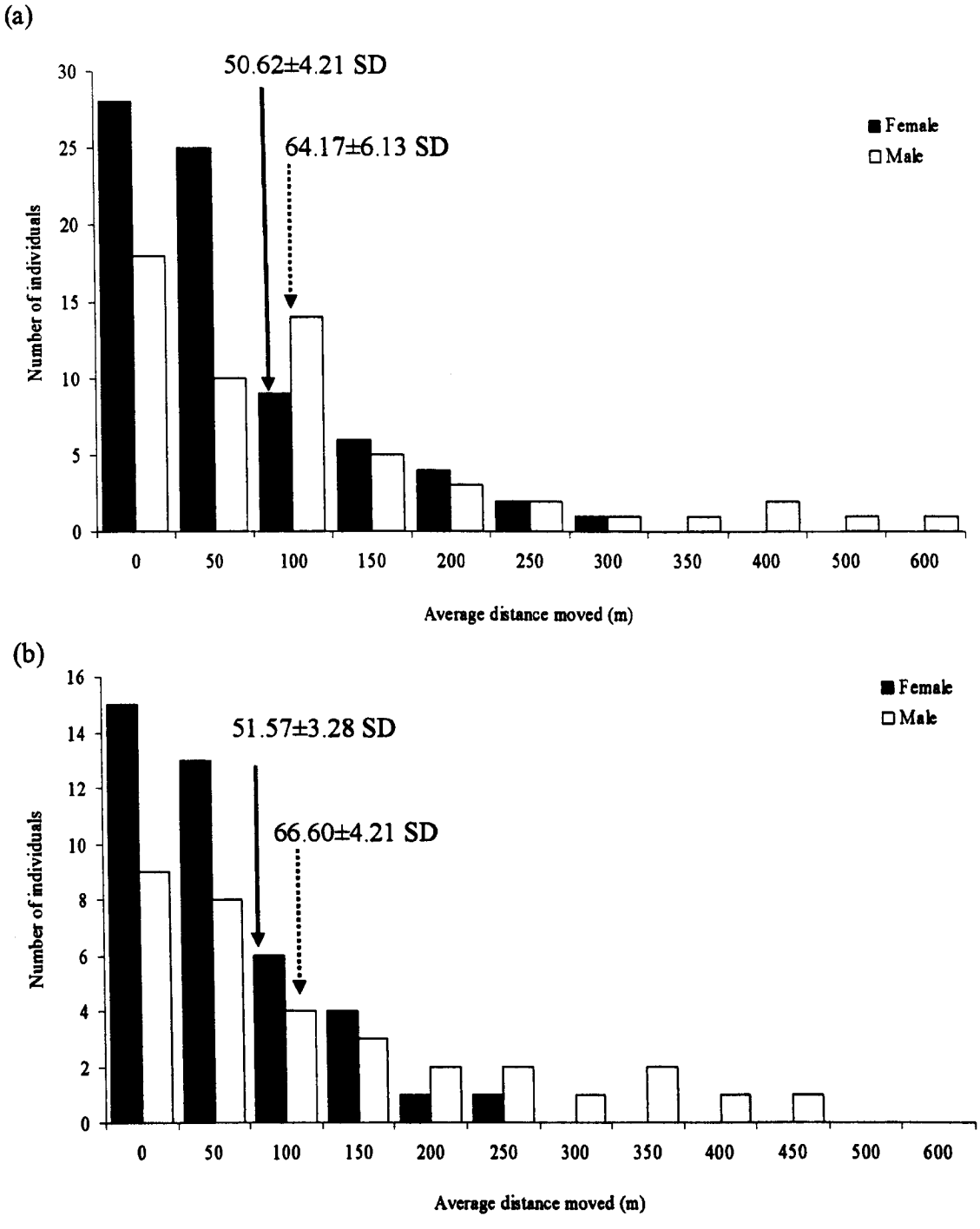


Figure 4.1 Frequency of cumulative lifetime movement of males and females adult *M. avellanarius* in (a) Bontuchel, and (b) Wych. Black (female) and dash (male) arrows indicate the average cumulative distance moved by each sex.

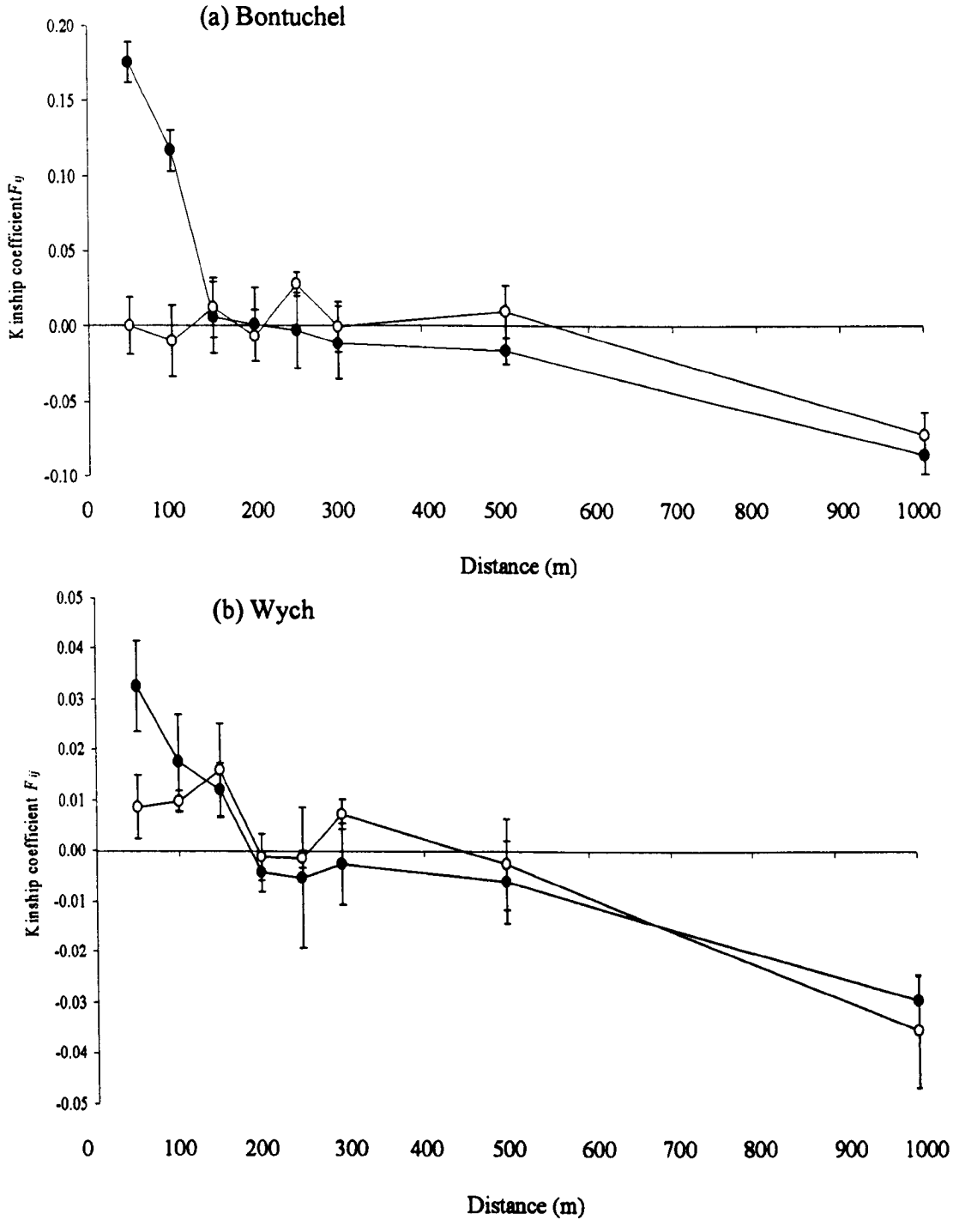
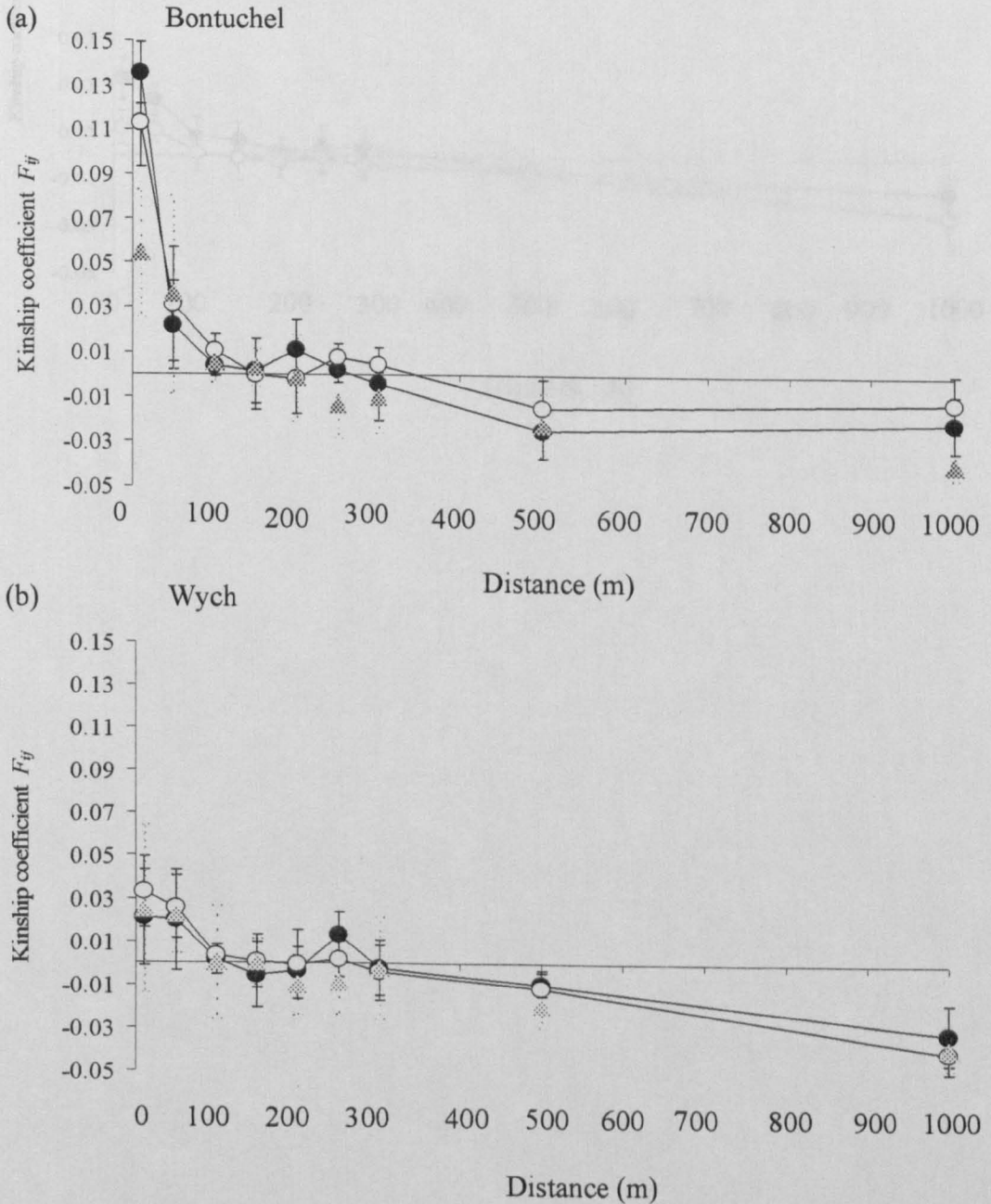


Figure 4.2 Spatial variation in average kinship (F_{ij}) (Loiselle et al. 1995) among pairs of adult males (white) and adult females (black) of *M. avellanarius* in (a) Bontuchel and (b) Wych. Standard error was obtained by jackknifing over 10 microsatellite loci.

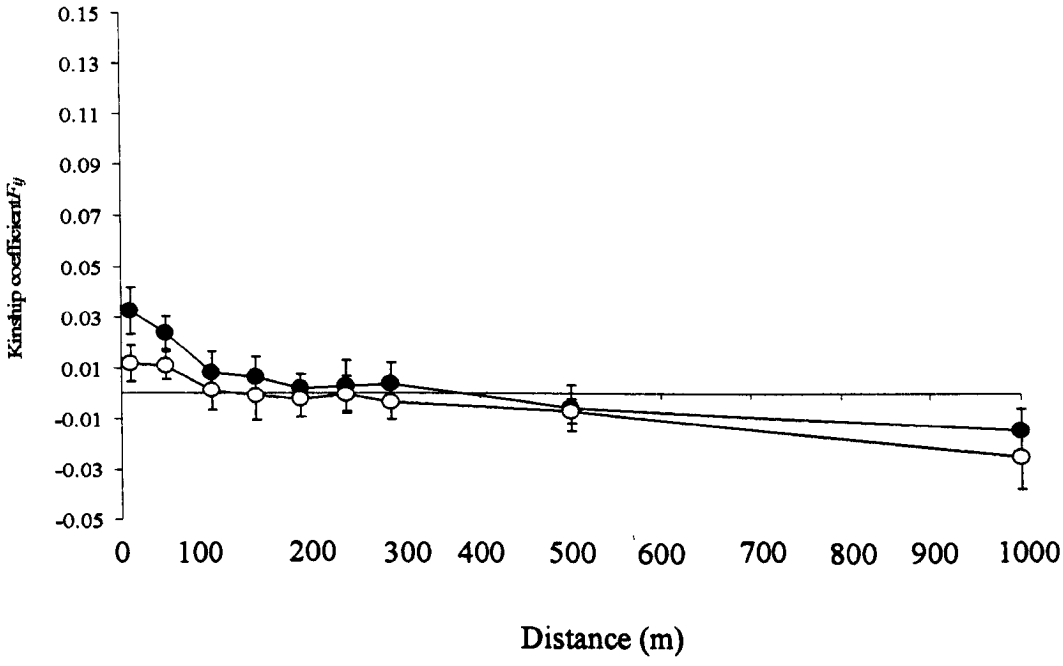
Appendix 4.1 Probability values for tests for a significant heterozygote excess indicative of a population bottleneck for samples of *M. avellanarius* using three models of microsatellite allele mutation (IAM, infinite allele model; TPM, two phase model; SMM, stepwise model) and two methods of analysis (Sign test and Wilcoxon sign-rank test). Bold indicates a significant ($P<0.05$) heterozygote excess, however none of the tests remain significant after a sequential Bonferroni correction for $k=3$.

		Sign test			Wilcoxon test		
Site	Year	IAM	TPM	SMM	IAM	TPM	SMM
Bontuchel	2006	0.1119	0.0884	0.2798	0.0610	0.0836	1
	2007	**0.0331**	0.2863	0.5718	**0.0259**	0.0637	0.7695
	2008	0.1184	0.1304	0.3807	**0.0261**	0.3661	0.0632
Wych	2006	**0.0312**	**0.0253**	0.1321	**0.0436**	0.0895	0.0563
	2007	**0.0411**	0.0695	0.1460	**0.0418**	0.0630	0.0855
	2008	0.0811	**0.0203**	**0.0305**	0.2198	0.0795	0.084

Appendix 4.2 Correlogram profiles of the variation in kinship (F_{ij}) (Loiselle et al. 1995) as a function of the average distance separating pairs of *M. avellanarius* from locations in both populations. Standard error was obtained by jackknifing over 10 microsatellite loci. (a) individual profiles for samples collected during 2006 (black), 2007 (white), 2008 (grey diamonds) in Bontuchel; (b) individual profiles for samples collected during 2006 (black), 2007 (white), 2008 (grey diamonds) in Wych; (c) combined individual profiles for samples collected across 2006-2008 in Bontuchel (black) and Wych (white).

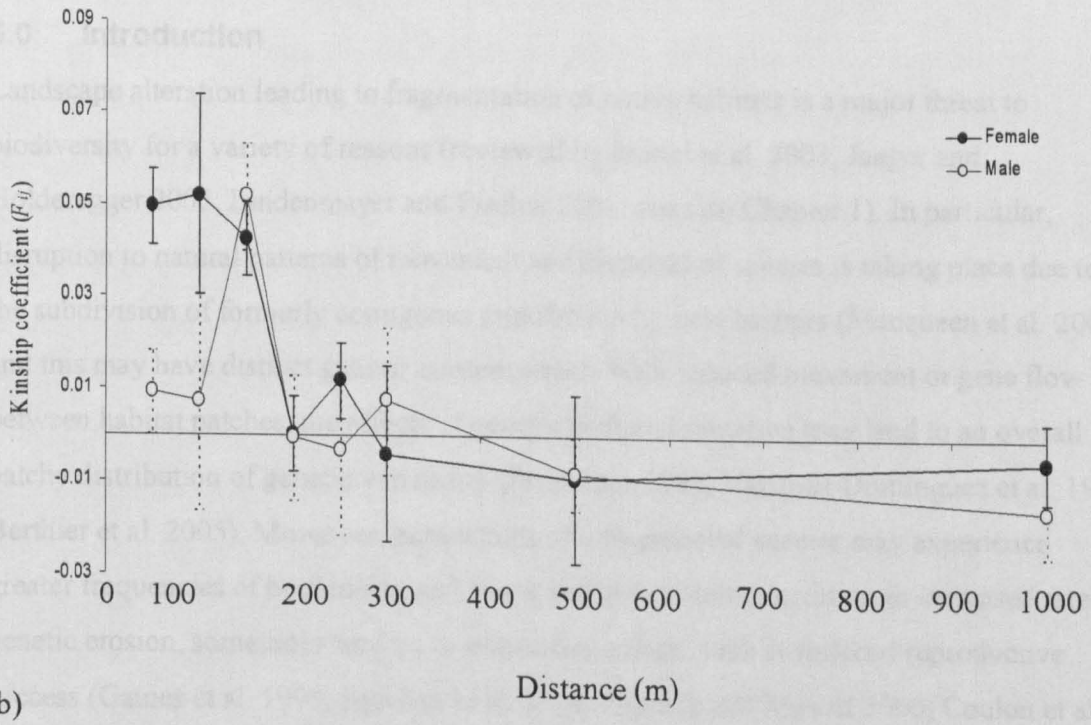


(c) Combined profiles

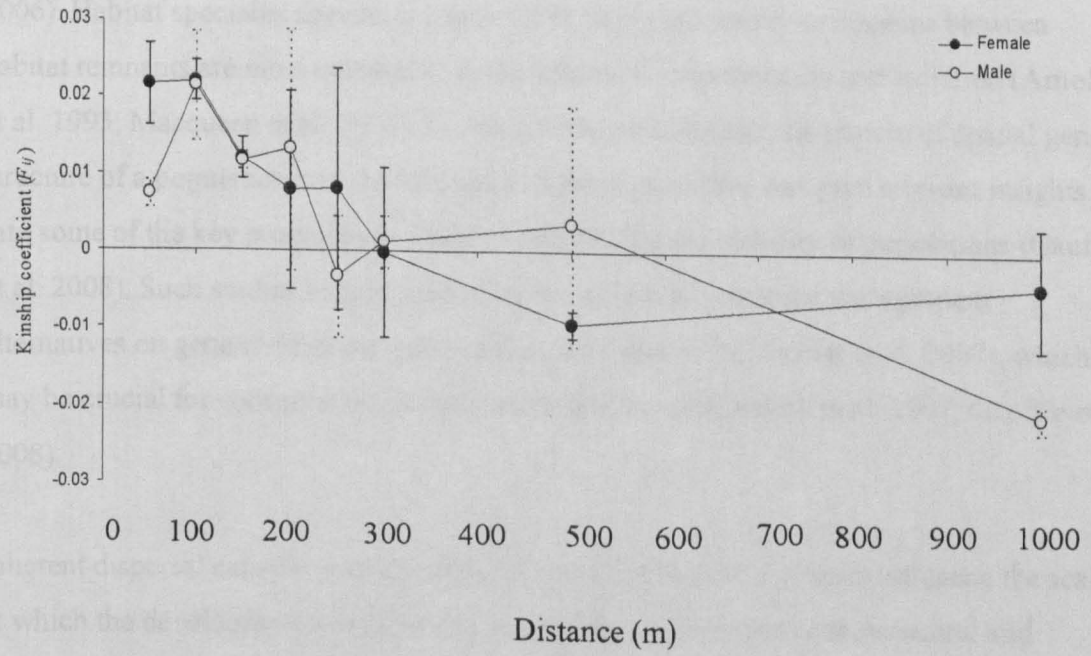


Appendix 4.3 Spatial variation in average kinship (F_{ij}) (Loiselle et al. 1995) among pairs of juvenile males (white) and juvenile females (black) of *M. avellanarius* in (a) Bontuchel and (b) Wych. Standard error was obtained by jackknifing over 10 microsatellite loci.

(a)



(b)



Chapter 5: Patterns of genetic divergence among populations of the common dormouse *Muscardinus avellanarius*.

5.0 Introduction

Landscape alteration leading to fragmentation of native habitats is a major threat to biodiversity for a variety of reasons (reviewed by Manel et al. 2003; Jaeger and Holderegger 2005; Lindenmayer and Fischer 2006; see also Chapter 1). In particular, disruption to natural patterns of movement and dispersal of species is taking place due to the subdivision of formerly contiguous populations by new barriers (Macqueen et al. 2008), and this may have distinct genetic consequences. With reduced movement or gene flow between habitat patches, the effects of genetic drift and mutation may lead to an overall patchy distribution of genetic variability (Frankham 1998; Vázquez-Domínguez et al. 1999; Berthier et al. 2005). Moreover, populations of a fragmented species may experience greater frequencies of bottlenecks and lower population sizes, resulting in increased rates of genetic erosion, sometimes leading to inbreeding effects such as reduced reproductive success (Gaines et al. 1996; Saccheri et al. 1998; Gerlach and Musolf 2000; Coulon et al. 2006). Habitat specialist species or species that have poor ability to disperse between habitat remnants are most vulnerable to the effects of fragmentation and isolation (Arnold et al. 1993; Macqueen et al. 2008). In this context, establishing the pattern of spatial genetic structure of a population and the rate and pattern of gene flow can give relevant insights into some of the key processes and factors influencing the viability of populations (Gauffre et al. 2008). Such studies help in predicting the effects of proposed management alternatives on genetic variation and population connectivity (Storfer et al. 2007), which may be crucial for conservation of the species concerned (Palsboll et al. 2007; Gauffre et al. 2008).

Inherent dispersal capacity and behavioural characteristics of a species influence the scale at which the development of spatial and genetic structure occurs both in natural and disturbed habitats (Chesser 1991a, b; Dobson et al. 1997; Dieckman et al. 1999; Loew et al. 2005; Macqueen et al. 2008). For example, female philopatry, which is common in mammalian species (Greenwood 1980; Clutton-Brock 1989; see also Chapter 3), may

increase the spatial genetic structure among females relative to males (see *e.g.* Hazlitt et al. 2004; Fredsted et al. 2007). On the other hand, spatial structure can also be considerably influenced by the environmental characteristics of a landscape (Manel et al. 2003; Jaeger and Holderegger 2005; Holderegger and Wagner 2006; Storfer et al. 2007; Holderegger and Wagner 2008; Balkenhol et al. 2009). This is particularly true for species inhabiting a fragmented habitat because an inhospitable matrix environment can substantially alter the successful movement between isolated patches of suitable habitat (Watts et al. 2004; Dixon et al. 2007; Bergl et al. 2008; Mitrovski et al. 2008). Landscape features that act as barriers to gene flow have been identified from the patterns of spatial population genetic structure in a wide range of small mammal species (Burnett 1992; Ouin et al. 2000; Roach et al. 2001; Johnson and Collinge 2004; Vuilleumier et al. 2007; Roedenbeck and Voser 2008; Lampila et al. 2009), particularly rodents (Garland and Bradley 1984; Clark et al. 2001; Goosem 2001, 2002; Rondinini and Doncaster 2002; Kozakiewicz et al. 2009b). For example, it has been demonstrated that landscape features such as rivers (Aars et al. 1998) or motorways (Gerlach and Musolf 2000) limit the dispersal of bank voles; creating distinct sub-populations. Thus, integrating landscape features with social and behavioural factors may provide complimentary information on the drivers of genetic structuring of populations.

The success of the utility of habitat corridors to facilitate movement and dispersal of animals is widely recognised (see *e.g.* Brinkerhoff et al. 2005; Lees and Peres 2008; Clarke and White 2008). For instance, habitat corridors have been shown to facilitate movement of small mammals in Bunyip State Park, Australia where corridors have supported a breeding population of a native small mammal, as well as promoting a mixture of different successional stages of small mammal life history (Clarke and White 2008). However, several studies have questioned the role of habitat corridors in facilitating connectivity among remnant fragments (*e.g.* Bennett 1999; Horskins 2005). Moreover, experimental evidence of the success of corridors in reducing population extinction rate and maintaining genetic diversity is rare (Forney and Gilpin 1989; Bolger et al. 2001). For example, a semi-natural riparian corridor linking two rainforests fragments has successfully expanded available habitat for the giant white-tailed rat *Uromys caudimaculatus*, but concerns regarding the maintenance of population genetic diversity of *U. caudimaculatus* has risen because the corridor has failed to promote gene flow due to high social factors that restricted gene flow in this species (Horskins 2005).

Muscardinus avellanarius is a good candidate for testing the influence of isolated habitat patches on patterns of genetic variation because of its close association with forest habitat. *Muscardinus avellanarius* is an important example of a "Flagship Species" (Morris 2003) and distributed across Europe and northern Asia Minor (IUCN 2009). Once widespread, *M. avellanarius* populations are dwindling in parts of its northern range (UK, Netherlands, Sweden, Germany, Denmark) and it has suffered a 64 % decline in numbers in Great Britain since the late 1970s (Bright et al. 2006). Internationally, *M. avellanarius* is categorised as 'least concern' on the Red List (IUCN 2009) and in the UK, it was granted full protection as a Schedule 5 species in 1986 (Morris 2004). Threats to *M. avellanarius* include unfavorable woodland management, a succession of poor breeding years and habitat fragmentation leading to increases in the isolation of populations (Bright et al. 1994; Bright and Morris 1996; Capizzi et al. 2002). Consequently, *M. avellanarius* is of conservation concern and is subject to protection throughout its range.

Conservation of *M. avellanarius* in UK has focused on *ex situ* programs. These have proved feasible with most reintroduced populations of *M. avellanarius* breeding and spreading into adjacent available habitat (Morris 2004; PTES 2009). Since 2007, the PTES has been promoting habitat enhancement, such as the establishment of dormouse 'bridges' between isolated habitat patches and launched the *Reconnecting the Countryside* competition to encourage farmers and landowners to carry out active conservation work that will connect up or create areas of woody habitat beneficial to *M. avellanarius* and to other wildlife. Despite all these conservation management, there is evident where *M. avellanarius* have been found crossing main roads (*i.e.* the A30 and A38) in Cornwall, UK (available at http://news.bbc.co.uk/1/hi/england/cornwall/8172460.stm). This implies that *M. avellanarius* may be more mobile and less susceptible to fragmentation than thought previously. However, clearly, the effective planning of the creation of future dispersal corridors for *M. avellanarius* is hampered by a lack of knowledge on how landscape features affect rates and patterns of dispersal in the field.

In this study, I compare patterns of population genetic differentiation at microsatellite loci in continuous and patchily distributed populations. This study aims to (1) describe and quantifying population structure at a landscape scale, (2) compare the level of genetic

variation in continuous habitat with that of fragmented/patchy habitat and (3) conduct an initial assessment of the effects of landscape features and barriers to gene flow.

5.1 Materials and methods

5.1.1 Description of study sites

Dormice samples were collected from two regions in Wales: (1) Bontuchel in Denbighshire (Latitude 53.109364 N, Longitude -3.370318 W; OS National Grid Reference, SJ082571) and (2) Afonwen in Gwynedd (Latitude 53.235309 N, Longitude -3.292935 W; OS National Grid Reference, SJ138007) (Figure 5.1). These regions are part of the monitoring programme for common dormouse by the Northwest Dormouse Partnership (also available at http://www.cheshirewildlifetrust.co.uk/proj_ dormouse_ partnership.htm). The distance between these regions is about 15 km and there is major road (A541) separating them. The Bontuchel region is represented by seven non-continuous suspected habitat patches that may accommodate common dormice - Coed Y Pennant (CYP), Coed Cooper (CC), Bill, Michael Adams (MA), Lady Bagots Drive (LBD), Coed Orlwyn (CO) and also Coed Tre Parc (CTP) - which are separated by about 0.3 km of agricultural habitat from Bontuchel wood. Also included in this region is Bontuchel wood (BON) a large, continuous habitat where most of the dormice were sampled in this study (see Chapters 3 and 4 for description of study site). Moving south of Bontuchel wood, there is a pasture area (*i.e.* 0.5 km) that separates this wood from two non-continuous populations, CC and CYP. Generally, Afonwen area is a fragmented habitat containing five non-continuous populations - Coed Bron Fadog (CBF), Bron Eiron (BE), Y Ddol Uchaf (YDU), Swan Wood (SW) and Fron Haul (FH). Most of the habitat patches within both regions consist of mixed broadleaves and conifers, with rivers and tracks crossing within regions. I treated Bontuchel wood as a continuous population and the remaining populations that surround Bontuchel as patchy, non-continuous populations. Similarly, all of the habitat patches within the Afonwen region are treated as non-continuous populations.

5.1.2 Sample collection

To monitor *M. avellanarius* in the general region, a total of 578 nestboxes were located in a number of sites. The focus of sampling has been some 250 nestboxes installed within Bontuchel wood; however recently (since 2007) an additional 44 nestboxes were installed at the edge of Bontuchel wood in close proximity to the surrounding sites. Of the remaining

284 nestboxes, 75 were located in eight sites that surround Bontuchel wood - CC, CTP, Bills, MA, LBD, CO, CYP and MA and the remaining (209) nestboxes were installed in CBF, BE, YDU, SW and FH. All nestboxes were situated on tree trunks, with the entrance facing the trunk at 1.5 m above ground level, and are spaced at approximately 20-40 m intervals. Nestboxes in Bontuchel wood were monitored in May, June, September and October while nestboxes in all patchy populations (in Bontuchel and Afonwen) were monitored twice a year (June and September). Data such as sex, weight, estimated age (brown young, juvenile, adult) and breeding status were recorded for all common dormice found in all study populations, but only common dormice found in Bontuchel Wood were scanned for a microchip before release to where they were captured. Common dormice without microchips that found in Bontuchel wood were anaesthetised and then microchipped using 8 mm microchips. The position of every encountered individual was recorded using a Global Positioning System (GPS). For genetic analyses, hair and buccal swab samples have been collected from all animals that were encountered.

5.1.3 Genetic analysis

5.1.3.1 DNA extraction and genotyping

Total genomic DNA was extracted from hair and buccal swab samples using CHELEX-100 (Walsh et al. 1991). All samples were then genotyped at ten polymorphic microsatellite loci designed for *M. avellanarius* (Md. Naim et al. 2009). Each 10 µl PCR reaction contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 3 mM $MgCl_2$, 1 µl extracted template DNA, 3 pmol of each primer and 10 µg BSA, 1.25 u DNA polymerase (ABgene). Thermal cycling conditions for all loci were 95°C 3 min, 6x [95°C 30s, T_a°C 45s, 72°C 45s], 25x [92°C 30s, T_a °C 45s, 72°C 55s], where T_a is the annealing temperature (either 53°C or 58°C, depending on the locus), on a Dyad Engine (MJ Research Inc). PCR products were then pooled with a 500 bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI3130*xl* and sized using GeneMapper software (Applied Biosystems).

5.1.4 Data analysis

5.1.4.1 General analysis of levels of genetic diversity

MICROCHECKER ver. 2.2.3 (Van Oosterhout et al. 2004) was used to identify any possible systematic genotyping errors (*i.e.* null alleles, large allele dropout and any mis-scoring of

stutter peaks). Genotypic linkage disequilibria between all pairs of loci, and deviation from Hardy-Weinberg equilibrium (HWE) for each locus separately and over all loci, were tested within each region (*i.e.* within Afonwen and Bontuchel) by exact tests using Markov chain methods implemented in the computer program GENEPOP ver. 3.1 (Raymond and Rousset 1995). Genetic diversity within each sample was quantified as observed heterozygosity (H_o), expected heterozygosity (H_e), allelic richness (A_R, number of alleles corrected for sample size) and Wright's (1951) inbreeding co-efficient (f). All parameters were calculated using computer software FSTAT ver. 2.9.3.2 (Goudet 2001). A sequential Bonferroni correction was applied to a significance level of 0.05 where appropriate (Rice 1989).

5.1.4.2 Genetic partitioning

Hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was employed to investigate the relative importance of temporal dynamics on genetic structure. Total genetic diversity was partitioned among temporal groups, among population and among individuals within populations in each region by carrying out a hierarchical AMOVA using ARLEQUIN ver. 2.0 (Schneider et al. 2000).

5.1.4.3 Detection of recent population bottleneck

To determine if there is a signature for recent bottleneck events, I used the software BOTTLENECK ver.1.2.02 (Piry et al. 1999) which compares observed (H_o) gene diversity with that expected (H_e) from the number of alleles per locus when population size remains constant and for a given mutation model. This test assumes mutation-drift equilibrium (Cornuet and Luikart 1996) with allelic diversity based on three different models of allelic mutation: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM). Both the Wilcoxon signed-rank test and a sign test were used to assess significance of whether the observed H_e is greater than expected under an equilibrium model.

5.1.4.4 Population genetic structure

To explore the genetic evidence for division among *M. avellanarius* between both regions and within each site, I employed the software STRUCTURE ver. 2.0 (Pritchard et al. 2000) that simultaneously identifies clusters (populations) and assigns individuals to populations

using a Bayesian approach. The Bayesian approach has the advantage of inferring populations based on the frequencies of the alleles, thus clustering individuals based on their genetic values (Bolstad 2004). STRUCTURE models K populations (where K may be unknown) that are characterized by the set of allele frequencies at each locus. K is defined by the user for each run of the programme. STRUCTURE does not provide a formal statistical procedure for evaluating the most appropriate value of K. However, Pritchard et al. (2000) has addressed this problem by placing a prior distribution on K and basing inference for K on the posterior distribution $\Pr(X|K) = \Pr(K|X) \Pr(K)$, where X is the multilocus genotype of individuals. The *ad hoc* measure, ΔK, which is the second order rate of change of Ln P(D) with respect to K (Evanno et al. 2005) was used as an estimator of K. The admixture of individuals regardless of the geographic locations of the samples was used for clustering all individuals from the study populations and ten independent runs of STRUCTURE were carried out for the total data set for K=1 to K=10. Simulations were carried out with the following settings: admixture model, correlated allele frequencies, 50,000 burn-in length, and MCMC repetitions of 500,000 iterations. All genotype data were used in this analysis including those from sites where too few individuals could be collected for analyses of differentiation among populations. Samples from Bontuchel and Afonwen were analysed separately.

Spatial genetic structure was examined using spatial autocorrelation analyses (Hardy and Vekemans 1999), which has the advantage over a Mantel test in providing results on the shape and pattern of the spatial relationship (Brouat et al. 2003). To investigate possible differences to the genetic structure of *M. avellanarius* in continuous and patchy habitats, I divided the samples into three groups: (1) Bontuchel wood (BON), (2) the populations that surrounded Bontuchel wood and (3) all samples in Afonwen (Figure 5.1). I treated Bontuchel wood as continuous habitat and the other groups (groups 2 and 3) as patchy habitat. All groupings were analysed separately. A computer software SPAGEDI ver.1.2 (Hardy and Vekemans 2002) was used to calculate the correlation in average kinship (F_{ij}, Loiselle et al. 1995) between pairs of *M. avellanarius* separated by a range of increasing spatial scales. I allowed SPAGEDI to assign distance categories that contained a similar number of pairwise comparisons to avoid a bias in the correlation coefficient due to unequal sample sizes within each spatial category (see Hardy and Vekemans 2002). To visualize SGS, kinship coefficients values were averaged over a set of distance intervals

and plotted against geographical distance. To test for SGS, spatial group locations were permuted 1,000 times and 95 % confidence intervals (CI) for multilocus kinship coefficients at each distance class were generated from the permutation. For all spatial statistics, Euclidian geographical distances between individuals and population centres were calculated from the GPS x and y coordinates.

5.1.4.5 Detection of migrants

The number of first-generation migrants was estimated with a frequency-based assignment provided in GENECLASS2 ver 1.0 (Piry et al. 2004). For each individual, I computed the likelihood of belonging to the population where it was sampled (*i.e.* L_h statistic) using the frequency method of Paetkau et al. (1995), which is the appropriate strategy recommended by Paetkau et al. (2004) when all potential source populations have not been sampled. The probability of an individual being resident was then assessed using a resampling procedure (Paetkau et al. 2004). Individuals with a probability of less than 0.05 were excluded as a resident.

5.2 Results

5.2.1 Sampling data

A total of 70 and 236 individuals were captured in Afonwen and Bontuchel region respectively from 2007 to 2009 (Table 5.1). Of these, 29, 18 and 3 samples from Afonwen in 2007, 2008 and 2009 respectively were successfully genotyped (n=50). For Bontuchel, 154, 71 and 7 samples were genotyped successfully for 2007, 2008 and 2009 respectively (n=232; Table 5.1).

5.2.2 Genetic diversity

Basic statistics summarising genetic diversity observed within the region are presented in Table 5.2. After sequential Bonferroni correction, none of the loci deviated significantly from Hardy-Weinberg equilibrium (Table 5.2). No significant linkage disequilibrium was found in any pairs of loci (P>0.05), so all ten microsatellite loci provide independent information. All microsatellite loci were polymorphic and, as expected from the difference in sample size, genetic variability was greater in Bontuchel region with the number of alleles per locus (N_a) ranging from 9-17, compared with 5-12 in Afonwen (Table 5.2). However, this difference was also shown by the sample size corrected mean allelic richness

(A_R, based on minimum sample size of 45 diploid individuals), which was greater in Bontuchel (mean =8.62) than in Afonwen (mean =6.16). The expected (H_e) heterozygosity in Bontuchel (H_e=0.700±0.01 SD) and Afonwen (H_e=0.586±0.03 SD) did not differ significantly (t-test, t=1.92, d.f.=18, P>0.05) (Table 5.2).

5.2.3 Genetic partitioning

AMOVA indicates that no significant genetic differences were attributed to variation among the temporal groups of samples with only 2 % and 1 % of the total genetic variance attributable to temporal genetic variation in Bontuchel and Afonwen respectively (Table 5.3). Approximately 19 % (Bontuchel) and 9 % (Bontuchel North) of the total genetic variance was attributed to differences among populations within each sample period and the majority of genetic variation occurred within populations. The lack of temporal variation among samples justifies pooling samples from the same site in different years in order to increase statistical power for analyses of population genetic structure in common dormice.

5.2.4 Detection of recent bottleneck

When testing for recent demographic change by looking for deviations from mutation-drift equilibrium under different mutation models using BOTTLENECK, we found no significant heterozygote excess (P<0.05) under all mutation models (Appendix 5.1), indicating that no recent bottleneck event has occurred in either population studied.

5.2.5 Population structure analysis

Posterior probability values from the STRUCTURE analysis suggest the data is best explained by the distribution of the samples into three clusters (K=3) for samples in Bontuchel and K=4 in Afonwen (Figure 5.1). These models were supported by the highest ΔK value (Evanno et al. 2005), implying the likely presence of several genetically distinct subpopulations (see also Appendix 5.2 for the graphical method for the detection of the true number of groups (K) in a sample). The assignment of individuals to populations in Bontuchel and Afonwen regions are presented in Figure 5.1 and Appendix 5.3. At a landscape scale, there is very clear division between Bontuchel and Afonwen region as show by STRUCTURE (Appendix 5.3). Within regions, there is no clear pattern of genetic grouping observed in Afonwen as a substantial proportion of individuals are inferred to have mixed ancestry and there is an approximately equal probability of membership to

either group (Figure 5.1 and Appendix 5.4a). By contrast, with K=3, three clusters were obtained in the Bontuchel, one comprising all the non-continuous populations (populations 1-9) that surround BON, while cluster two and three comprised an admixed samples within BON (Figure 5.1; Appendix 5.4b). This indicates that there was no clear population structure between the two model clusters generated by this software. However, when analysing the results from STRUCTURE more deeply, the patchy populations (population 1, 2 and 3) within Bontuchel region have some shared ancestry with the population in Bontuchel wood (see also Appendix 5.4b). Moreover, the distance between these populations and Bontuchel wood is quite close (Figure 5.1). In contrast, the other populations within this region have an average probability of membership of nearly 100 % (see Appendix 5.4b) and are quite isolated from Bontuchel wood. I also detected one immigrant in Bontuchel wood (individual 77; Figure 5.1)

Genetic autocorrelation analysis displayed significant (P<0.05) positive kinship coefficients (F_{ij}) for both continuous and non-continuous/patchy groups of samples up to distances of 800 m, with average kinship between common dormice from Bontuchel wood significantly greater than between individuals from the other two groups (non-continuous populations in Bontuchel and Afonwen) (Figures 5.2a-c). All the three correlograms show a similar pattern, with positive kinship coefficients at the smallest distances (less than 1 km). The decline in spatial autocorrelation, which is an indication of isolation-by-distance (IBD) (Sokal and Wartenberg 1983; Sokal et al. 1997), occurs rapidly over short distances of 500-800 m in all groups analysed, (Figure 5.2a-c) but there is a slight extra tail at Bontuchel wood (BON) (Figure 5.2c). This shows that there is only a slight difference between the patchy populations and the continuous population in BON.

5.2.6 Detection of immigrants

Assignment tests revealed that a high proportion of individuals were assigned as residents in the area at which they were caught. Overall, 255 individuals from all populations studied (97.9 %, n=282) were assigned as residents with more than 95 % likelihood. There were 4 (1.72 %, n=232) and 2 (4.0 %, n=50) individuals (2.13 % of the total sample of 282 individuals) in Bontuchel and Afonwen identified as first-generation migrants respectively. Of these, four migrants were males and two were females.

5.3 Discussion

The main outcomes of this study are that: (1) high genetic differentiation between Afonwen and Bontuchel is probably due to a very low gene flow among populations as these populations are separated at a landscape scale (*i.e.* more than 15 km), (2) there is limited gene flow between habitat patches at fine geographical scale (*i.e.* about 1 km), but the detection of occasionally rare inter-patch movement (*i.e.* through dispersal corridor) shows that common dormice are probably less sedentary than previously thought, (3) the apparent IBD pattern within continuous habitat patch (BON) is not strong enough to develop discrete subpopulations. Moreover, I also detect a relatively low genetic heterogeneity in patchy populations in Afonwen, but this is more likely an artifact of small sample size rather than effect of habitat fragmentation.

5.3.1 Patterns of genetic diversity

It is hard to fully interpret patterns of diversity across species (*cf.* Spielman et al. 2004, who found less diversity in threatened taxa compared with related non-threatened taxa), but the high level of genetic diversity (see Table 5.2) implies that dormice have not suffered a severe population decline. The range of heterozygosity (H_e=0.37-0.82) in this study is comparable to those at microsatellite loci in other small mammal species (e.g. Latch et al. 2008; Lecis et al. 2008; Zalewski et al. 2009), including rodents (e.g. Schulte-Hostedde et al. 2001; Crawford et al. 2009). It has to be remembered that estimates of genetic structure may be ambiguous if samples are collected at a single point in time as it will reflect the social organisation of a population at the time of sampling (Latch and Rhodes 2006) – see Chapter 4 for structure of BON for each year separately. However, in this study, due to the temporal stability found in the genetic structure of sampled populations in the data set (also reported in Chapter 4), pooling samples across years is acceptable at this landscape level (Frantz et al. 2008).

Overall genetic diversity of common dormice in Bontuchel was higher than in Afonwen region (Table 5.2) but no substantial erosion of genetic diversity via a recent genetic bottleneck in either region was apparent (Appendix 5.1). This could be due to the fact that the current statistical tests to detect bottlenecks only have the power to detect very severe and extended reductions in population size, and are insensitive to the effect of a transient reduction in population size, with recent gene flow further diminishing the powerof these

tests (Cornuet and Luikart 1996). To some extent, lower diversity may be due to the relatively small sample size in Afonwen (n=50 in total) compared with Bontuchel (n=232 in total) and thus reflect the lower sampling effort within this area. However, there is a difference between sites in their allelic richness (A_R), a statistic designed to account for potential biases of unequal sample size (Table 5.2), which likely reflects differences in the abundance of *M. avellanarius* between these two areas. Numerous studies have reported a positive correlation between population size and within-population genetic diversity (see *e.g.* O'Ryan et al. 1998; Knaepkens et al. 2004; White and Searle 2007). Thus, a plausible explanation for the apparent pattern is populations within Afonwen are smaller, either because of a slow decline (not detected by the bottleneck analysis) or because this area always had less genetic variability than those in Bontuchel through historical differences in abundance related to patterns of colonisation. Additional sampling of dormice from North Wales, combined with phylogeographic markers (see Chapter 6) would help to examine and clarify such ideas.

Moreover, in Afonwen, common dormice were mostly sampled within an area of mixed woodland which has a major track through part of the woodland on the side of a main road (the A541, Figure 5.1). Thus, it is possible that dormice in this region have been fragmented, leading to reductions in dispersal rates and low levels of genetic variation. The low genetic diversity in this region is also evident in the relatively high average inbreeding (f) (Table 5.2). I did not uncover any evidence of substantial population substructure in this region (see below for further discussion), so an unidentified Wahlund effect was unlikely to occur (Law et al. 2003); rather, I predicted that a strong family structure as a result of recent habitat fragmentation probably explains this phenomena. This could make common dormice in this region vulnerable to inbreeding depressions and loss of adaptive potential as low levels of heterozygosity are associated with reduced survival and fecundity during environmental changes (Saccheri et al. 1998). Thus, at present, the mechanism behind the low level of microsatellite polymorphism in common dormice in the Afonwen region is unknown. Continued monitoring of these new sites, and additional sampling elsewhere is required.

5.3.2 *Gene flow between populations and barrier effects*

Landscape features may strongly influence spatial genetic structure and patterns of gene flow (Manel et al. 2003). Thus, a patchy distribution of suitable habitats is expected to create a patchy genetic structure, particularly in poorly dispersing species (Gauffre et al. 2008). It was predicted that landscape structure (*i.e.* arable farmland, grazing pasture, roads, river) within both regions (Bontuchel and Afonwen) would restrict the dispersal of common dormice between populations (but see Introduction about common dormice crossing roads), thus contributing to strong genetic structuring of this species, as observed in many other small mammals (see *e.g.* Gerlach and Musolf 2000; Orlowski and Nowak 2006; McGregor et al. 2008; Rodenbeck and Voser 2008; Lampila et al. 2009). In this study, STRUCTURE shows that there is strong differentiation between the Afonwen and Bontuchel regions despite some evidence of admixture in some patches in Afonwen (Appendix 5.3). Thus, differences between these two regions were expected as both regions were separated by some 15 km of generally non-hospitable habitat, a distance sufficient to largely prevent migration among populations. Importantly, both regions were separated by roads, which are known to act as barriers to movement and have a strong negative effect on animal populations as well as disturbing the surrounding habitat through noise and pollutants which may reduce the densities of animals (see *e.g.* Rodenbeck and Voser, 2008; Shepard et al. 2008; Kerth and Melber 2009), including small mammals (*i.e.* Gerlach and Musolf 2000; Orlowski and Nowak 2006; McGregor et al. 2008; Rodenbeck and Voser 2008; Lampila et al. 2009). These analyse therefore confirm that dormice inhabiting patches separated at the landscape scale (*i.e.* < 20 km) will be isolated. Evidence of shared ancestry among some patches in Afonwen may be an artefact of small sample size, or is possibly a historical signature reflecting that Bontuchel region is the source for populations in Afonwen. Whether the separation between these regions is due to the landscape structure of the regions or because of the large distance of separation *per se* is not clear. However, it is crucial to consider these areas as two separate samples until more populations (i.e. intermediate between the two areas) can be examined. Moreover, mitochondrial DNA (mtDNA) analysis shows that both regions harboured different and unique haplotypes, suggesting a historical population genetic structure (see Chapter 6).

Comparing the genetic structure of animals between fragmented and continuous habitat can provide insight into a better understanding of the evolutionary impact of habitat

fragmentation (Berthier et al. 2005; Kraaijeveld-Smit et al. 2007; Norris et al. 2008; Chavez and Ceballos 2009; Kozakiewicz et al. 2009b). Unfortunately, the lack of field data in the patches surrounding the main Bontuchel wood (*i.e.* tagging data) limits the conclusion on whether individuals are able to move between habitat patches. However, I observed a low level of migration (2.13 % of the total sample of 282 individuals) as evidenced by both STRUCTURE (Figure 5.1) and assignment tests (Table 5.4), with the proportion of male immigrants being greater than females. This may reflect the male-biased dispersal (MBD; described in Chapter 3). MBD is common in polygamous animals (Greenwood 1980; Clutton-Brock 1989; Devillard et al. 2004; Lacey and Sherman 2007; Nunes 2007), particularly rodents (see *e.g.* Lacey and Wieczorek 2004; Cutrera et al. 2005; Gauffre et al. 2009), and recently has also been detected both in a wild and reintroduced population of common dormice (see Chapter 4). As common dormice are generally perceived to be reluctant to cross open ground (Capizzi et al. 2002; Morris 2004; Bright and Morris 2002; Bright et al. 2006), this raises an issue on how this species moves between the habitat patches and to what extent.

The vegetation and native forest surrounding Bontuchel wood (Figure 5.1) may serve as dispersal pathways connecting the non-continuous populations of common dormice. For instance, in Bontuchel region, there is *ca.* 0.4 km of overlapping and interconnecting tree lines that link BON wood and CTP (population 1) at the west (Rhian Hughes, North Wales Wildlife Trust (NWWT), pers. comm.). There is also a good connection between CYP (population 2) and CC (population 4) at southern Bontuchel wood (Figure 5.1). Moreover, STRUCTURE shows a probability of shared ancestry between these populations (populations 1-3) with Bontuchel wood, suggesting that gene flow may occur between these populations. Corridors have been promoted widely as a conservation strategy (reviewed by Lindenmayer 1994; Hess and Fischer 2001; Chetkiewicz et al. 2006; Davies and Pullin 2007; Beier et al. 2008; Kadoya 2009), with the purpose of countering the effects of habitat loss and fragmentation (Sih et al. 2000) by increasing the movement of individuals among isolated populations (i.e. Gilbert et al. 1998; Gonzalez et al. 1998; reviewed in Chetkiewicz et al. 2006). This highlights the importance of habitat connectivity across large scales to enhance, or at least maintain genetic diversity of a species. The existence of potential immigrants in Afonwen raises the possibility of neighbouring dormice populations. The lack of data, however, limits an attempt to identify the specific source of immigrants. Due to the low

level of migration detected, I propose that very low levels of inter-population gene flow and exchanges characterise common dormice populations in patchy landscapes scale. Thus, a clear next step to this work is to study additional patchy populations of dormice to identify specific landscape features that could facilitate and hinder dispersal.

In addition to the observed rare movement between habitat patches and detection of immigrants within both regions, alternatively, this pattern of regular short movements and occasional long-distance (*i.e.* > 600 m) movements is consistent with the existing literature on small mammals (*e.g.* Martinse 1968; Liro and Szacki 1987; Gentile and Cerquira 1995; Wells et al. 2008). This pattern of dispersal is probably natal dispersal, defined by Greenwood (1980) as dispersal from the birth site to that of first reproduction or potential reproduction, and is often the only long-distance movement an animal will make (Dahl and Willebrand 2005 and references therein).

5.3.3 Population genetic structure within and among habitat patches

STRUCTURE clustered the patchy, non-continuous populations within Bontuchel region into a single hypothetical population, and similarly no apparent genetic division was observed among patches within Afonwen (Figure 5.1). On the one hand this may reflect mobility by dormice. Equally, however, the observed genetic homogeneity between populations in both regions may reflect a recent colonisation of common dormice into these regions, either from the original habitat or through a small number of founders and a lack of time for the patches to diverge (see *e.g.* Heaney et al. 2005; Barker et al. 2009; Drury et al. 2009; Franzen and Nilsson 2010). More likely, it should be remembered that the observed lack of structure should be interpreted cautiously because of the relatively small sample size of populations within both regions. The result of STRUCTURE can be sensitive to the sampling scheme when there is clinal variation in allele frequencies (Falush and Pritchard 2003; Falush et al. 2003). Clearly, additional sampling in both regions is required to identify the population genetic structure in Afonwen and fragmented populations in Bontuchel.

STRUCTURE suggests the occurrence of two subpopulations within BON (Figure 5.1) which is further supported by AMOVA where most of the total variance was attributable to among individuals within populations (Table 5.3). However, this result should be treated cautiously as the subdivision is quite weak and was not strongly supported genetically

where the average probability of membership shows a very high admixture among individuals (Appendix 5.4b) implying high gene flow between these subpopulations. Moreover, several studies (*i.e.* Bamshad et al. 2003; Rosenberg et al. 2003; Ramachandran et al. 2004; Evanno et al. 2005) have raised a question about the reliability of the STRUCTURE results in cases of complex genetic structure (Camus-Kulandaivelu et al. 2007). The absence of population subdivision within BON implies that movement is unlikely to be affected by any single barrier within a large habitat patch, thus suggesting that the pattern of dispersal is sufficient to prevent the formation of discrete subpopulations within the large continuous habitat patch of 2.5 km.

The most striking result in this study was the apparent of very slight extra tail in the IBD pattern in the continuous population in Bontuchel wood (Figure 5.2c). This result was unexpected as this shows that despite the difference between the patchy populations in both regions and in continuous population which suggest a very low inter-patch movement, the difference is very weak. This contrasts with other work that found greater effect of habitat fragmentation in non-continuous habitat (see e.g. Watts et al. 2004; Noël et al. 2007). Fragmentation leads to overall reductions in population size for most species, and to reduced migration (gene flow) among patches (Frankham et al. 2002). The impact of habitat fragmentation on movement of animals has been reported in many studies, particularly in small mammals (see *e.g.* Goheen et al. 2003; Saavedra and Simonetti 2005; Elliott and Root 2006; Bentley 2008; Gauffre et al. 2008; Marchesan and Carthew 2008).

Theoretical and empirical studies have shown that genetic structuring is largely influenced by a combination of dispersal patterns and mating systems (see *e.g.* Chesser 1991; Sugg et al. 1996; Dobson 1998; Matocq and Lacey 2004; Norton and Ashley 2004; Fredsted et al. 2007). Consequently, small mammals that are incapable of traversing long distances, typically exhibit high levels of genetic differentiation (*i.e.* Borkowska and Ratkiewicz 2004; Schweizer et al. 2007). Social organisation and the possibility of kin selection and cooperation have been studied in numerous mammalian systems (de Ruiter and Geffen 1998; Gagneux et al. 1999; Hazlitt et al. 2004; Gauffre et al. 2009), and this has been recently shown in common dormice (see Chapter 3). Common dormice typically form a crèche and exhibit communal nesting behaviour and remain in their natal group during the breeding season, whilst finding mating opportunities in neighbouring groups (Chapter 3).

Thus, it is expected that one social group could be sampled within a small area. Thus, the combination of localised breeding (see Chapter 3), high site fidelity (philopatry) in females and short range dispersal in males (Chapter 4) may have contributed to the formation of genetic structuring at small spatial scale (*i.e.* less than 1 km).

5.4 Implications for conservation

Management of common dormice populations for conservation requires effective population monitoring and understanding of the biology and ecology of the species. The findings of this study provide a perspective on contemporary population structure and dispersal within and between fragmented habitat patches obtained through indirect observations (though direct observation is not impossible as demonstrated in Chapter 4, but lack of sampling data limits this analysis and this is a consequence of the recent establishment of nestboxes). Indeed, results from this study will aid in the future management of this species, by providing knowledge of the geographical scale at which common dormice populations are genetically structured and distinct. Several important observations emerge from this finding: (1) patterns of high population differentiation between regions (Afonwen and Bontuchel) are almost certainly a consequence of very low gene flow among these geographically distinct populations (*i.e.* more than 15 km). Consequently, management regimes will need to focus on a regional scale rather than a local scale to effectively manage these populations. (2) The apparent genetic structuring within and between habitat patches suggests that overall levels of gene flow generally are limited at a fine geographical scale (*i.e.* about 1 km). This has implications for reintroductions of animals and/or translocation. (3) The detection of immigrants within Bontuchel and Afonwen reflect the probability of occasional long-distance movements of individuals from neighbouring areas probably through dispersal corridors; however, limited data precluded the identification of source populations. However, this highlights the importance of habitat connectivity in facilitating movement between populations. Even though poor quality habitat is not suitable for breeding, by enabling dispersal it may be crucial for increasing landscape connectivity (Wiegand et al. 2005; Lampila et al. 2009). In future, further studies on additional populations and extend geographic area are required to explore the consequences of these findings in more detail.

Table 5.1 Number of samples collected in each year. Number in bracket indicates number of samples genotyped successfully and was used for population genetic analysis.

		Years collected		
Region	Samples	2007	2008	2009
Bontuchel	CTP	8 (8)	5 (4)	
	CYP		2 (2)	1 (1)
	Bill	1 (1)		3 (3)
	CC	6 (5)		5 (3)
	MA		3 (3)	
	LBD		2 (2)	
	CO		2 (2)	
	BON	140 (140)	58 (58)	
Afonwen	CBF	33 (21)	15 (9)	
	BE/BFL		2 (2)	1 (1)
	YDU		5 (3)	
	SW	8 (8)	4 (4)	
	FH			2 (2)

Table 5.2 Genetic characteristics of studied populations including number of samples (n), number of allele (N_a), observed (H_o) and expected (H_e) heterozygosity, allelic richness (A_R) and inbreeding coefficient (f).

Population group	n		Locus G3	E3	F10	B5	A5	G6	G9	H3	C4-2	F1-2	Average
Bontuchel	232	N_a	10	13	11	9	12	13	9	9	16	17	12
		H_e	0.706	0.675	0.766	0.526	0.722	0.739	0.685	0.597	0.767	0.819	0.700
		H_o	0.680	0.659	0.706	0.476	0.642	0.724	0.696	0.535	0.709	0.671	0.649
		A_R	8.11	8.98	9.40	7.07	9.22	7.74	6.50	6.03	10.34	12.82	8.62
		f	0.038	0.024	0.078	0.095	0.111	0.021	-0.016	0.105	0.076	0.181	0.071
Afonwen	50	N_a	6	5	6	5	7	5	5	5	12	7	6
		H_e	0.772	0.369	0.520	0.387	0.489	0.704	0.629	0.428	0.791	0.771	0.586
		H_o	0.706	0.245	0.413	0.389	0.347	0.617	0.725	0.222	0.698	0.520	0.488
		A_R	6.00	4.70	5.98	2.97	6.84	5.00	4.88	5.00	11.20	8.99	6.16
		f	0.086	0.338	0.207	-0.005	0.292	0.124	-0.154	0.484	0.118	0.328	0.182

Table 5.3 Analysis of molecular variance (AMOVA) in *M. avellanarius*. d.f., degrees of freedom; SS, sum of squares; MS, mean squares; Est. var., estimated variance; % D, distribution of total variance.

Source	d.f	SS	MS	Est. var.	% D	*P*
Bontuchel						
Among temporal groups	2	33.863	16.932	0.171	2.00	<0.05
Among populations	7	168.216	24.031	1.878	19.00	0.00
Within populations	224	1757.206	7.845	7.845	79.00	<0.01
Afonwen						
Among temporal groups	2	16.333	8.167	0.091	1.00	<0.05
Among populations	4	57.102	14.275	0.833	9.00	0.00
Within populations	50	404.262	8.085	8.085	90.00	<0.05

Table 5.4 Percentage of individuals correctly assigned to population within both regions.

Region	Population of origin	*n*	Number of individuals assigned correctly (%)
Bontuchel	BON	198	0.99
	CO	2	1.00
	LBD	2	1.00
	MA	3	1.00
	BILL	4	0.75
	CC	8	1.00
	CTP	12	0.92
	CYP	3	1.00
Afonwen	CBF	30	0.97
	BFL	3	1.00
	SW	12	0.92
	YDU	3	1.00
	FH	2	1.00

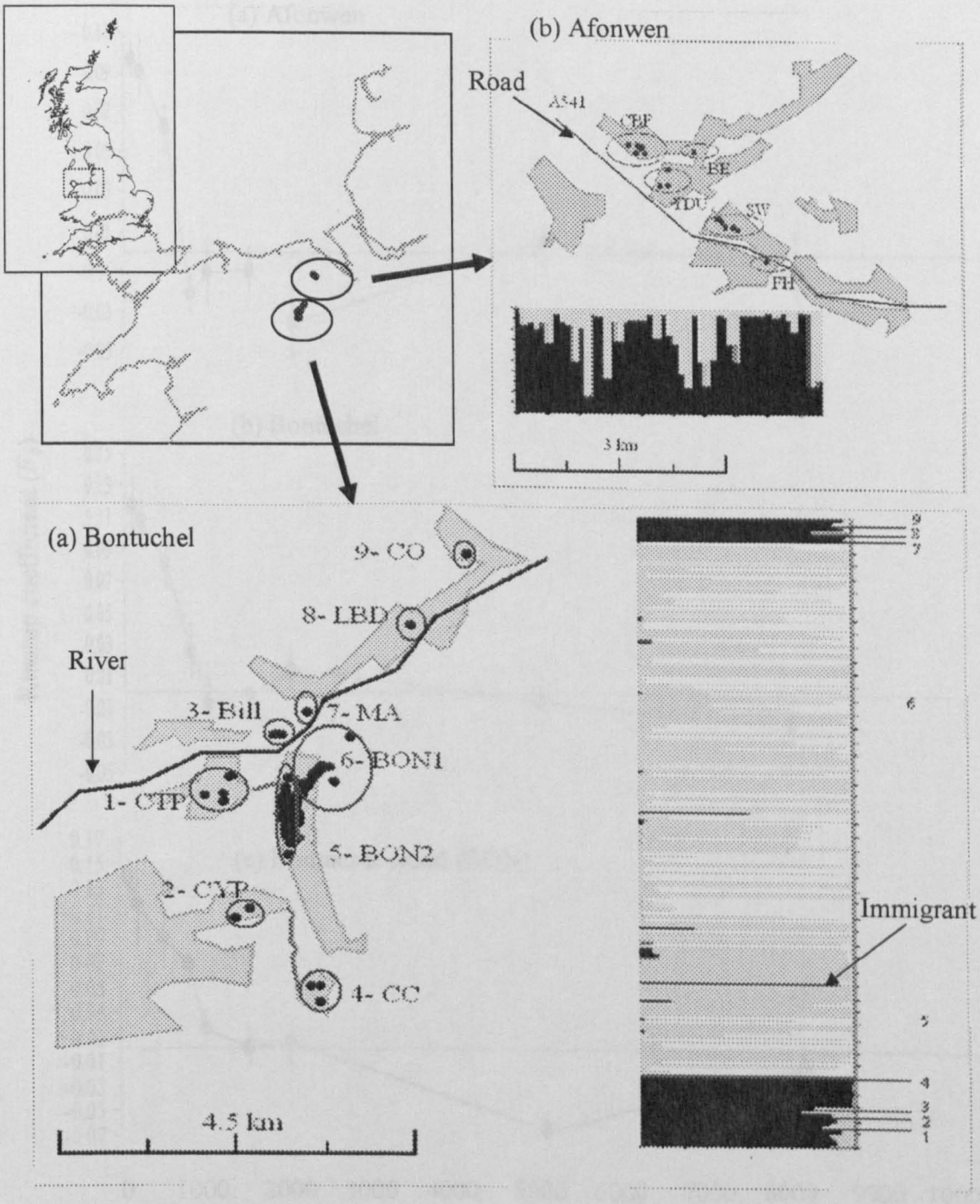


Figure 5.1 Location of study sites; (a) Bontuchel, (b) Afonwen. Bar plot obtained from STRUCTURE, analysing the probability of individual membership to clusters with K=3 inferred clusters in Bontuchel and K=4 in Afonwen. Each bar represents one individual and the proportion of the bar that is black, white, grey and light grey represents the proportion of assignment to cluster one, two, three or four respectively. Green line shows a possibility of a good connection between habitats.

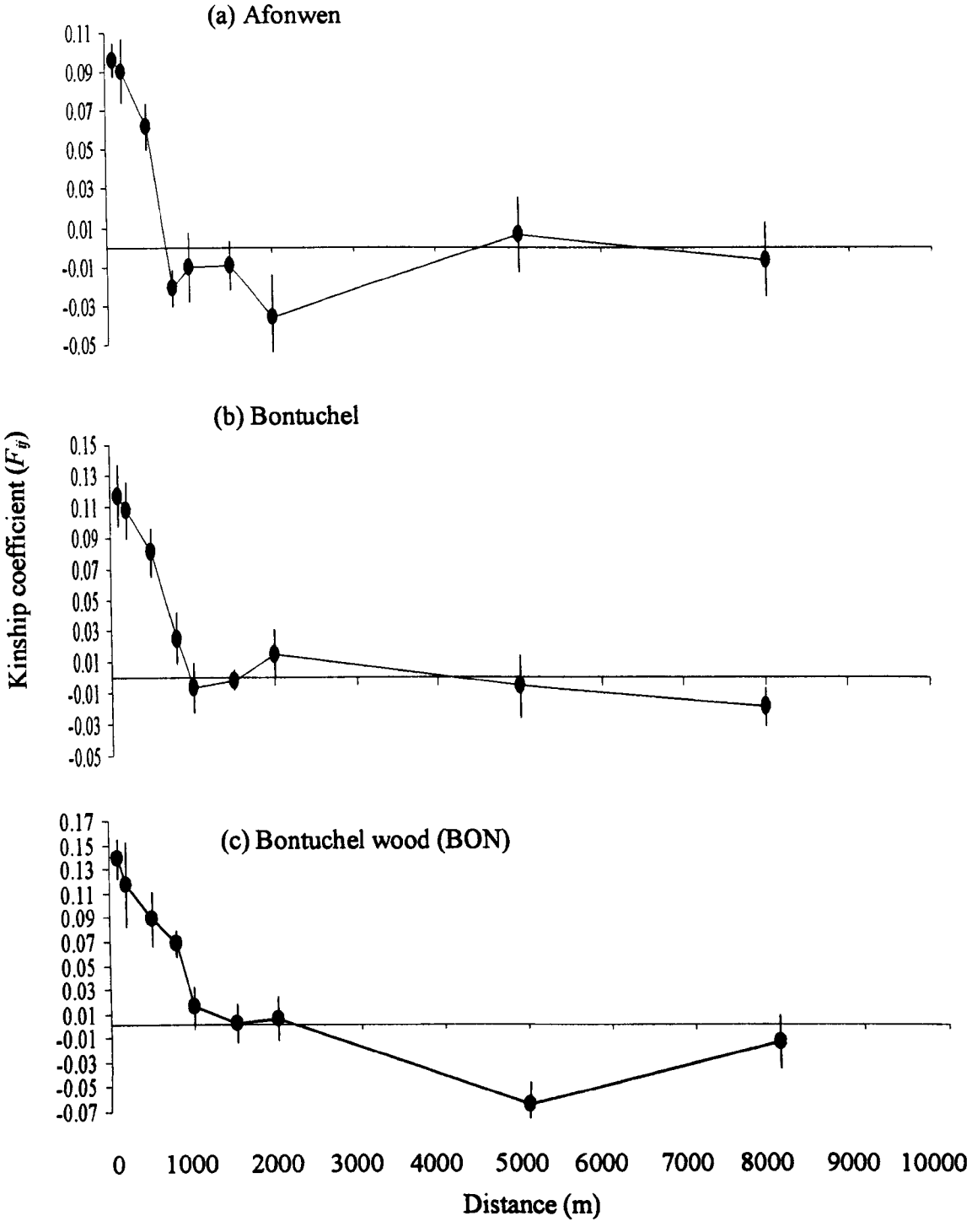
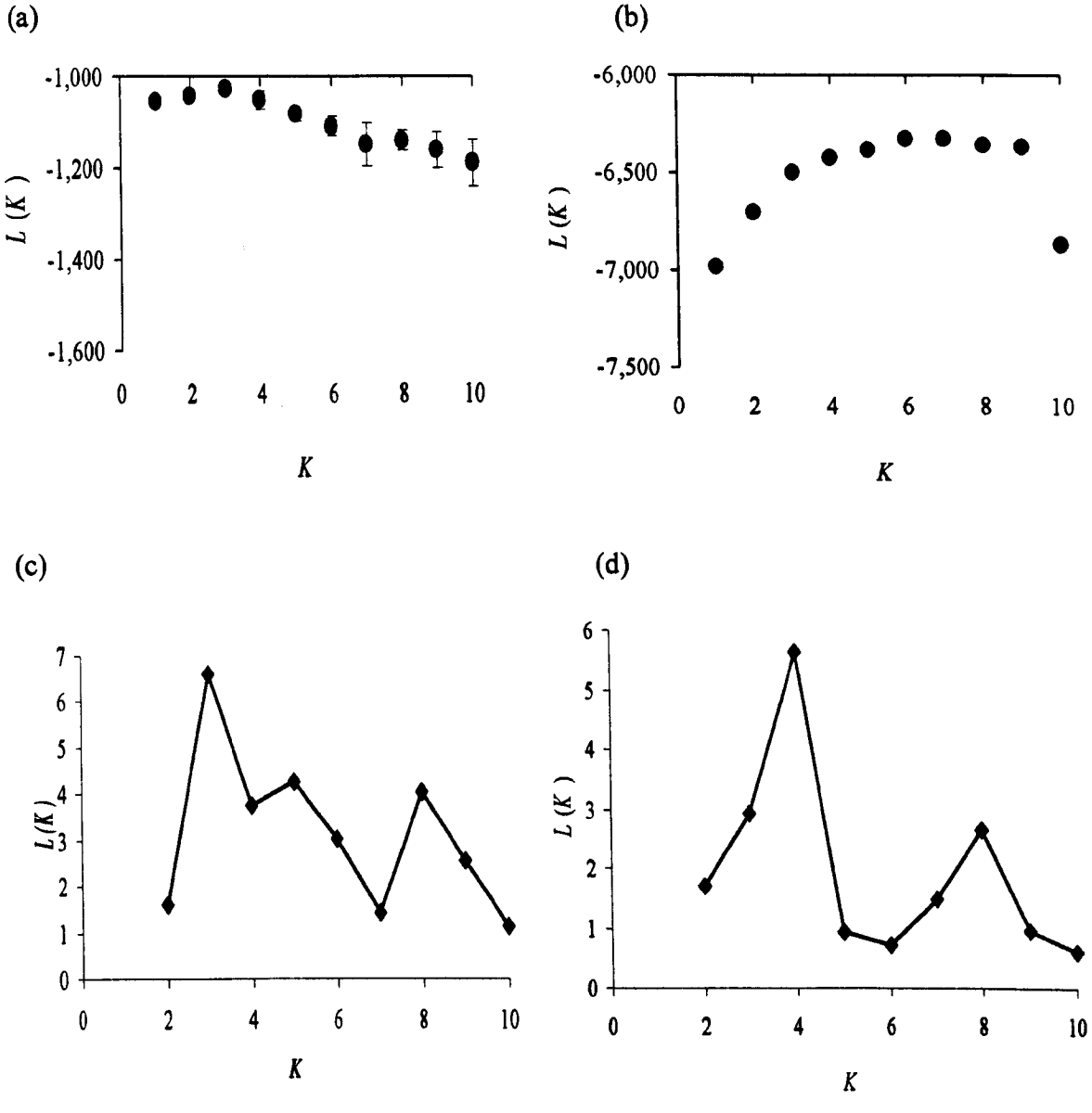


Figure 5.2 Correlogram showing the combined genetic correlation in kinship kinship (F_{ij}) (Loiselle et al. 1995) among pairs of *M. avellanarius* from (a) Afonwen, (b) Bontuchel and (c) Bontuchel wood (BON). Standard errors were obtained by jackknifing over 10 microsatellite loci.

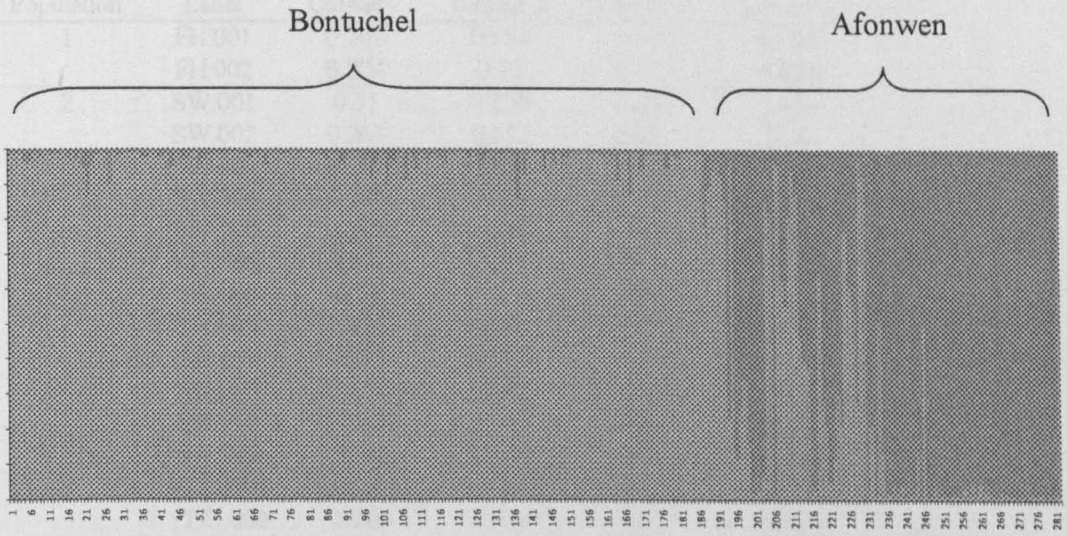
Appendix 5.1 Probability values for tests for a significant heterozygote excess of a population bottleneck for samples of *M. avellanarius* using three models of microsatellite allele mutation (IAM, infinite allele model; TPM, two phase model; SMM, stepwise model and two methods of analysis, Sign test and Wilcoxon sign-rank test.

	Sign test			Wilcoxon test		
Site	IAM	TPM	SMM	IAM	TPM	SMM
Afonwen	0.2162	0.1102	0.2511	0.1621	1.000	1.000
Bontuchel	0.3001	0.1351	0.0656	0.2488	0.8748	0.9362

Appendix 5.2 Description of the two steps for the graphical method that allows detection of the true number of groups (K) in a sample: mean L(K) (±95 % CI) over 10 independent runs for each K value in a) Afonwen and (b) Bontuchel; ΔK calculated according to Evanno et al.'s (2005) formula as $\Delta K = m|L''(K)|s[L(K)]$ for (c) Afonwen and (d) Bontuchel. The modal value of this distribution represents either the true value of K or the uppermost level of genetic structure.



Appendix 5.3 Bar plot obtained from STRUCTURE, analysing the probability of individual membership to clusters with K=2 inferred clusters between Bontuchel and Afonwen region. Each bar represents one individual and the proportion of the bar that is red and blue represents the proportion of assignment to cluster one (Afonwen) and cluster two (Bontuchel).



Appendix 5.4 Average probability of membership for populations of *M. avellanarius* to clusters derived using STRUCTURE in (a) Afonwen and (b) Bontuchel. Populations with average membership >0.6 to a cluster are highlighted bold.

(a) Afonwen

		Probability of assignment			
Population	Label	Cluster 1	Cluster 2	Cluster 3	Cluster 4
1	FH.001	0.207	0.134	0.031	**0.629**
	FH.002	0.204	0.13	0.027	**0.639**
2	SW.001	0.31	0.255	0.221	0.214
	SW.002	0.261	0.317	0.262	0.16
	SW.003	0.236	0.26	0.264	0.24
	SW.004	0.289	0.259	0.297	0.155
	SW.005	0.318	0.252	0.219	0.212
	SW.006	0.221	0.201	0.156	0.422
	SW.007	0.22	0.205	0.188	0.386
	SW.008	0.232	0.341	0.31	0.116
	SW.009	0.239	0.304	0.347	0.111
	SW.010	0.231	0.248	0.18	0.341
	SW.011	0.244	0.266	0.215	0.274
	SW.012	0.242	0.289	0.242	0.226
3	YDU.001	0.316	0.241	0.242	0.202
	YDU.002	0.229	0.163	0.127	0.481
	YDU.003	0.207	0.135	0.039	0.619
4	BFL.001	0.247	0.263	0.388	0.102
	BFL.002	0.237	0.336	0.308	0.119
	BFL.003	0.242	0.301	0.273	0.185
5	CBF.001	0.26	0.321	0.28	0.139
	CBF.002	0.216	0.269	0.359	0.155
	CBF.003	0.297	0.272	0.241	0.19
	CBF.004	0.262	0.313	0.3	0.126
	CBF.005	0.272	0.292	0.305	0.131
	CBF.006	0.239	0.307	0.361	0.093
	CBF.007	0.308	0.259	0.258	0.175
	CBF.008	0.256	0.277	0.367	0.101
	CBF.009	0.247	0.333	0.3	0.12
	CBF.010	0.256	0.271	0.264	0.209
	CBF.011	0.228	0.307	0.346	0.12
	CBF.012	0.287	0.262	0.334	0.117
	CBF.013	0.298	0.24	0.225	0.237
	CBF.014	0.246	0.259	0.431	0.064
	CBF.015	0.235	0.327	0.319	0.119
	CBF.016	0.238	0.286	0.311	0.166
	CBF.017	0.245	0.261	0.421	0.073
	CBF.018	0.255	0.29	0.305	0.151
	CBF.019	0.253	0.262	0.38	0.106
	CBF.020	0.211	0.176	0.11	0.503
	CBF.021	0.264	0.301	0.328	0.107
	CBF.022	0.262	0.27	0.268	0.199
	CBF.023	0.26	0.291	0.286	0.164
	CBF.024	0.243	0.314	0.32	0.124
	CBF.025	0.251	0.271	0.358	0.12
	CBF.026	0.234	0.341	0.309	0.116

Appendix 5.4 *cont.*

Population	Label	Probability of assignment			
		Cluster 1	Cluster 2	Cluster 3	Cluster 4
	CBF.027	0.256	0.291	0.358	0.095
	CBF.028	0.208	0.152	0.062	0.578
	CBF.029	0.205	0.145	0.054	0.596
	CBF.030	0.221	0.251	0.289	0.24

(b) Bontuchel

Population	Label	Probability of assignment		
		Cluster 1	Cluster 2	Cluster 3
1	CTP.001	0.139	0.021	**0.839**
1	CTP.002	0.122	0.013	**0.865**
1	CTP.003	0.053	0.012	**0.936**
1	CTP.004	0.128	0.009	**0.863**
1	CTP.005	0.109	0.009	**0.882**
1	CTP.006	0.075	0.014	**0.911**
1	CTP.007	0.088	0.014	**0.898**
1	CTP.008	0.107	0.01	**0.884**
1	CTP.009	0.117	0.015	**0.868**
1	CTP.010	0.056	0.01	**0.934**
1	CTP.011	0.075	0.02	**0.905**
1	CTP.012	0.084	0.031	**0.885**
2	CYP.001	0.033	0.226	**0.741**
2	CYP.002	0.048	0.148	**0.804**
2	CYP.003	0.043	0.151	**0.805**
3	Bill.001	0.013	0.01	**0.977**
3	Bill.002	0.008	0.01	**0.982**
3	Bill.003	0.008	0.007	**0.984**
3	Bill.004	0.021	0.007	**0.972**
4	CC.001	0.012	0.007	**0.982**
4	CC.002	0.012	0.012	**0.976**
4	CC.003	0.006	0.007	**0.987**
4	CC.004	0.01	0.006	**0.984**
4	CC.005	0.004	0.003	**0.993**
4	CC.006	0.006	0.007	**0.987**
4	CC.007	0.009	0.006	**0.986**
4	CC.008	0.007	0.005	**0.987**
5	Bon.001	0.03	**0.969**	0.001
5	Bon.002	**0.988**	0.008	0.004
5	Bon.003	**0.683**	0.299	0.018
5	Bon.004	**0.987**	0.012	0.001
5	Bon.005	**0.962**	0.037	0.002
5	Bon.006	**0.911**	0.087	0.002
5	Bon.007	0.148	**0.851**	0.001
5	Bon.008	0.035	**0.965**	0.001
5	Bon.009	0.109	**0.89**	0.001
5	Bon.010	**0.936**	0.062	0.002
5	Bon.011	**0.884**	0.034	0.083
5	Bon.012	**0.944**	0.053	0.003
5	Bon.013	0.053	**0.946**	0.001

Appendix 5.4 *cont.*

Population	Label	Probability of assignment		
		Cluster 1	Cluster 2	Cluster 3
5	Bon.014	**0.97**	0.028	0.002
5	Bon.015	**0.976**	0.022	0.003
5	Bon.016	0.022	**0.977**	0.001
5	Bon.017	0.101	**0.898**	0.001
5	Bon.019	**0.978**	0.02	0.002
5	Bon.020	**0.985**	0.014	0.001
5	Bon.021	**0.945**	0.054	0.001
5	Bon.022	**0.976**	0.022	0.001
5	Bon.023	**0.987**	0.012	0.001
5	Bon.024	**0.803**	0.195	0.002
5	Bon.025	**0.786**	0.061	0.153
5	Bon.026	**0.977**	0.022	0.001
5	Bon.027	**0.978**	0.018	0.004
5	Bon.028	**0.971**	0.023	0.005
5	Bon.029	**0.975**	0.021	0.003
5	Bon.142	**0.687**	0.294	0.018
5	Bon.143	**0.885**	0.033	0.082
5	Bon.145	**0.97**	0.021	0.009
5	Bon.146	**0.977**	0.021	0.001
5	Bon.148	**0.976**	0.023	0.002
5	Bon.151	0.104	**0.896**	0.001
5	Bon.153	**0.98**	0.015	0.005
5	Bon.155	0.029	**0.97**	0.001
5	Bon.156	**0.975**	0.023	0.001
5	Bon.159	0.374	**0.623**	0.002
5	Bon.161	**0.978**	0.02	0.002
5	Bon.164	**0.98**	0.019	0.002
5	Bon.167	**0.976**	0.022	0.002
5	Bon.168	**0.836**	0.163	0.001
5	Bon.173	**0.988**	0.008	0.003
5	Bon.177	**0.987**	0.012	0.001
5	Bon.187	**0.962**	0.037	0.002
5	Bon.192	**0.979**	0.02	0.001
5	Bon.195	**0.897**	0.048	0.055
5	Bon.196	**0.93**	0.015	0.055
5	Bon.197	**0.878**	0.062	0.059
5	Bon.198	0.059	0.017	**0.924**
6	Bon.018	0.177	**0.822**	0.001
6	Bon.030	**0.766**	0.23	0.004
6	Bon.031	**0.845**	0.153	0.002
6	Bon.032	0.468	0.513	0.019
6	Bon.033	**0.939**	0.029	0.032
6	Bon.034	**0.971**	0.029	0.001
6	Bon.035	**0.99**	0.009	0.001
6	Bon.036	**0.733**	0.259	0.008
6	Bon.037	0.097	**0.902**	0.001
6	Bon.038	**0.98**	0.019	0.001
6	Bon.039	0.072	**0.927**	0.001
6	Bon.040	0.019	**0.98**	0.001
6	Bon.041	0.043	**0.957**	0.001
6	Bon.042	0.142	**0.857**	0.001
6	Bon.043	**0.809**	0.184	0.007

Appendix 5.4 *cont.*

Population	Label	Probability of assignment		
		Cluster 1	Cluster 2	Cluster 3
6	Bon.044	0.025	**0.974**	0.001
6	Bon.045	0.023	**0.976**	0.001
6	Bon.046	0.258	**0.741**	0.001
6	Bon.047	0.052	**0.94**	0.008
6	Bon.048	0.019	**0.98**	0.001
6	Bon.049	**0.665**	0.326	0.009
6	Bon.050	0.016	**0.983**	0.001
6	Bon.051	0.138	**0.86**	0.002
6	Bon.052	0.164	**0.835**	0.001
6	Bon.053	**0.981**	0.018	0.001
6	Bon.054	0.056	**0.941**	0.003
6	Bon.055	**0.977**	0.021	0.002
6	Bon.056	0.027	**0.972**	0.001
6	Bon.057	**0.701**	0.038	0.26
6	Bon.058	**0.965**	0.034	0.001
6	Bon.059	0.031	**0.968**	0.001
6	Bon.060	**0.701**	0.296	0.003
6	Bon.061	0.329	**0.669**	0.002
6	Bon.062	**0.905**	0.094	0.002
6	Bon.063	0.549	0.449	0.003
6	Bon.064	0.139	**0.859**	0.002
6	Bon.065	**0.984**	0.015	0.001
6	Bon.066	0.053	**0.946**	0.001
6	Bon.067	0.061	**0.938**	0.001
6	Bon.068	**0.972**	0.026	0.002
6	Bon.069	0.025	**0.974**	0.001
6	Bon.070	**0.85**	0.148	0.002
6	Bon.071	**0.944**	0.054	0.002
6	Bon.072	0.057	**0.942**	0.001
6	Bon.073	**0.971**	0.027	0.002
6	Bon.074	**0.987**	0.012	0.001
6	Bon.075	0.057	**0.942**	0.002
6	Bon.076	0.029	**0.969**	0.001
6	Bon.077	**0.978**	0.021	0.001
6	Bon.078	**0.985**	0.015	0.001
6	Bon.079	**0.979**	0.02	0.001
6	Bon.080	0.034	**0.966**	0.001
6	Bon.081	**0.699**	0.299	0.002
6	Bon.082	**0.728**	0.268	0.004
6	Bon.083	0.048	**0.951**	0.001
6	Bon.084	**0.968**	0.03	0.002
6	Bon.085	**0.979**	0.018	0.003
6	Bon.086	0.058	**0.941**	0.001
6	Bon.087	0.124	**0.874**	0.002
6	Bon.088	**0.975**	0.022	0.002
6	Bon.089	0.035	**0.962**	0.002
6	Bon.090	0.016	**0.983**	0.001
6	Bon.091	**0.954**	0.044	0.002
6	Bon.092	**0.983**	0.016	0.001
6	Bon.093	**0.946**	0.053	0.001
6	Bon.094	**0.849**	0.147	0.005
6	Bon.095	**0.801**	0.195	0.003

Appendix 5.4 *cont.*

Population	Label	Probability of assignment		
		Cluster 1	Cluster 2	Cluster 3
6	Bon.096	**0.976**	0.023	0.001
6	Bon.097	**0.944**	0.052	0.004
6	Bon.098	**0.988**	0.011	0.001
6	Bon.099	**0.969**	0.031	0.001
6	Bon.100	**0.926**	0.07	0.003
6	Bon.101	**0.966**	0.033	0.001
6	Bon.102	0.094	**0.904**	0.002
6	Bon.103	**0.969**	0.03	0.001
6	Bon.104	**0.988**	0.009	0.002
6	Bon.105	**0.854**	0.138	0.009
6	Bon.106	**0.972**	0.025	0.003
6	Bon.107	**0.958**	0.041	0.001
6	Bon.108	**0.979**	0.019	0.002
6	Bon.109	**0.986**	0.013	0.001
6	Bon.110	**0.975**	0.02	0.005
6	Bon.111	0.388	**0.611**	0.001
6	Bon.112	**0.943**	0.055	0.002
6	Bon.113	0.264	**0.734**	0.002
6	Bon.114	**0.989**	0.01	0.001
6	Bon.115	**0.963**	0.035	0.002
6	Bon.116	**0.98**	0.012	0.008
6	Bon.117	**0.989**	0.009	0.002
6	Bon.118	**0.986**	0.013	0.001
6	Bon.119	**0.85**	0.089	0.061
6	Bon.120	0.024	**0.975**	0.001
6	Bon.121	**0.871**	0.126	0.003
6	Bon.122	**0.67**	0.312	0.018
6	Bon.123	0.071	**0.926**	0.003
6	Bon.124	**0.646**	0.348	0.006
6	Bon.125	**0.964**	0.033	0.003
6	Bon.126	0.034	**0.966**	0.001
6	Bon.127	**0.891**	0.107	0.002
6	Bon.128	0.112	**0.887**	0.001
6	Bon.129	**0.846**	0.153	0.001
6	Bon.130	**0.653**	0.344	0.003
6	Bon.131	0.355	**0.644**	0.001
6	Bon.132	**0.938**	0.058	0.004
6	Bon.133	**0.963**	0.036	0.001
6	Bon.134	**0.976**	0.022	0.002
6	Bon.135	0.22	**0.776**	0.004
6	Bon.136	**0.979**	0.019	0.002
6	Bon.137	**0.962**	0.033	0.005
6	Bon.138	**0.974**	0.025	0.001
6	Bon.139	**0.723**	0.275	0.001
6	Bon.140	0.019	**0.98**	0.001
6	Bon.141	0.452	0.04	0.508
6	Bon.144	0.025	**0.974**	0.001
6	Bon.147	**0.926**	0.07	0.004
6	Bon.149	**0.965**	0.032	0.003
6	Bon.150	**0.977**	0.02	0.003
6	Bon.152	**0.975**	0.023	0.003
6	Bon.154	**0.977**	0.021	0.001

Appendix 5.4 *cont.*

		Probability of assignment		
Population	Label	Cluster 1	Cluster 2	Cluster 3
6	Bon.157	**0.924**	0.072	0.004
6	Bon.158	**0.791**	0.207	0.002
6	Bon.160	**0.984**	0.015	0.001
6	Bon.162	0.554	0.443	0.003
6	Bon.163	**0.977**	0.021	0.002
6	Bon.165	**0.981**	0.017	0.002
6	Bon.166	0.381	**0.617**	0.002
6	Bon.169	**0.982**	0.017	0.001
6	Bon.170	0.315	**0.667**	0.017
6	Bon.171	**0.968**	0.031	0.001
6	Bon.172	0.354	**0.645**	0.001
6	Bon.174	0.054	**0.945**	0.001
6	Bon.175	**0.989**	0.01	0.002
6	Bon.176	**0.962**	0.033	0.005
6	Bon.178	**0.97**	0.03	0.001
6	Bon.179	**0.99**	0.009	0.001
6	Bon.180	0.019	**0.98**	0.001
6	Bon.181	0.019	**0.98**	0.001
6	Bon.182	**0.695**	0.303	0.002
6	Bon.183	0.016	**0.983**	0.001
6	Bon.184	0.027	**0.973**	0.001
6	Bon.185	0.026	**0.973**	0.001
6	Bon.186	0.142	**0.857**	0.001
6	Bon.188	0.046	**0.953**	0.001
6	Bon.189	0.02	**0.979**	0.001
6	Bon.190	0.034	**0.966**	0.001
6	Bon.191	**0.74**	0.253	0.008
6	Bon.193	**0.962**	0.014	0.024
6	Bon.194	**0.963**	0.027	0.01
7	MA.001	0.012	0.012	**0.977**
7	MA.002	0.026	0.017	**0.957**
7	MA.003	0.018	0.017	**0.965**
8	LBD.001	0.083	0.107	**0.811**
8	LBD.002	0.029	0.035	**0.936**
9	CO.001	0.063	0.037	**0.901**
9	CO.002	0.031	0.014	**0.955**

Chapter 6: Phylogeography and mitochondrial DNA (mtDNA) diversity of the common dormouse *M. avellanarius* in the UK.

6.0 Introduction

A frequent observation in phylogeographic studies is that the modern distribution of genetic diversity retains a historical signature with the geographic landscape in which species evolved (Avise 2000; Hewitt 2000; Van Tuinen et al. 2008). The retreat of the ice sheet at the end of the last Pleistocene glaciation as a consequence of quaternary climate change (Bennett 1997) had a strong impact in structuring genetic diversity (Hewitt 1999) and shaping the evolutionary history and phylogeographic structure in most European taxa (Hewitt 1999, 2000; Jaarola and Searle 2004; Piertney et al. 2005). Palaeoecological evidence indicates that most of Northern Eurasia and North America was covered by treeless vegetation during glacial periods and distributions of forest species contracted to refugial areas that were free of ice (Fedorov et al. 2008), or the regions of tundra at the glacial margins (Lunt et al. 1998). Thus, survival of European temperate species during this postglacial period was conditioned by the ability of populations to track favourable habitats (Deffontaine et al. 2009), and as the ice sheet retreated, vacant habitat was gradually re-exposed, allowing populations to colonise new areas, later expanding and shifting their distributions (Hewitt 2000; Piertney et al. 2005).

Several scenarios have been proposed to explain the contemporary patterns of the distribution of genetic structure of postglacial species such as the rate of population expansion, vicariance events and/or refugial isolation (Hewitt 1996; Ibrahim et al. 1996). Most phylogeographic studies have suggested that Southern Europe and its three Mediterranean peninsulas (*i.e.* Balkans, Italy and Iberia) have acted as core areas or glacial refugia for the survival of temperate plants and animals throughout the entire last 2 million years, by offering a way to escape from the prevailing steppic and cold conditions (Deffontaine et al. 2009), although there is evidence that some species survived in refugia to the north and east (see *e.g.* Bilton et al. 1998; Jaarola and Searle 2002; Kotlik et al. 2006). More recently, with the evidence from many European species from Iberia and Italy (*i.e.* Vila et al. 2005; Bella et al. 2007; Terrab et al. 2008), it is now well established that

each Mediterranean peninsula actually provided multiple glacial refugia instead of a single broad one ('refugia within refugia' concept, see Gomez and Lunt 2007 for review).

Rodents offer interesting models to infer phylogeographic history from contemporary patterns of genetic variation as their relatively limited dispersal ability on a large geographical scale preserves the genetic signature of historical events against erosion by substantial gene flow (Fedorov et al. 2008). Despite the large number of studies investigating the phylogeography of widespread rodent taxa (see *e.g.* Conroy and Cook 2000; Haynes et al. 2003; Fink et al. 2004; Piertney et al. 2005; Krystufek et al. 2007; Searle et al. 2009), no attention has been given to the common dormouse *Muscardinus avellanarius*. This species is a cryptic mammal that inhabits areas of deciduous forest with a thick layer of scrub plants and underbrush (Bright et al. 2006). *M. avellanarius* is distributed from the Mediterranean to southern Sweden, eastward to Russia (excluding Iberia) and into parts of northern Asia Minor (Juškaitis 2007; IUCN 2009), and reaches the northwest limit of its European range in the UK, where it can be found over much of southern England and in isolated patches in northern Wales (Bright et al. 2006). The detrimental effects of loss and fragmentation of forest habitat on *M. avellanarius* populations have been highlighted in the UK, where this species has suffered a 64% decline in numbers since the late 1970s (Bright et al. 2006). *M. avellanarius* is now regarded as a "Flagship Species" for nature conservation and a bioindicator of woodland quality (Morris 2004; Bright et al. 2006). The status of *M. avellanarius* in Europe varies, depending on the country (Vilhelmsen 2003; Hofmann 2004; Morris 2004; Foppen et al. 2002) but generally this species is categorised as 'least concern' on the Red List (IUCN 2009). In the UK it is listed as a Schedule 5 species and was placed on the English Nature's Species Recovery Programme in 1992 (Macdonald and Tattersal 2003).

Captive breeding and reintroduction programmes have been developed as part of a conservation effort for common dormice, which are aimed at conserving and protecting common dormice in the UK. By 2008, a total of 635 captive-bred dormice had been released in 16 reintroduction sites across the UK (PTES 2009). Evolutionary Significant Unit (ESU) has been recommended by Moritz (1994) and become a common approach in a reintroduction effort as it can demonstrate the degree of population differentiation and genetic structure (Schwartz 2005). Thus, as there is no recorded data on the evolutionary

genetic structure of common dormice in UK, and in regards to reintroduction, this delineation deemed necessary. To qualify as ESU, a populations should show phylogeographic differentiation for mtDNA variants and significant divergence of allele frequencies (Moritz 1994).

Owing to its high conservation profile, it is surprising that to date, no study on the phylogeography of *M. avellanarius* has been conducted. Indeed, the taxonomy of this species is still disputed, and recently this species has been reclassified into a different subfamily in Gliridae that is Lethiinae and Glirinae (Wahlert et al. 1993; Daams and De Bruijn 1995; Nunome et al. 2007). The oldest fossil of Glirids comes from deposits of early Eocene age (*i.e.* 50 million years ago (Mya); Daams and De Bruijn 1995; Daams 1999), while the oldest fossil record for *Muscardinus* species was found in Spain and dates back 18 Mya (Daams and De Bruijn 1995; Daams 1999). Thus, the phylogeographic study of *M. avellanarius* is crucial to help enlighten insights into the evolutionary complexity and conservation importance of this species. Moreover, mitochondrial DNA (mtDNA) has proven to be a powerful tool for phylogenetic reconstruction (Avise 2000), thus facilitating the inference of demographic and evolutionary history.

In this study I examined mtDNA phylogeography and diversity in *M. avellanarius* from UK, specifically to (1) examine the genetic structure and biogeographical pattern of genetic variation, (2) identify major phylogenetic lineages across the UK population, and (3) compare the pattern of intra-populational mtDNA diversity estimates between regions to identify the effect of the last glaciation on the level and distribution of genetic variation.

6.1 Materials and methods

6.1.1 Sample collection

I utilised 161 samples of common dormice that were collected in 2007 and 2008 from 15 localities distributed across UK, and also 7 samples from Lithuania that were kindly provided by Rimvydas Juškaitis from Vilnius University, Lithuania. These samples were collected at Šakiai district in south-western Lithuania (55°03'N, 23°04'E) (Figure 6.1). All samples are individuals from natural populations except those from Wych, which was established from a mixture of 29 (1996) and 24 (1997) captive breeding individuals (see Chapters 3 and 4). Samples from Lithuania were used as an outgroup. All samples were

screened for sequence variation at parts of the mitochondrial d-loop and cytochrome oxidase I (COI) genes.

6.1.2 DNA extraction, amplification and sequencing

Total genomic DNA was extracted from hair and buccal swab samples using a CHELEX-100 protocol (Walsh et al. 1991). Polymerase chain reaction (PCR) was used to amplify the target region of the d-loop and COI gene in the mtDNA genome of *M. avellanarius*; the 495 bp target region in the d-loop was amplified using the primer pair designed by Stacy et al. (1997) (M15997: 5'-TCCCCACCATCAGCACCCAAAGC-3' and H16401: 5'-TGGGCGGGTTGTTGGTTTCACGG-3'), whilst the primer pair described by Pfunder et al. (2004) (RonM 5'-GGMGCMCCMGATATRGCATTCCC-3' and NancyM 5'CCTGGGAGRATAAGAATATAWACTTC-3') were used to amplify an 490 bp region of the COI. Each PCR reaction mixture contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, either 1.5 or 3.0 mM $MgCl_2$, 5-50 ng template DNA, 10 pmol each primer and sterile distilled water to a final volume of 10 µl per reaction. Amplification consisted of an initial 3 min of denaturation (95°C) followed by 6 cycles of (95°C for 30 s, 54°C for 30 s, 72°C for 45 s), 36 cycles of (92°C for 30 s, 54°C for 30 s, 72°C for 55 s) with a final extension cycle of 10 min at 72°C, and hold temperature at 4°C. PCR products were then purified with ExoSAP-IT (Amersham) following manufacturer's standard protocol. DNA sequencing was performed using a BigDye v1.1, v3.1 terminator (Applied Biosystems) sequencing kit, with approximately 20-30 ng of cleaned PCR products and 1.6 pmol of primer (forward and reverse separately) in each reaction. Sequencing products were cleaned and then electrophoresed on an ABI 3100*xl* capillary sequencer following standard protocols.

6.1.3 Sequence analysis

The program Sequencing Analysis ver. 5.2 (Applied Biosystems) was used to visualise and align all forward and reverse sequences. The resulting consensus sequences for each individual were then aligned using CLUSTAL W ver. 2.0.12 (Thompson et al. 1994) and were manually checked and trimmed in the BIOEDIT ver. 7.0.4 sequence editing program (Hall 1999); alignments were then subsequently revised by eye in an effort to maximize positional homology.

6.1.4 Phylogenetic analysis

The model of DNA substitution that best fitted the data was selected based on Akaike's Information Criterion (AIC) with the program MODELTEST ver. 3.06 (Posada and Crandall 1998), and was subsequently used to calculate pairwise genetic distances among haplotypes. The phylogenetic relationships among haplotypes were reconstructed using neighbour-joining (NJ) methods implemented by PAUP* ver. 4.0b10 (Swofford 2002) assuming the best model of evolution selected by MODELTEST; the reliability of branches was estimated using non-parametric bootstrap re-sampling procedure with 10,000 replicates. The maximum parsimony (MP) method was also used to reconstruct a phylogenetic tree using the heuristic search algorithm and boostrap analysis comprised 1,000 replicates as implemented in MEGA ver. 4.1 (Tamura et al. 2007). Both trees were rooted using the common dormice samples from Lithuania as an outgroup. Using TCS ver. 1.21 (Clement et al. 2000), a 95% set of plausible haplotypes networks of the haplotypes were computed based on statistical parsimony. A median-joining network was also generated for all dormouse haplotypes using the program NETWORK ver. 4.2.0.1 (www.fluxus-engineering.com) as another way of visualizing relationships among haplotypes (Bandelt et al. 1999). Levels of total and net divergence (D_{xy} and D_a respectively; Nei 1987) were calculated between phylogroups as the number of nucleotide substitutions per site using DNASP ver. 4.10 (Rozas et al. 2003).

To test for the reliability of common dormouse from Lithuania as an outgroup, I reconstructed MP tree implemented in MEGA ver. 4.1 (Tamura et al. 2007) for a total of 15 individuals of common dormice from each localities and rooted the tree using edible dormouse (*Glis glis*) as an outgroup. Bootstrap analysis comprised 1000 replicates and using the heuristic search algorithm.

6.1.5 Geographical distribution of genetic variability

The frequency of each haplotype, haplotype diversity (h) (*i.e.* the probability that two randomly selected haplotypes are present in the sample) and the nucleotide diversity (π) within populations and geographical regions was estimated using DNASP ver. 4.10 (Rozas et al. 2003) based on the number of polymorphic sites (S) and the mean number of pairwise differences respectively. To measure the extent of genetic structuring among samples, I performed an analysis of molecular variance (AMOVA; Excoffier et al. 1992) using the

method implemented by the ARLEQUIN ver. 2.0 (Schneider et al. 2000) software. The analysis was performed among regions (corresponding to the observed lineage), within region and among localities within each region.

I also tested for a possible signature of historical demographic expansion by calculating Tajima's D and Fu's F_s statistics (Tajima 1989; Fu 1997) using DNASP ver. 4.10 software (Rozas et al. 2003), and with 1,000 simulations used to evaluate significance. Negative values of these statistics indicate an excess of rare alleles in the geneology, which can be taken as evidence of either a population expansion or for genetic hitchhiking (in response to selection).

6.1.6 Divergence time estimation

The divergence time (T) between phylogroups was estimated as $T=D_a/2\mu$, where 2μ is the divergence rate (see Ramakrishnan and Mountain 2004). I considered the standard divergence rate for mammalian mtDNA of 2% per million years (Myr) which is on the Quaternary timescale and thus should minimise potential overestimation of recent divergence times (Wilson et al. 1985; Avise et al. 1998; Ho et al. 2005).

6.2 Results

6.2.1 Sequence data

Of all dormice samples examined (n=161), DNA from a total of 97 samples (including all seven Lithuanian samples) was successfully extracted to allow PCR-amplification of both d-loop and COI sequences, and which produced 401 base pairs (bp) and 432 bp fragments respectively. There were no insertions, deletions or stop codons present in the sequences obtained. A total of 32 different and unique haplotypes were identified among the 90 UK samples from both the d-loop and COI gene sequences (Tables 6.1a, b), with 46 (11.5%) and 87 (20.1%) polymorphic sites in the d-loop and COI sequences respectively that represented a total of 56 (d-loop) and 125 (COI) mutations. Most of the samples do not share haplotypes, with the exception for samples from Nash Wood and Burfa Wood where haplotypes n13 and n14 (d-loop) and n11 (COI) were shared among populations (Table 6.2). Consistent with the greater number of variable sites in COI compared with the d-loop sequences, the parsimony informative sites was also higher in COI (87 sites; 20.1%) than the d-loop (41 sites, 10.2%) with the overall transition/tranversion bias for COI sequences

calculated to be R=0.485. The forward and reverse sequences of each sample yielded consistent sequences, confirming sequencing errors should not have contributed to the observed spatial variation.

6.2.2 *Phylogenetic analysis*

For the d-loop sequences, the Hasegawa-Kishino-Yano plus Gamma model (HKY+I+G) was chosen as the most appropriate for the data set as determined by MODELTEST, with an unequal base gamma, distributed shape parameter (α) of 0.87 or 1.26 (with or without outgroup). The Tamura-Nei plus Gamma (TrN+G) with gamma distribution shape parameter equal to 0.15 was the best model determined by MODELTEST for COI data set.

The evolutionary relationship among the 32 d-loop and COI haplotypes was first illustrated by the NJ and MP trees. Both the NJ and MP analyses using the d-loop and COI (Figures 6.2a, b; Appendix 6.2) sequence data yielded very similar tree topologies and recovered well-structured trees with two main groups (*i.e.* lineages) that cluster according to the distinct geographic separation of samples - Lithuania and the UK. The UK group was further divided into three lineages that again clustered according to geography, forming Northwestern, Central and Southern lineages (Figures 6.1, 6.2, 6.3). For both the d-loop and COI sequences, the bootstrap support for branch nodes ranged from medium to high (>50% to 100%) in both the NJ and MP trees (despite few clades in MP tree have lower bootstrap support compare to NJ tree). The Northwestern lineage (n=50), comprising the eight geographically distinct populations to the northwest of the UK (Figure 6.1), could be further divided into three (Northwestern A, B and C) geographically separate sub-lineages (see Figures 6.2a, b; Appendix 6.2). Sub-lineage A contained all samples from the North Wales, while sub-lineage B comprised samples that are separated by about 15 km from sub-lineage A (see also Chapter 5). Samples in sub-lineage C are located at English-Welsh border (Figure 6.1). Support for these sub-lineages is moderate for d-loop haplotypes (76%) but only few of the internal branches had low bootstrap support (<60%). There is high bootstrap support in COI haplotypes (82-100%). The Central lineage (n=12) comprised samples from the central (Wyre Forest, Worcestershire) and east England (Bedfordshire and Suffolk). The Southern lineage (n=33) comprises samples from a large geographical area, extending from Somerset to the southwest UK in Cornwall (Figure 6.1), but could be divided further into two sub-lineages that were geographically distinct; South

A comprised samples from Somerset and Dorset and South B contains samples from Devon and Cornwall. The bootstrap support for both sub-lineages was moderate to high in both d-loop (ranged=53-81%) and COI (ranged=69-100%) sequences.

Similarly, the unrooted 95% parsimony haplotype network (Figures 6.3a, b) revealed three main clusters with some sub-division within the Northwestern and Southern lineages. The clades representing central and southern regions are separated by a minimum of five and nine mutational steps from the northwestern region in d-loop and COI gene respectively. The rest of the haplotypes (*i.e.* samples within regions) were less divergent and differed by one, two or three mutational steps only. The median-joining network was also support the division where it shows clearly that the haplotypes from the three regions (Northwestern, Central and Southern regions) are separated to each other by a relatively long branches (Appendix 6.3). The one exception to the strict phylogeographic pattern of samples in UK was the reintroduced population of dormice at Wych, where both the phylogenetic tree and haplotype network indicated that this captive-bred population was derived from the southern region (Figures 6.2a, b; Figures 6.3a, b).

The divergence estimates between phylogenetic lineages is summarised in Table 6.3. Generally, for the d-loop sequence, the phylogeographic lineages between Lithuania and both Northwestern and Central lineages emerged with a net sequence divergence (D_a) of between 1.40% and 1.97%; D_a between Lithuania and the Southern lineage was 1.95%. Divergence estimates were slightly greater for the COI sequence, where the net divergence between Lithuania and all of the UK lineages (Northwestern, Central, Southern lineage) was 2.87%, 2.53% and 3.08%, respectively. D_a between all main lineages within UK was less, with estimates of sequence divergence varying between 0.64-0.90% (d-loop) and between 1.22-1.48% (COI) (Table 6.3).

The preliminary analyses on the status of outgroup shows that common dormice from Lithuania are considerably reliable as outgroup population in this study as evident by the MP tree in which most of the branches have 100% supported by the boostrap analysis (see Appendix 6.4).

6.2.3 *Nucleotide diversity and genetic structure*

Overall, the level of haplotype diversity (h) observed in these dormice samples was high (Table 6.2), with greatest diversity observed in the Lithuanian samples (d-loop, h=0.968±0.103 SD) and (COI, h=1.000±0.000 SD). The haplotype diversity for the three UK phylogenetic lineages for the d-loop and COI sequences was also high (h=0.758-0.945 & h=0.712-0.951 respectively) but decreased with latitude - that is, haplotype diversity was greatest in the Southern lineage, slightly lower in the Central lineage and lowest in the Northwestern lineage at both genes (Table 6.2). For both loci, the nucleotide diversity per site (π) based on the mean number of pairwise differences was highest in the Lithuanian samples (π=0.021±0.000 SD, d-loop; π=0.040±0.000 SD, COI), followed by the UK samples. The same pattern of decreasing haplotype diversity from Southern towards Northwestern lineage was also observed in nucleotide diversity of UK samples; Southern (π=0.014±0.000, d-loop; π=0.031±0.00, COI), Central (π=0.006±0.001, d-loop; π=0.029±0.001, COI) and Northwestern (π=0.004±0.000, d-loop; π=0.012±0.004, COI).

AMOVA showed that the majority of the total mtDNA sequence variation (>87.5% for both gene sequences) occurred among the three main UK regions. Similarly, significant sequence variation could be attributed to differences among localities within each region while the rest of the variation was distributed within localities (Table 6.3). This was further supported by the high degree of sequence similarity that was observed among phylogenetic sub-lineages that resulted in the observed pattern of geographic partitioning among phylogeographic regions (see Figures 6.2 and 6.3).

Over all of these UK populations and for both mitochondrial gene sequences there was an qualitative excess of mutations that were likely either rare or recently derived, as indicated by the negative values of both Tajima's D (D-loop: -0.39748; COI: -0.20493) and Fu's F_s (D-loop: -4.409; COI: -14.580); however, all of the values did not significantly differ from neutral expectations (P>0.10).

6.2.4 *Divergence times*

Applying a 2% divergence rate to the net divergence estimates (D_a) for the d-loop sequences suggests that Lithuania and each of the phylogeographic lineages within UK (*i.e.* Northwestern, Central and Southern) putatively diverged approximately 0.70, 0.99 and 0.98

Mya, respectively, substantially less than the earliest known fossil record of species from this genus (see Daams and De Bruijn 1995; Daams 1999). Greater sequence divergence at the COI increases the estimate to between 1.27 and 1.54 Mya. Corresponding divergence estimates among the three phylogenetic lineages within UK vary from 0.32 Mya up to 0.45 Mya (d-loop) and between 0.55 Mya and 0.74 Mya (COI).

6.3 Discussion

This study has highlighted that common dormice populations in the UK are characterised by a strong geographical subdivision, based on sequence variation at two mtDNA genes that divide UK populations into at least three distinct phylogenetic lineages: (1) Northwestern, (2) Central and (3) Southern lineage. I have also identified strong divergence between UK dormice populations and dormice from Continental Europe (Lithuania) that probably occurred during the Pleistocene age (0.7-1.5 Mya), which predates the last glacial period (~10,000 BP). I found that the reintroduced dormice in Wych derived from the southern region in UK and therefore resulted in the arrival of new unique haplotypes in the northern area. The occurrence of at least three phylogenetic lineages within UK suggests that they should be considered as Evolutionary Significant Units (ESUs). As defined by Dimmick et al. (2001), ESU is an approach to defining units of conservation that emphasizes the identification of adaptive differences between populations.

6.3.1 Divergence of UK common dormouse populations

The strict phylogeographic pattern (*i.e.* no overlapping pattern) as demonstrated by the distribution of haplotypes (Table 6.1), the partitioning of molecular variances (*i.e.* AMOVA, Table 6.4) and the genealogical divergence between three mtDNA phylogenetic lineages in UK (Figures 6.1, 6.2 and 6.3; see also Appendix 6.2 and 6.3) could be taken for the existence of the influence of more than one glacial refugium affecting the evolution of UK dormice (see also Hewitt 2000; Durka et al. 2005). However, confirming this, and more importantly identifying the locations of these areas (*i.e.* recolonisation routes) is not possible due to the lack of appropriate sampling of this species throughout Europe; indeed, to date, there is no information on the origin of common dormice in the UK. However, there is evidence of colonisation of other rodent species into UK from several areas, notably the Iberian Peninsula and/or eastern refugia (Piertney et al. 2005; Searle et al. 2009)

and also from Italian refugia (Zeisset and Beebee 2001). Indeed, most phylogeographical evidence for rodents in Europe demonstrate the importance of southern and eastern refugia (see *e.g.* Ehrich et al. 2000; Brunhoff et al. 2003; Haynes et al. 2003; Jaarola and Searle 2002; Piertney et al. 2005; Mora et al. 2007). Thus, although speculative, it is plausible to postulate that the founder populations of common dormice were from southern and eastern refugia. Sampling from the rest of southern and Eastern Europe would verify the relationship between the populations and therefore more conclusively identify the refugia/refugium for common dormice.

The relative efficiencies of the NJ and MP (as well as the maximum-likelihood method) in obtaining the correct topology for phylogenetic inference were studied by computer simulation (Tateno et al. 1994). The NJ method gives a correct topology even when the distance measures used are not unbiased esimators of nucleotide substitutions, while for the MP method, both the weighted and unweighted parsimony are generally less efficient than the NJ method even in the case where the MP mehod gives a consistent tree (Tateno et al. 1994). However, the NJ and MP analysis in my study returned a similar tree topology (see Figure 6.2 and Appendix 6.2 respectively), as demonstrated by several other studies at different taxa (see *e.g.* Monteiro et al. 2000; Ge et al. 2002; Takezaki and Nei 2008). Thus, in addition to the strict phylogenetic pattern in this study, the same tree topology (although different and low bootstrap values for some of the clades) that demonstrated by both of the phylogenetic trees has support the strong divergent between the UK population, and between the UK and Lithuanian dormice.

Tests for population expansion (originally derives as tests for selective neutrality) indicated a qualitative support for slight population expansion (*i.e.* negative D and F_s), by populations of common dormice in the UK; however, these values were not significantly different from zero, so it appears likely the population size of the species have remained more or less constant, which may be a consequence of the combined effects of this species' decline and the recent conservation afforts to augment populations (possible expansion). Thus, I suggest that the climatic changes and contractions to refugia during Pleistocene, and the changes in distribution of suitable habitats that common dormice populations have experienced during several glacial-interglacial events may have contributed to the mtDNA divergence in common dormice. Additionally, the topography of UK (*i.e.* uplands and

several major rivers) and unsuitable habitat has been considered as effective geographic barriers for many mammals by constituting a significant barrier for migration (Searle et al. 2009). For species such as field voles and shrews, such barriers are considered to be located around the north, west and south of UK, (see Searle et al. 2009) and in Scotland, Wales and southern UK for Eurasian otters *Lutra lutra* (Dallas et al. 2002).

Although deep phylogenetic divergences generally result from historical isolation as a result of geographical or environmental barriers to gene flow and dispersal (Avise 2000), recent study has demonstrated that such phylogeographical breaks can arise in the absence of physical barriers to gene flow, and this is true particularly in taxa that have restricted dispersal (Irwin 2002). For example, common dormice typically undertake only relative short range movements (*i.e.* less than 1 km; see Chapter 4) while longer movements at landscape scale (*i.e.* more than 1 km) do occur, but only rarely (see Chapters 4 and 5). Consequently, during interglacial periods, common dormice are unlikely to be sufficiently mobile enough to expand and mix fully, thus avoiding intermixing of populations that would obscure past phylogeographical structure of this species. This is in contrast to a species such as the wolf (*Canis lupus*), where high dispersal rates have accelerated the decay of historical divergence (Vila et al. 1999). The finding that significant proportions of variance in haplotypes frequency are among populations within lineages (Table 6.4) is also consistent with restricted dispersal in common dormice.

6.3.2 Divergence between UK and Lithuanian common dormouse populations

The relatively deep gene trees (see Figure 6.2; Appendix 6.2) clearly show a large divergence between the UK and Lithuanian common dormouse populations. A previous study of roe deer found that most of the mtDNA haplotypes found in Lithuanian populations were private, demonstrating a very deep genetic divergence compared to other populations of the species (see Lorenzini and Lovari 2006). Other studies, particularly in small mammals also show a large genetic divergence among populations (see *e.g.* Conroy and Cook 2000; Cook et al. 2001; Jaarola and Searle 2004; Krystufek et al. 2007; Krystufek et al. 2009). Although southern Europe clearly served as a very important refugium (Taberlet et al. 1998; Hewitt 1999, 2004), species that are more tolerant to low temperatures may have survived in more northern refugia (Hewitt 2004) and the possible existence of such refugia serves as an alternative to the hypothesis of rapid long-distance

colonisation (Pinceel et al. 2005), particularly for species that have low dispersal ability. Clearly, much more work is required to understand the evolutionary history of *M. avellanarius*.

In addition to the large divergence found in this study, particularly in the COI gene, one potential cause of these large differences is the presence of DNA sequences homologous to mtDNA within the nuclear genome, which refer to as pseudogenes or NUMTs (Lopez et al. 1994; Collura and Stewart 1995; Sunnucks and Hales 1996; Sorenson and Quinn 1998; Bensasson 1999). NUMTs have been reported in a broad range of animal species and because of the similarity between NUMTs' sequences with functional mitochondrial genes, they can be accidentally amplified together with mtDNA amplicons (Bensasson et al. 2001). Alternatively, the pattern of large divergence detected in this study could be due to the multiple copies of mtDNA that present in a single animal resulting from the parental and maternal inheritance of a mitochondrial genome (e.g. Skibinski et al. 1994). However, these are not fully explain for such result. Clearly, more samples are needed to strengthen the results in this study.

6.3.3 mtDNA diversity and divergence time

The high level of genetic diversity in common dormice in the Southern UK lineage suggests relatively large population sizes, compared with other areas (Jaarola and Searle 2002). In contrast, the low levels of genetic diversity observed in the northwestern UK populations are potentially a result of small population size, possibly due to a bottleneck or founder event (Stevens et al. 2007). Interestingly, my mtDNA data exhibit a geographical trend of decreasing within-lineage sequence variation from the Southern towards the Northwestern lineages (Table 6.2), suggesting a consistent pattern with the models of postglacial colonization by successive founder events (Hewitt 1996; Taberlet et al. 1998; Petit et al. 2003; Hewitt 2004). The level of genetic diversity in this study is as high as, or higher than other mammal species studied in the UK (see e.g. Jaarola and Searle 2002; Stanton et al. 2009) and generally across their European range (*e.g.* Haynes et al. 2003; Krystufek et al. 2007; Deffontaine et al. 2009), which is perhaps unexpected given the declining population size and expected loss of genetic diversity.

To determine minimum population divergence times, I used net divergence (D_a; Table 6.3) under the assumption that the ancestral effective population sizes were the average of the current populations sizes (see Edwards and Beerli 2000; Brunhoff et al. 2003). This study indicates that divergence between the Lithuania lineage and the phylogeographic lineages in UK (0.7-1.5 Mya) occurred during the Pleistocene age (1.8 Mya to 10,000 BP; Webb 1990); while the divergence between phylogenetic lineages in UK is more recent (Table 6.3), but still predates the last glacial age; if these data are correct then processes that occurred in the last glaciations have left a strong genetic imprint. Approximately similar estimates of divergence time have been obtained in other rodent species (*i.e.* Conroy and Cook 2000; Jaarola and Searle 2004; Krystufek et al. 2007; Mouline et al. 2008; Brouat et al. 2009). However, this result should be interpreted cautiously as the application of a molecular clock for mtDNA is controversial due to the high variation in the rate of sequence evolution and the high rate of heterogeneity observed among mammalian taxa (Excoffier and Yang 1999; Larizza et al. 2002; Durka et al. 2005).

6.3.4 Evolutionary significant unit (ESU) and reintroduction of common dormice

Genetic data provide a means for assessing the evolutionary distinctiveness of populations of conservation concern, and this concept can be used to identify evolutionary significant units (ESUs) (Waples 1991; Moritz 1994; Crandall et al. 2000), an approach used in the designation and reintroduction of threatened or endangered taxa (Schwartz 2005). The general principle for this identification is to find a population unit that merits separate management due to its reproductive isolation from other populations and its unique adaptations (Waples 1991; Crandall et al. 2000). The three phylogroups of common dormice in this study form a hierarchical set of reciprocally monophyletic units (Figures 6.2a, b; Appendix 6.2), and thus qualify under a relative simple definition of an ESU and this issue, presently ignored, deserves consideration for conservation management.

The identification of ESUs in common dormice has relevance for ongoing programmes of common dormice reintroduction. As the goal of reintroduction efforts should be to reintroduced individuals as closely related genetically, behaviourally and morphologically to the existing population, the best source population is likely one that is within the same ESU (Schwartz 2005). Thus, in common dormice, future reintroduction efforts should take into account the three identified ESUs (Northwestern, Central and Southern phylogenetic

groups). For example, clearly, population from northern UK could not be translocated and reintroduced in southern region in UK. Of course, such view is overly-simplistic as the presence of genetic divergence at one or few genetic markers does not necessarily mean that there are adaptive (i.e. functional) differences that would affect fitness.

An important element for the understanding and successful implementation of a reintroduction of captive breeding program is the extent of adaptive divergence between source populations and whether this should be maintained in the new populations (Ralls and Ballou 1986; Moritz 1999). The release of potentially admixed (the origins of animals used for breeding have not been kept) captive bred founder populations (see Methods in this chapter and also Chapter 3) of common dormice in Wych, with a maternal component that is derived from the Southern lineage in UK has resulted in the arrival of new unique haplotypes (Table 6.1), which could certainly increase the genetic diversity of populations in the region; however, whether this has fitness consequences (*e.g.* minimises the chance of inbreeding depression or even causes outbreeding depression by introducing non-adapted genomes; see Edmands and Timmerman 2003; Edmands 2007; Pertoldi et al. 2007; Hoelzer et al. 2008) clearly requires dedicated further research. At the present, however, these new haplotypes apparently do not influence other populations within northern region (*i.e.* there is no detected shared haplotypes – see Figures 6.2a, b; Figures 6.3a, b). Moreover, the reintroductions are apparently successful in the absence of any genetic management.

The use of molecular genetics can provide powerful tools to aide in the reintroduction and augmentation of wildlife populations by (1) understanding the population structuring of the target species for source selection (Earnhardt 1999; McDougall et al. 2006), (2) confirming one is really doing a reintroduction and not an augmentation (Teixeira et al. 2007), and (3) assisting in captive breeding efforts (Schwartz 2005). Despite the difficulties in setting minimum successful criteria for reintroduction (Kleiman et al. 19994), however with several criteria successful (*i.e.* occurrence of genetic introgression, successful breeding by the first wild-born population; the establishment of a self-sustaining wild population – see Jule et al. 2008) that are generally agreed upon as indicating project success, successful reintroduction have been reported in several taxa (*e.g.* Vandel et al. 2006; Hannon and Hafernik 2007; Bertolero and Besnard 2007; Narum et al. 2007; Jacobson et al. 2008). Most of the animals used in reintroduction were either captive-born or brought into

captivity due to their near extinct status, including common dormice in Wych (Morris 2004; Mitchell-Jones and White 2009). Although the demography data for reintroduced population in Wych shows a gradual increase across years (1996-2008; unpublished data), however, this raise a question on how long this established population could survive in the wild.

6.4 Conclusions

I have presented the first phylogeographic study of the common dormouse, using mtDNA sequence data to demonstrate three clear phylogenetic lineages across much of its distribution in the UK, as evident by both phylogenetic trees and networks. The divergence between these lineages was estimated to have occurred during the Pleistocene period but prior to the last Ice Age. The existence of these three major phylogenetic lineages in UK is concordant with the idea that dormice diverged in multiple refugia during glacial periods, perhaps matching the southern and eastern refugium identified by studies of some other species. However, the limited dispersal ability of this species may have left strong genetic structuring. It appears that there is no evident of population contraction or expansion, so the population size of dormice in UK remained constant. Overall, climatic changes, historical changes and isolation, geography and species' limited vagility and low dispersal ability appear to have had a profound impact on the macro- and micro-geographic genetic divergence in common dormice. This study allows the recognition of at least three ESUs for common dormice in UK, as well as identifying the regional source for captive bred populations of common dormice that were reintroduced in Wych – interestingly, the source does not match the genetic make-up of the northern region. Future work needs to determine the appropriateness of genetic considerations for the conservation management of this species.

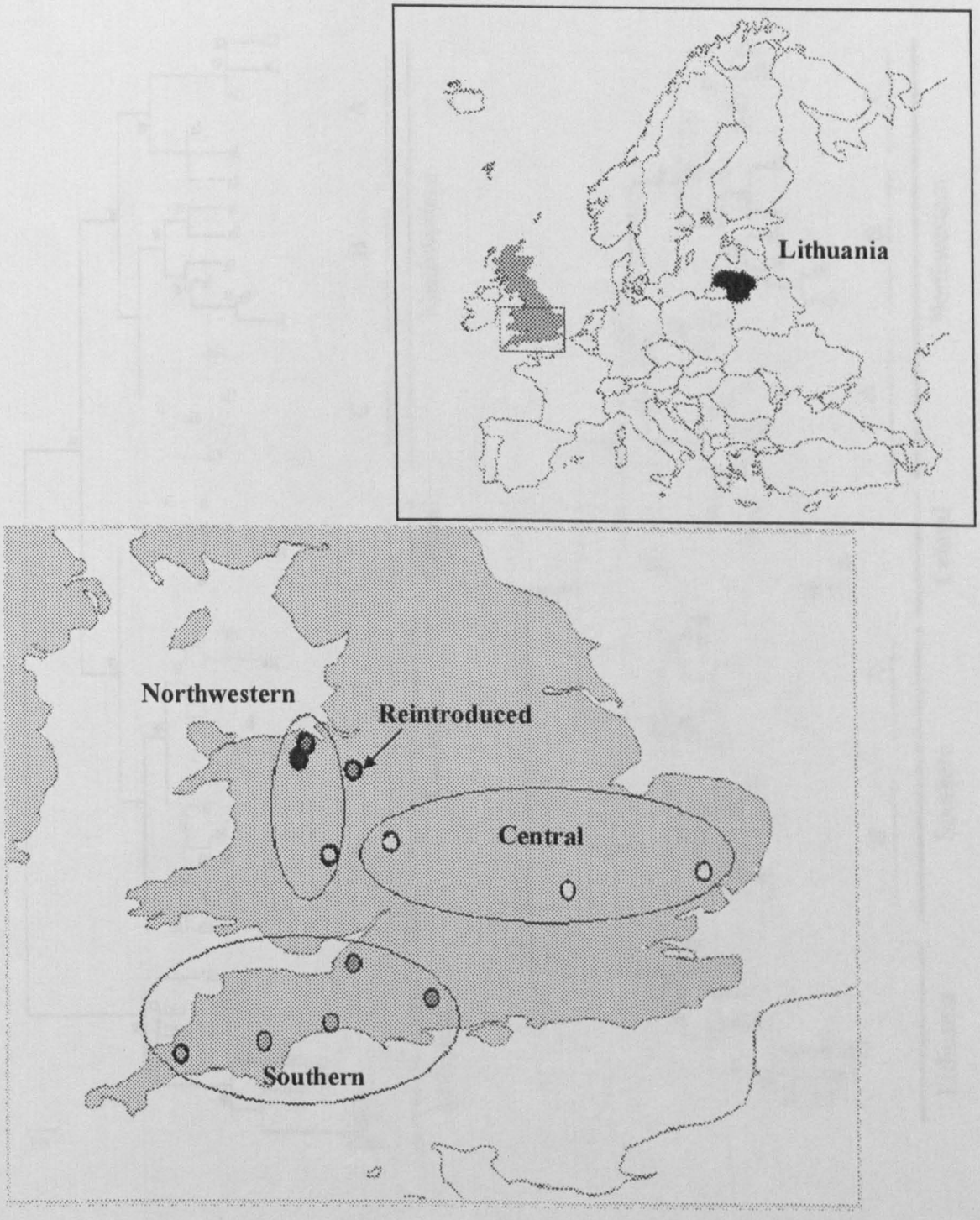


Figure 6.1 Distribution of the locations of dormice samples. (a) Lithuania and the UK; (b) distribution of mtDNA haplotype lineages. The colours equate to the clades identified in Figure 6.2. Circled haplotypes represent the haplotypes from the Northwestern, Central and Southern lineage.

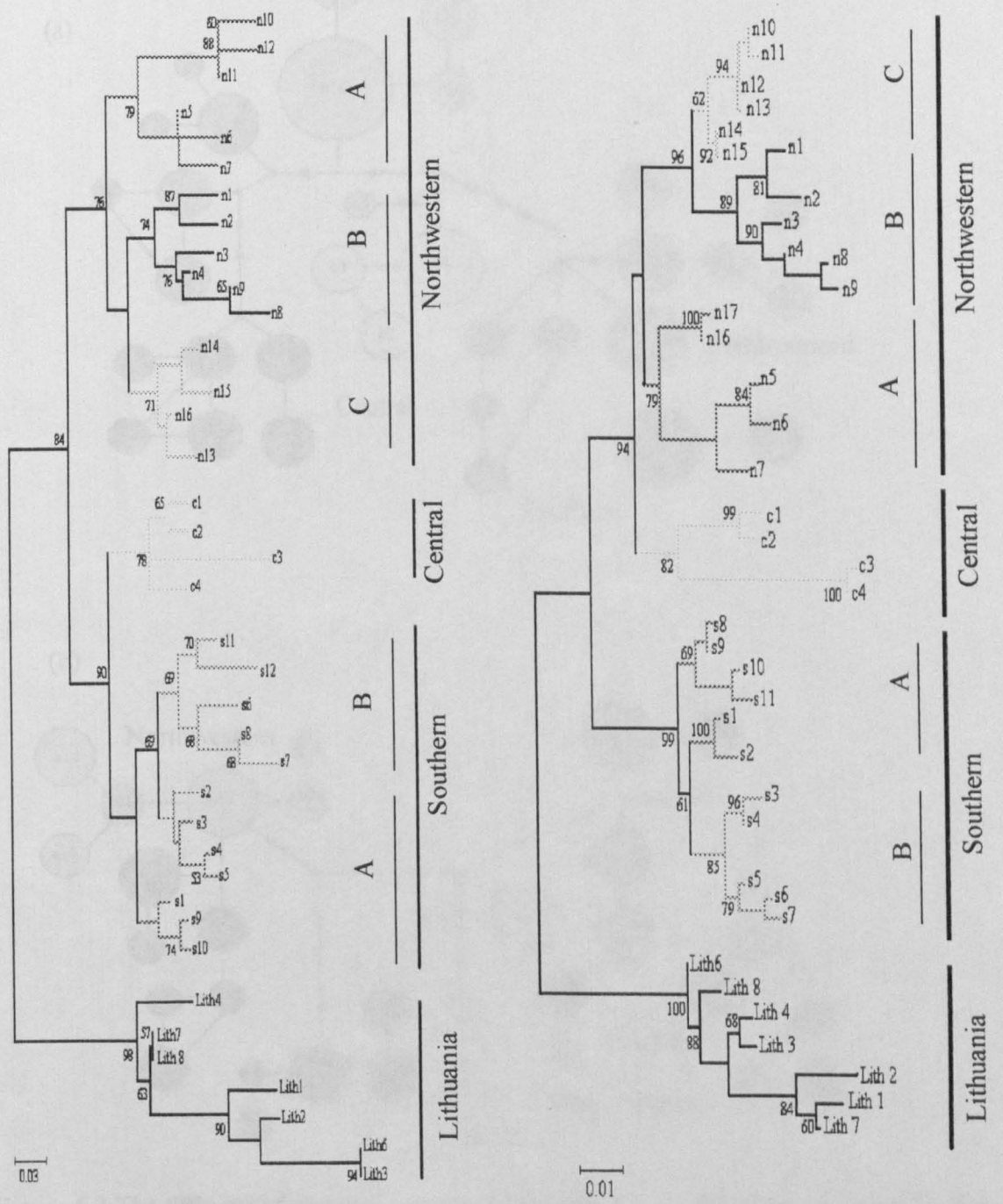


Figure 6.2 Neighbour-joining (NJ) tree illustrating the phylogenetic relationships among the 32 common dormice (a) d-loop and (b) COI haplotypes respectively. Values above and below branches indicate percentage bootstrap support for the NJ tree. Numbers indicate haplotypes localities and correspond to Appendix 6.1. Branch colours correspond to localities in Figure 6.1.

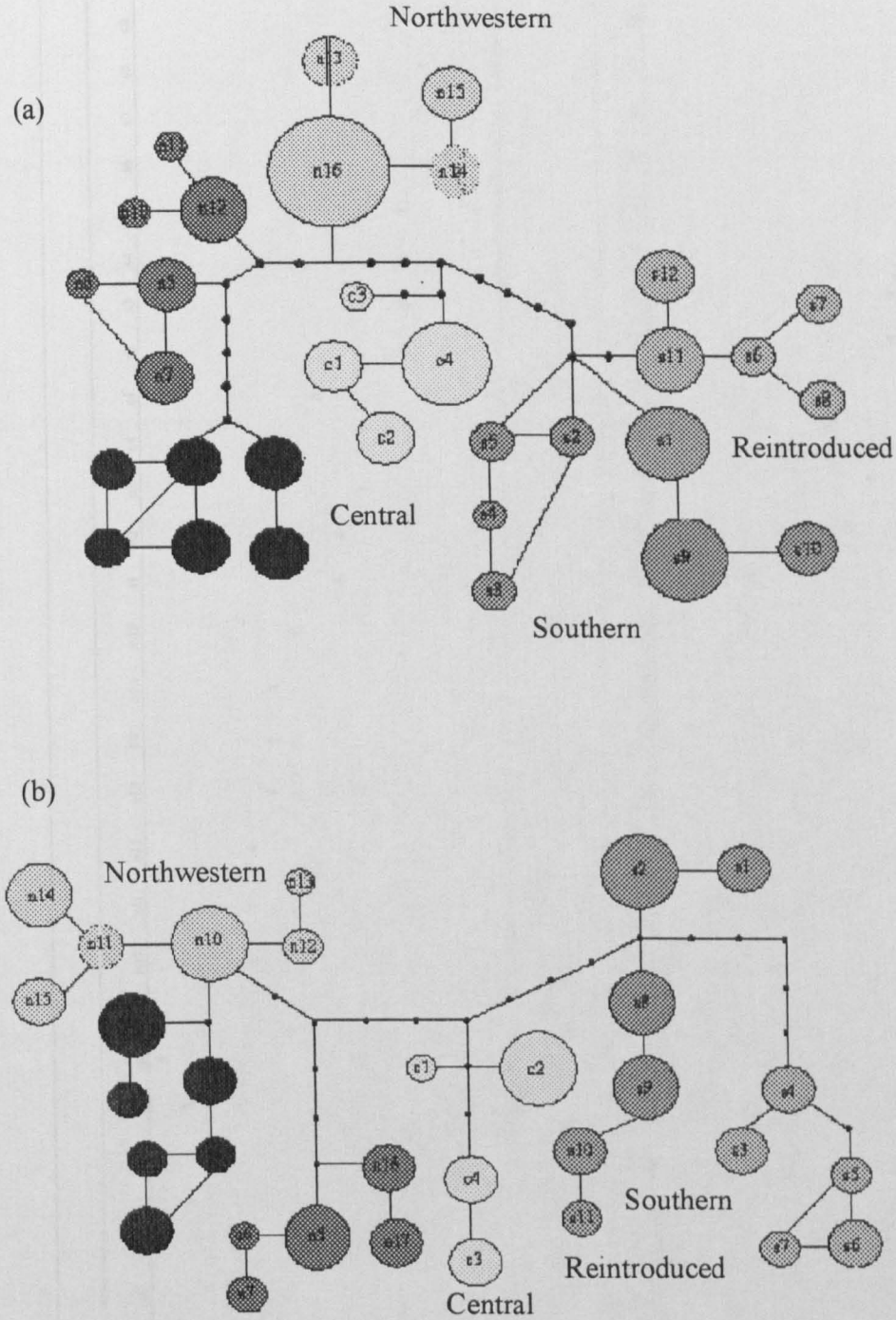


Figure 6.3 The 95% set of plausible unrooted haplotypes networks of *M. avellanarius* from (a) d-loop and (b) COI sequence as calculated by TCS ver. 1.21 (Clement et al. 2000). Each circle represents a unique haplotypes and the numbers indicate haplotypes identification. Proportion of each haplotypes in the different geographic regions is specified as the total number of individuals carrying these haplotypes. The area of the circles denotes the number of individuals that contain that haplotype (see also Table 6.1). Small, black circles represent hypothetical haplotypes not found in the samples separated by one mutational step. Shared haplotypes within subregions are represented by fragmented circle. Colours represent localities as per Figure 6.1.

Table 6.1 Frequencies and distribution of the different and unique mtDNA (a) d-loop and (b) COI haplotypes in 15 localities of *M. avellanarius* in UK.

(a)

			Haplotype																												
Region	Site	*n*	n1	n2	n3	n4	n5	n6	n7	n8	n9	n10	n11	n12	n13	14	n15	n16	c1	c2	c3	c4	s1	s2	s3	s4	s5	s6	s7	s8	s9	s10	s11	s12
North	Bontuchel (BON)	5								3	2																							
	Coed Bron Fadog (CBF)	7					3	1	3																									
	Coed Cooper (CC)	6	3	3																														
	Coed Tre Parc (CTP)	5			3	2																												
	Swan Wood (SW)	6										1	1	4																				
	Nash Wood (NW)	6													2	1	3																	
	Burfa Wood (BW)	10													2	2		6																
	Wych (WYC)	5																					5											
Central	Wyre forest (WF)	6																	3	3														
	Bedfordshire (BED)	1																			1													
	Suffolk (SUF)	5																				5												
South	Somerset (SOM)	7																						2	2	1	2							
	Cornwall (COW)	6																										2	2	2				
	Devon (DEV)	7																															4	3
	Dorset (DOR)	8																													5	3		

(b)

			Haplotype																													
Region	Locality	*n*	n1	n2	n3	n4	n5	n6	n7	n8	n9	n10	n11	n12	n13	n14	n15	n16	n17	c1	c2	c3	c4	s1	s2	s3	s4	s5	s6	s7	s8	s9	s10	s11
North	Bontuchel (BON)	5								2	3																							
	Coed Bron Fadog (CBF)	7					4	1	2																									
	Coed Cooper (CC)	6	2	4																														
	Coed Tre Parc (CTP)	5			3	2																												
	Swan Wood (SW)	6																3	3															
	Nash Wood (NW)	6										5	1																					
	Burfa Wood (BW)	10											1	2	1	4	2																	
	Wych (WYC)	5																															3	2
Central	Wyre forest (WF)	6																				3	3											
	Bedfordshire (BED)	1																		1														
	Suffolk (SUF)	5																			5													
South	Somerset (SOM)	7																						3	4									
	Cornwall (COW)	6																								3	3							
	Devon (DEV)	7																										2	3	2				
	Dorset (DOR)	8																													4	4		

Table 6.2 Indices of mtDNA molecular diversity (at two loci) for samples of the common dormouse *M. avellanarus* from Lithuania and three lineages of *M. avellanarius* in the UK. *n*=sample size, π=nucleotide diversity, *h*=haplotype diversity.

Primer	mtDNA lineage	*n*	Number of haplotypes	$\pi \pm$ SD	*h* ± SD
D-loop	Northern region	48	17	0.004 ± 0.000	0.758 ± 0.081
	Central region	12	5	0.006 ± 0.001	0.926 ± 0.022
	Southern region	33	11	0.014 ± 0.000	0.945 ± 0.013
	Lithuania	7	5	0.021 ± 0.000	0.968 ± 0.103
COI	Northern region	48	18	0.012 ± 0.004	0.712 ± 0.105
	Central region	12	5	0.029 ± 0.001	0.915 ± 0.019
	Southern region	33	10	0.031 ± 0.000	0.951 ± 0.011
	Lithuania	7	7	0.040 ± 0.000	1.000 ± 0.000

Table 6.3 Estimates of raw (D_{xy}; above matrix) and net (D_a; below matrix) divergence between Lithuania and UK regions in numbers of nucleotide substitution per site as calculated following Nei (1987).

(a)	Lithuania	Northwestern	Central	Southern
Lithuania		0.0198	0.0211	0.0201
Northwestern	0.0140		0.0081	0.0097
Central	0.0197	0.0078		0.0071
Southern	0.0195	0.0090	0.0064	

(b)	Lithuania	Northwestern	Central	Southern
Lithuania		0.0293	0.0298	0.0314
Northwestern	0.0253		0.0124	0.0153
Central	0.0287	0.0110		0.0137
Southern	0.0308	0.0148	0.0122	

Table 6.4 Analysis of molecular variance (AMOVA) for *M. avellanarius* in the three regions in the UK. SS=sum of squares; Est. var.=estimated variance;% D=distribution of total variance.

	D-loop				COI			
Source	SS	Est. var.	% D	*P*	SS	Est. var.	% D	*P*
Among regions	95.487	4.734	87.50	<0.0001	69.532	3.945	89.45	<0.0001
Within regions	31.554	1.143	12.50	<0.0005	34.254	1.116	10.55	<0.0005
Northwestern region								
Among localities	29.343	6.153	78.19	<0.0005	41.243	5.476	84.12	<0.0005
Within localities	1794.118	1.904	21.81	>0.05	1422.324	1.399	15.88	>0.05
Central region								
Among localities	32.320	7.904	98.21	<0.01	30.115	8.079	97.02	<0.050
Within localities	767.497	0.153	1.79	>0.05	700.010	0.254	2.98	>0.05
Southern region								
Among localities	38.234	8.254	88.31	<0.050	35.164	7.587	90.32	<0.050
Within localities	967.497	0.079	11.69	0.000	976.587	1.412	9.68	>0.05

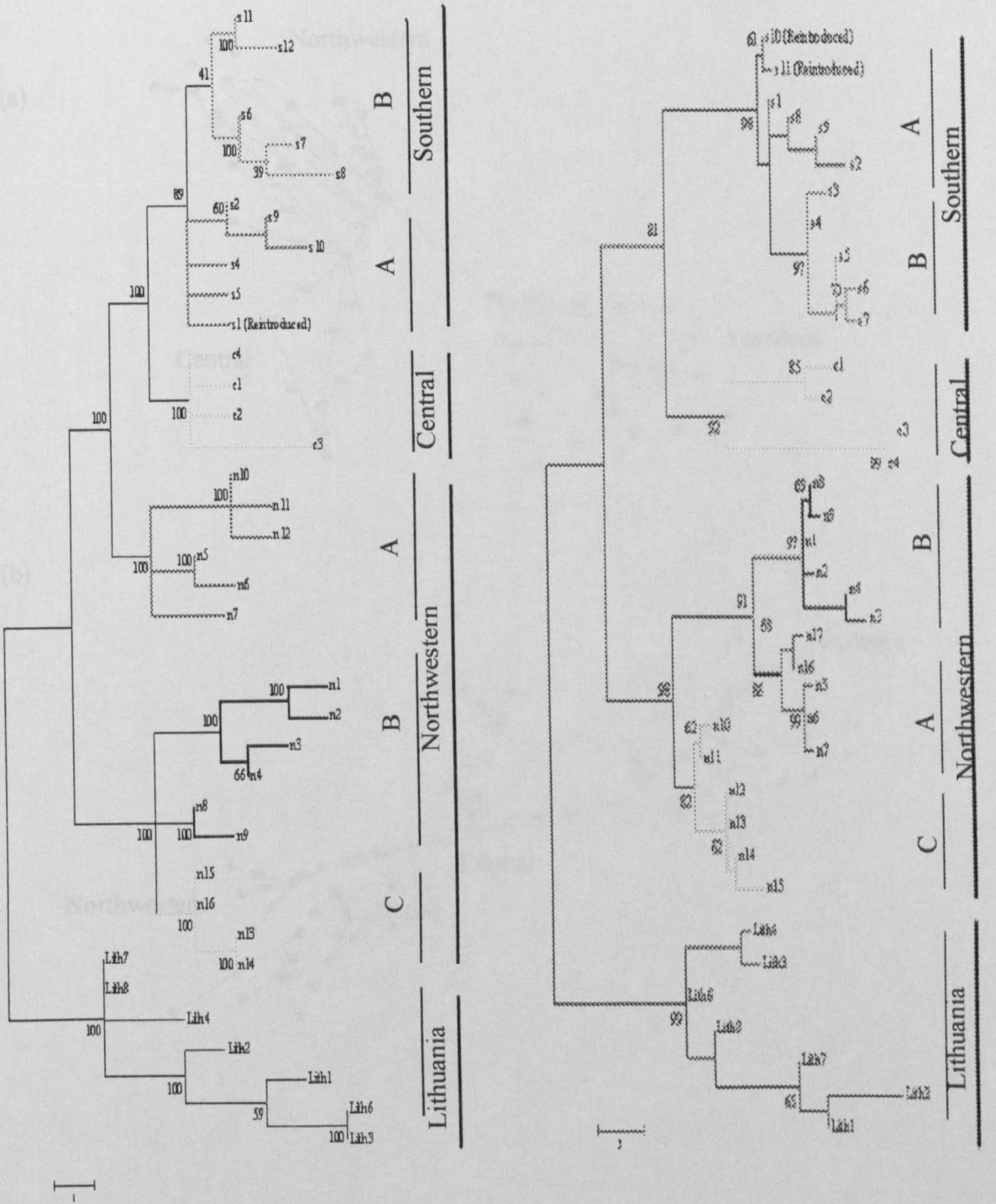
Appendix 6.1 List of samples included in the mtDNA analysis of *M. avellanarius*, haplotypes distribution and coordinate of each haplotypes.

Region	Subregion	Localities	Individual	Haplotype D-loop	Haplotype COI	Coordinate Latitude	Coordinate Longitude
Northwestern	A	Coed Bron Fadog (CBF)	1	n5	n5	53.23531	-3.29293
			2	n5	n5	53.23531	-3.29293
			3	n5	n5	53.23531	-3.29293
			4	n6	n5	53.23531	-3.29293
			5	n7	N6	53.23528	-3.29129
			6	n7	n7	53.23580	-3.29186
			7	n7	n7	53.23528	-3.29271
	A	Swan Wood (SW)	1	n10	n16	53.22709	-3.27398
			2	n11	n16	53.22790	-3.27692
			3	n12	n16	53.22790	-3.27692
			4	n12	n17	53.22740	-3.27622
			5	n12	n17	53.22740	-3.27622
			6	n12	n17	53.22740	-3.27622
	B	Bontuchel	1	n8	n8	53.10145	-3.37041
			2	n8	n8	53.10145	-3.37041
			3	n8	n9	53.10145	-3.37041
			4	n9	n9	53.10145	-3.37041
			5	n9	n9	53.10163	-3.37051
	B	Coed Cooper (CC)	1	n1	n1	53.08250	-3.37196
			2	n1	n1	53.08072	-3.37041
			3	n1	n2	53.08072	-3.37041
			4	n2	n2	53.08072	-3.37041
			5	n2	n2	53.08072	-3.37041
			6	n2	n2	53.08250	-3.37196
	B	Coed Tre Parc (CTP)	1	n3	n3	53.10307	-3.38157
			2	n3	n3	53.10127	-3.38152
			3	n3	n3	53.10127	-3.38152
			4	n4	n4	53.10217	-3.38155
			5	n4	n4	53.10217	-3.38155
	C	Nash Wood (NW)	1	n13	n10	52.25800	-3.02697
			2	n13	n10	52.25800	-3.02697
			3	n14	n10	52.25800	-3.02697
			4	n15	n10	52.25800	-3.02697
			5	n15	n10	52.25800	-3.02697
			6	n15	n11	52.25800	-3.02697
	C	Burfa Wood (BW)	1	n13	n11	52.24252	-3.04858
			2	n13	n12	52.24252	-3.04858
			3	n14	n12	52.24252	-3.04858
			4	n14	n13	52.24252	-3.04858
			5	n16	n14	52.24252	-3.04858
			6	n16	n14	52.24252	-3.04858
			7	n16	n14	52.24252	-3.04858
			8	n16	n14	52.24252	-3.04858
			9	n16	n15	52.24252	-3.04858
			10	n16	n15	52.24252	-3.04858
	Introduced	Wych (WYC)	1	s1	s10	52.99499	-2.77452

Appendix 6.1 *cont.*

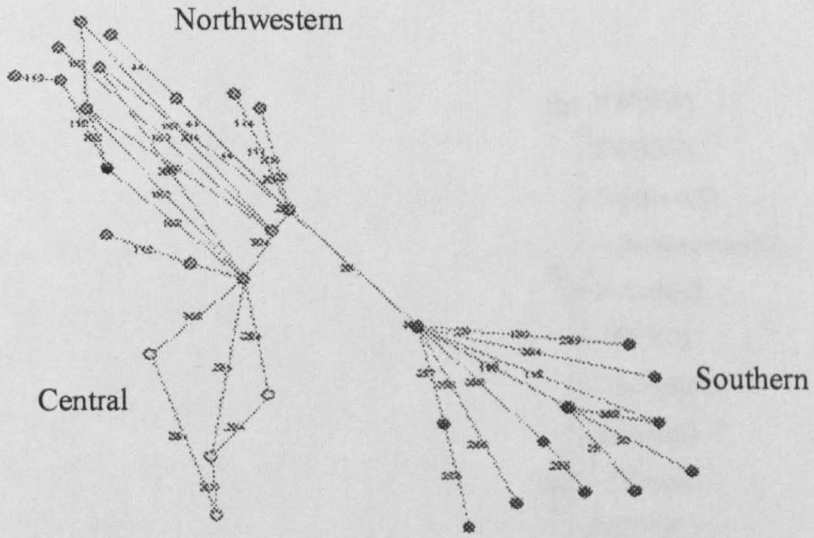
Region	Subregion	Localities	Individual	Haplotype D-loop	Haplotype COI	Coordinate Latitude	Coordinate Longitude
			2	s1	s10	52.99499	-2.77452
			3	s1	s10	52.99877	-2.77299
			4	s1	s11	52.99513	-2.78015
			5	s1	s11	52.99561	-2.78003
Central		Wyre forest (WF)	1	c1	c3	52.36347	-2.37003
			2	c1	c3	52.36347	-2.37003
			3	c1	c3	52.36347	-2.37003
			4	c2	c4	52.36347	-2.37003
			5	c2	c4	52.36347	-2.37003
			6	c2	c4	52.36347	-2.37003
		Bedfordshire (BED)	1	c3	c1	51.95654	-0.45049
		Suffolk (SUF)	1	c4	c2	52.12730	1.03146
			2	c4	c2	52.12730	1.03146
			3	c4	c2	52.12730	1.03146
			4	c4	c2	52.12730	1.03146
			5	c4	c2	52.12730	1.03146
South	A	Somerset (SOM)	1	s2	s1	51.29688	-2.78736
			2	s2	s1	51.29688	-2.78736
			3	s3	s1	51.29688	-2.78736
			4	s3	s2	51.29688	-2.78736
			5	s4	s2	51.29688	-2.78736
			6	s5	s2	51.29688	-2.78736
			7	s5	s2	51.29688	-2.78736
	A	Dorset (DOR)	1	s9	s8	50.99378	-1.94438
			2	s9	s8	50.99378	-1.94438
			3	s9	s8	50.99378	-1.94438
			4	s9	s8	50.99378	-1.94438
			5	s9	s9	50.99378	-1.94438
			6	s10	s9	50.99378	-1.94438
			7	s10	s9	50.99378	-1.94438
			8	s10	s9	50.99378	-1.94438
	B	Cornwall (COW)	1	S6	s3	50.50130	-4.67916
			2	S6	s3	50.50327	-4.67644
			3	S7	s3	50.50168	-4.67931
			4	S7	s4	50.49925	-4.68301
			5	S8	s4	50.50353	-4.67535
			6	S8	s4	50.50353	-4.67535
	B	Devon (DEV)	1	s11	s5	50.77693	-3.04098
			2	s11	s5	50.60896	-3.76937
			3	s11	s6	50.60896	-3.76937
			4	s11	s6	50.60896	-3.76937
			5	s12	s6	50.60896	-3.76937
			6	s12	s7	50.60896	-3.76937
			7	s12	s7	50.60896	-3.76937

Appendix 6.2 Maximum parsimony (MP) tree illustrating the phylogenetic relationships among the 32 common dormice (a) d-loop and (b) COI haplotypes respectively. Values above and below branches indicate percentage bootstrap support for the MP tree. Numbers indicate haplotypes localities and correspond to Appendix 6.1. Branch colours correspond to localities in Figure 6.1.

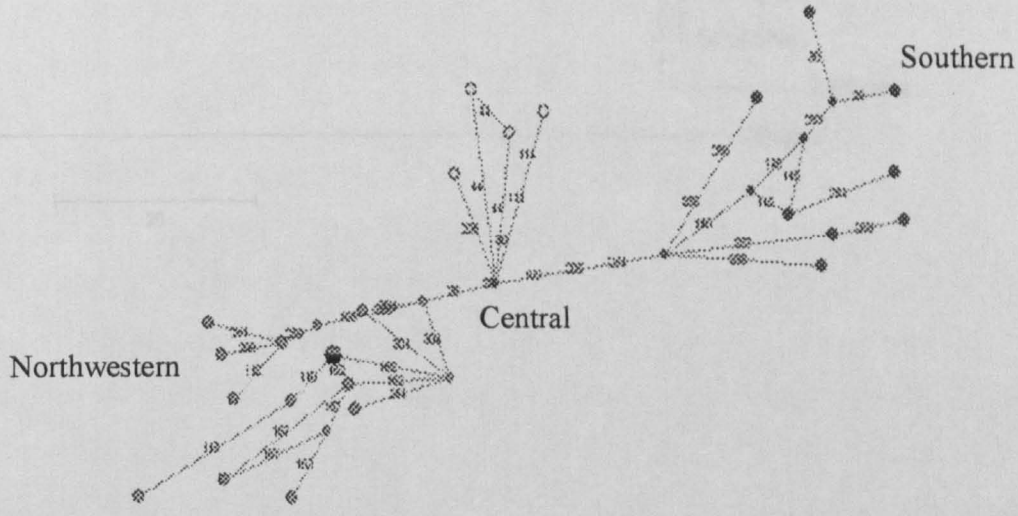


Appendix 6.3 Median-joining networks for the (a) d-loop and (b) COI mtDNA haplotypes of *M. avellanarius*. The numbers on the connecting line determine the number of mutated position estimated by NETWORK ver. 4.1.1. Colours of the circles presented haplotypes in separate regions; blue (Northwestern), yellow (Central) and red (South). Shared haplotypes within subregions are represented by fragmented circle.

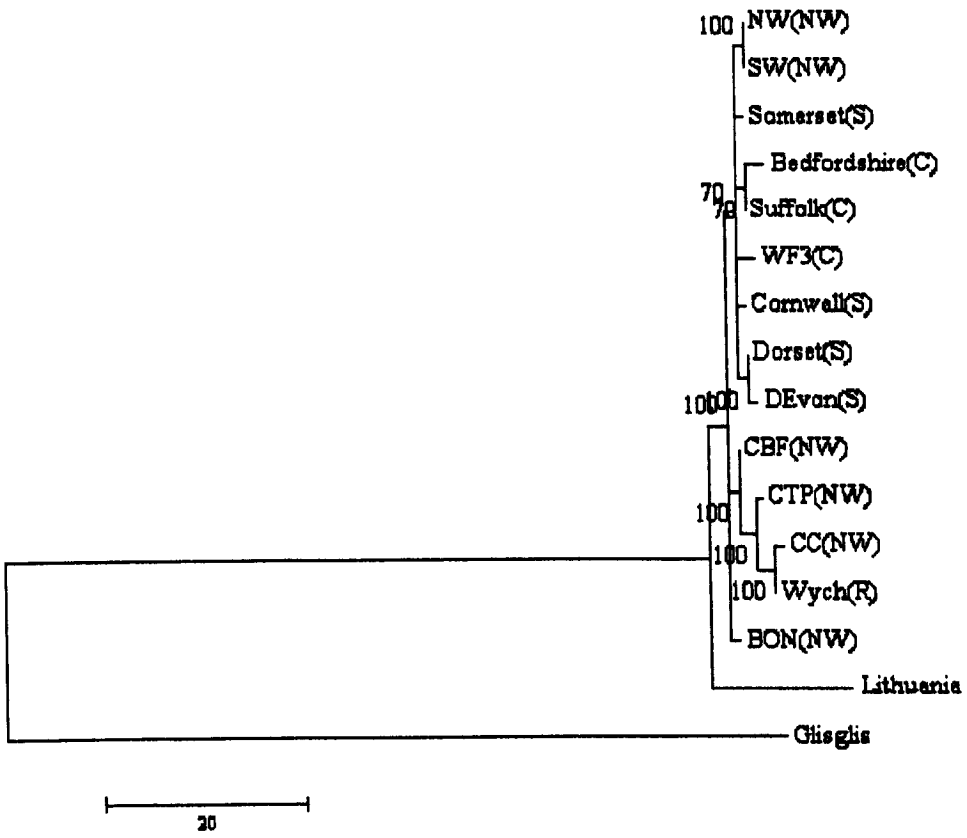
(a)



(b)



Appendix 6.4 Maximum parsimony (MP) tree illustrating the phylogenetic relationships among the 15 individuals of common dormice d-loop sequences from the Southern (S), Central (C), Northwestern (NW), reintroduced (R) dormouse, including individual from Lithuania, and the position of edible dormouse (*Glis glis*) as an outgroup. Values above and below branches indicate percentage bootstrap support for the MP tree.



Chapter 7: Overview and general discussion

7.0 Introduction

This thesis provides the first comprehensive study of spatial and temporal patterns of genetic diversity of populations of the common dormouse, *Muscardinus avellanarius*, in UK, with specific reference to investigate the breeding structure and patterns of gene flow, both in natural and reintroduced populations. Furthermore, this thesis clearly exemplified that microsatellite DNA markers can be extremely informative in determining how a behavioural trait may act to shape the current structure of genetic variation within and among populations of *M. avellanarius*. Additionally, this thesis used patterns of variation at two regions of mitochondrial DNA (mtDNA) to characterize broader geographic divergence among UK populations of *M. avellanarius*. The results of these studies will not only contribute to the understanding of dispersal characteristics and how this process structured the population at a small and large study scales, but also add significantly to our biological and evolutionary understanding on *M. avellanarius* and this can be directly applied to the ongoing conservation and management of this species.

7.1 Chapter overviews and future directions

7.1.1 Chapter 3: Prevalence of multiple mating by female common dormice (Muscardinus avellanarius) in natural and reintroduced populations.

Both manual and statistical methods used to infer parentage in *M. avellanarius* have revealed a prevalence of multiple mating by females with a high proportion (42-76 %) of litters sired by multiple males. Interestingly, these estimates are among the highest reported in such studies particularly in rodents (*e.g.* Dean et al. 2006; Kennis et al. 2008; Borkowska et al. 2009), suggesting that most females may be able to monopolise resources critical to reproduction probably because of lack of territoriality by males, raising the question as to what costs or benefits are associated with this behaviour. A striking result in this study is that multiple mating is also adopted by released animals even though enforced monogamy has been practiced in *ex situ* captive bred programme, making this study the first to report the similarity of mating behaviour in wild and reintroduced populations.

Common dormice typically form a crèche and exhibit communal nesting behaviour and remain in their natal group during the breeding season, whilst finding mating opportunities in neighbouring groups. The apparent of such behaviour is very interesting as this probably could maximize the evolutionary fitness of offspring (Hamilton 1964; Maynard Smith 1964). While numerous investigations have implicated that multiple paternity improved offspring fitness (see *e.g.* Tregenza and Wedell 1998; García and Simmon 2005; Fisher et al. 2006; Klemme et al. 2006, 2007), however, this was not possible in this study due to the lack of information on offsprings' lifetime reproductive performance (see Klemme et al. 2008). This examination of offsprings' lifetime reproductive success would have allowed the characterization of offspring that have single or multiple sired. This could therefore be an area of further study with the future monitoring expected to continue for some time.

An additional area for study could be that of examining the extent of female choice in driving mating behaviour. In this chapter, there is perhaps some evidence that male *M. avellanarius* prefer larger females for mating (Appendix 3.3 in Chapter 3). However, the observed relationship (refer to Appendix 3.3 in Chapter 3) could be due to the fact that large females have larger litters in this study, thus increasing the chances for mating. The positive correlation between body weight and reproductive success is common in small mammals, particularly in rodents (*e.g.* Wauters and Dhondt 1989; Schulte-Hostedde et al. 2002; Holt et al. 2004). However, a burgeoning literature has shown that female mate choice also has a significant impact on mating behaviour (see *e.g.* Manno et al. 2008; Byers and Kroodsma et al. 2009; Gershman 2009; Kozakiewicz et al. 2009a) – thus a clear future direction is to clarify the extent of male versus female choice in mating behaviour. For example, in this chapter, even though females in both study sites (Bontuchel and Wych) apparently show a preference towards unidentified males, this result however may not represent the actual scenario due to the high proportion of males that successfully sired offspring were not present in my samples. Further studies to augment the sample size along with more detailed behavioural analyses (*e.g.* automatic records of chipped animals entering nestboxes) would represent an effective method of further exploring this finding.

The Major Histocompatibility Complex (MHC) genes are well suited particularly to the study of mate choice (Penn and Potts 1999; Piertney and Oliver 2006). MHC has been implicated as a possible source of individual specific body odours in mice and humans,

provideing the basis for individual MHC profile recognition (Penn and Potts 1999; Pfau et al. 2001; Sommer et al. 2002; Stockley 2003; Sommer 2005). By a combination of different MHC profiles in different mates, it is widely thought that an individual can provide a progeny with the best immune response (Gillingham et al. 2009), thus enhancing offspring fitness and survival. Thus, analysis of MHC gene diversity would be the next step to examine mate choice in *M. avellanarius*.

7.1.2 Chapter 4: Dispersal characteristics of natural and reintroduced populations of the common dormouse (*Muscardinus avellanarius*).

Understanding the evolutionary causes of dispersal pattern in *M. avellanarius* is crucial for the conservation of the species, particularly when the dispersal differ between genders, as it can give insight into better management strategies. In this study, both direct (*i.e.* tagging) and indirect (*i.e.* genetic) methods in quantifying dispersal shows that male *M. avellanarius* tend to move further (up to 600 m) than females – and this is consistent with general pattern of sex-biased dispersal in mammalian species. However, because this result only covered a relatively small habitat patch (approximately 2.5 km long) there is a potential for sampling bias (for example, overlooking extremely long-distance dispersal by females) and this raises the question as to whether the dispersal characteristics of *M. avellanarius* at larger scales (and in particular between isolated habitat patches) will demonstrate the same pattern (see Chapter 5). Moreover, recently, there was evidence that *M. avellanarius* crossing a dual carriage way in Cornwall (available at http://news.bbc.co.uk/1/hi/england/cornwall/8172460.stm), further support the relevance study in Chapter 5.

Another major finding in this study was that genetic evidence revealed the occurrence of sex-biased dispersal in *M. avellanarius* with females philopatric and males mobile - certainly at a local scale. Accordingly, gene flow within habitat patches is mediated largely by male dispersal. I proposed that male dispersal was driven by inbreeding avoidance and kin competition (*e.g.* see Gauffre et al. 2009), although there is clearly a large amount of further work required to examine this hypothesis. In contrast, besides factors such as resource abundance and distribution, philopatry in *M. avellanarius* was highly linked with communal-nesting behaviour exhibited by females during post-mating and they may use a crèche to support their young. However, this raises a question as to whether female mate-choice drives the evolution of male-biased dispersal in *M. avellanarius* (see Honer et al.

2007; Perez-Gonzalez and Carranza 2009). The restricted dispersal and philopatry have contributed to the fine-scale spatial genetic structure (a pattern of isolation-by-distance, IBD) in less than 1 km scale in *M. avellanarius*, both in wild and reintroduced population. This tendency for short movement behaviour has important implications for the conservation of the species as it could increase the vulnerability of populations to extinction, particularly when the habitat becomes increasingly fragmented (Lawes et al. 2000).

Interestingly, I found a similar estimates of effective population size N_e at both wild (Bontuchel) and reintroduced (Wych) sites, despite reduced genetic diversity being observed in Wych. This, together with the absence of genetic bottleneck (Appendix 4.1) clearly shows that reintroductions of *M. avellanarius* in Wych have been apparently successful. Demographic data reported by the Northwest Dormouse Partnership (NWDP) has confirmed this finding (NWDP unpubl. data). However, this single generation N_e estimate is not reliable due to lack precision and additional genetic samples from several generations in the future should prove more reliable (*e.g.* see Frankham 1995; Wang 2005; Waples 2006). Thus, further study with more adult samples and using more precise estimators of N_e would provide insights into the relative influences of demographic factors that determine the successful breeding population. In addition, the greater length of time post-reintroduction would allow a more accurate appraisal of the long-term viability of the reintroduced population.

7.1.3 Chapter 5: Population genetic structure of common dormouse (*Muscardinus avellanarius*)

My analysis of gene flow and the pattern of population genetic structure was expanded in Chapter 5 to quantify the level of dispersal at larger scale, among isolated habitat patches, in order to describe geographical barriers to gene flow. This study focused on population of *M. avellanarius* in North Wales which is divided into two different study sites, namely Bontuchel and Afonwen as they were separated by some 15 km and it is evident that they are not connected. Bontuchel comprises populations that inhabit large continuous habitat (BON) and several patchy populations, meanwhile all populations in Afonwen are treated as discrete populations. The most important findings of this chapter are: (1) autocorrelation analysis suggests that gene flow was restricted within and also among populations (*i.e.*

between habitat patches) at a scale of less than 1 km, and Bayesian analysis (STRUCTURE) detected the occurrence of a sharp genetic division among populations in Bontuchel, but no significant differentiation was apparent among the samples in Afonwen, (2) fragmented populations in Afonwen were less genetically diverse compared with populations in Bontuchel, indicating that they are smaller. I also found a strong significant isolation-by-distance pattern at a fine-scale (less than 1 km) within continuous habitat (BON) and between habitat patches (Bontuchel except BON, and Afonwen). Male-biased dispersal could be interpreted by the male-biased proportion of immigrants in samples, but statistic shows that this proportion is not significant. I suggest that intense small-scale dispersal within continuous habitat and very low inter-patch movement of *M. avellanarius* between habitat patches is associated with social structure and restricted dispersal in this species rather than the observed physical barriers.

Population genetic theory predicts that in the absence of gene flow, small populations will lose genetic diversity at a rate proportional to their effective population size (Slatkin 1985; Hastings and Harrison 1994; Frankham et al. 2002; Lenormand 2002). Through the combination effects of erosion of genetic diversity (because of small effective population sizes) in small habitat patches and without immigration to introduce genetic diversity, the fragmented area, and particularly the smaller fragments, is potentially on an extinction risk (Newmark 1995; Turner 1996; Turner and Corlett 1996; Kuussaari et al. 2009; Nicholson et al. 2009). The effect of smaller fragment on genetic diversity on common dormice could be found in this study where I detected reduced heterogeneity in patchy populations in Afonwen compare to populations in Bontuchel (see Table 5.2 in Chapter 5). However, this result could be an artifact of a small sample size within this region.

Individuals in isolated populations that are separated by inhospitable matrix may be reluctant to disperse (see *e.g.* Banks et al. 2005; Blaum and Wichmann 2007; Rizkala and Swihart 2007), leading to increased relatedness (Sunnucks 2004) and disruption of sex-biased dispersal as well as altered mate choice decisions (Banks et al. 2007; Fromhage et al. 2009). Surprisingly, I detected immigrants in Bontuchel wood but due to limited sampling data, the specific source of immigrants could not be identified. The detection of immigrants implies the possibility of movement of *M. avellanarius* between habitat patches. However, the overall frequency of migration was very low. Moreover, the autocorrelation analysis

pattern shows only slight difference between the patchy populations (within Afonwen and Bontuchel) and continuous population in Bontuchel wood, suggesting the occurrence of very low inter-patch movement. This, together with reduced densities in small fragments is likely to reduce mating chances and probability for mate choice. Thus, estimation of fitness (*e.g.* litter size) in *M. avellanarius* is needed to determine if inbreeding depression could occur within population.

Given the short movement of *M. avellanarius* (see Chapter 4), the apparent low inter-patch movement gives a new knowledge that *M. avellanarius* is less susceptible to habitat fragmentation than what I previously thought. However, this pattern of movement is considered as an occasional long range movement in *M. avellanarius*. This also implies the possibility of utilisation of habitat corridor by *M. avellanarius* as a pathway of movement between habitats. Future work needs to expand the sampling to obtain more robust estimates of genetic structure and identify the source populations.

7.1.4 *Phylogeography and mitochondrial DNA (mtDNA) diversity of common dormouse* (Muscardinus avellanarius) *in the UK.*

In this final results chapter, I evaluated how historical processes have influenced the distribution of genetic variation in *M. avellanarius* in UK and proposed how evolutionary forces (*i.e.* gene flow), behaviour (i.e. mating system, dispersal) and environmental factors (*i.e.* topography) may have been responsible in the population divergence in *M. avellanarius*. My data contribute to understanding the complex nature of glacial refugia and how the evolutionary processes associated with such events could have impacted *M. avellanarius*.

An especially important discovery in this study was the division of *M. avellanarius* in UK into three highly divergent allopatric phylogenetic clusters, namely Northwestern, Central and Southern lineages, which has never been studied using molecular genetic data or non-genetic method previously on this species. The genealogical evidence suggests that colonization of UK from at least two refugia (*i.e.* southern and eastern refugia). This has important conservation implications as the results clearly indicate that conservation of populations from all of the observed clusters is warranted.

Decreased genetic diversity from Southern towards the Northwestern lineage was observed and this is consistent with postglacial colonization by successive founder events. Interestingly, genealogical evidence revealed that the source populations of captive bred *M. avellanarius* that released in Wych are from the southern UK. The arrival of unique haplotypes in Wych apparently not influences the haplotypes distribution in other region in UK due to the absence of between haplotypes intermixing. However, this result should be treated cautiously as we are not attempt to determine the haplotypes distribution of resident *M. avellanarius* in Wych (if any). However, if this is true, the reintroduced population of common dormice in Wych might represent a potentially high value and highly diverse candidate for sourcing founders for future reintroduction programme. The next step is to conform these finding with nuclear markers and to characterise the pattern of genetic structure at the other introduced sites. Such additional information would help to identify the origins of the animals used for captive breeding. Future work could then attempt to correlate whether genetic background (*i.e.* the lineage) used for reintroduction plays a role in the success of the reintroduction programme; at present we do not know whether the observed phylogeographic genetic differentiation has a functional genetic component.

According to Waples (1991), the Evolutionary Significant Unit (ESU) can be defined as a population or population group with historical isolation and adaptive distinction from other populations within the species, and therefore representing a significant portion of the evolutionary legacy of the species. This approach is typically the first approach for reintroduction of a species (Schwartz 2005). For a population to qualify as an ESU (Waples 1991), several criteria should be satisfied: (1) it must substantially reproductively isolated from other conspecific populations units, and (2) it must represent an important component of the evolutionary legacy of the species. The identification of ESUs in common dormice has relevance for ongoing programmes of common dormice reintroduction. Having satisfied all these criteria, I detected at least three ESUs of *M. avellanarius* in UK, that is the Northwestern, Central and Southern groups. Future reintroduction efforts should take into account these three identified ESUs. For example, clearly, population from northern UK could not be translocated and reintroduced in southern region in UK as reintroduced animal should be genetically and behaviourally similar to that existed populations. However, more samples are needed to possibly identify another ESU across *M. avellanarius* range in UK and also in Europe.

7.2 Conservation of *M. avellanarius* and management recommendations

Muscardinus avellanarius is a useful model for conservation biology and conservation genetics especially with regard to the effect of habitat fragmentation this species are an excellent woodland indicator for biodiversity. Over the last century, this species has experienced a relatively severe reduction in population numbers due to anthropogenic factors that have resulted in fragmentation and loss of suitable habitat (Morris 2004; Bright et al. 2006). Accordingly, *M. avellanarius* categorised as 'least concern' on the Red List (IUCN 2009) and much effort has been placed towards an extensive programme of habitat restoration and population reintroduction – somewhat surprisingly, given the many studies based on molecular ecological techniques, none of these procedures have utilised any basic genetic information to inform conservation management practice.

The most crucial management priorities for the long-term viability of *M. avellanarius* populations are to increase, or at least sustain the present level of genetic diversity (see Chapters 3, 4, 5 and 6) within population by increasing connectivity of existing populations to facilitate gene flow, increase local population sizes and to increase the quantity of available high quality habitat. These objectives could be achieved by linking existing isolated habitat fragments using wildlife corridors. In Bontuchel, connectivity studies have been undertaken in 2007 to assess the quality of wildlife corridor for *M. avellanarius* existing between Bontuchel woodland and surrounding remnant habitats. Preliminary results suggest that *M. avellanarius* observed in the surrounding woodlands are separate populations from the main Bontuchel woodland and this is mostly due to the lack of connections between these habitat patches (Jones 2007). Having realised the importance of habitat corridor for movement of *M. avellanarius*, People Trust of Endangered Species (PTES) has launched the *Reconnecting the Countryside* competition to encourage farmers and landowners to carry out active conservation work that will connect up or create areas of woody habitat beneficial to *M. avellanarius* and to other wildlife.

However, while the utility of habitat corridors to provide additional habitat is encouraging and widely recognised (see *e.g.* Brinkerhoff et al. 2005; Stenberg and Judd 2006; Lees and Peres 2008; Clarke and White 2008), several authors (*e.g.* Bennett 1999; Horskins 2005) however, argued the function of habitat corridor in facilitating connectivity among remnant fragments and experimental evidence of the success of corridors in reducing population

extinction rate and maintaining genetic diversity is rare (Forney and Gilpin 1989; Bolger et al. 2001). For example, a semi-natural riparian corridor that linking two rainforests fragments has successfully provided habitat for the giant white-tailed rat *Uromys caudimaculatus*, but it was functionally failed as a gene flow corridor due to high social factors that restricted gene flow (Horskins 2005). Thus, in the of *M. avellanarius*, a high quality dispersal habitat (*i.e.* food resources) is crucial to maintain gene flow between fragmented habitat patches.

An understanding of the organismal units involved in conservation studies is often critical to the proper implementation of captive breeding and reintroduction programs (Amato 1994, Amato et al. 1995; Wyner et al. 1999). Several authors have suggested that captive breeding that designed specifically for a reintroduction effort should have some additional aspects than a more general captive breeding programme (Snyder et al. 1996), including utilizing single-species facilities in range countries to reduce the likelihood of disease transmission, specific management that encourages more natural behaviour, and a consideration of the genetic introgression at the subspecific/evolutionary significant units (ESUs) (Moritz 1994) hierarchical level (Wyner et al. 1999). Thus, in a reintroduction programme, particularly one that designed for population reinforcement as in the case of *M. avellanarius*, a greater emphasis might be put on having the captive population more carefully match the specific local genetic type in order to allay fears of outbreeding that may result in reduced fitness (Templeton 1986; Lynch 1996 and see Chapter 6).

7.3 Concluding remarks

This study has shown that the combination of ecological and genetic data have provided new insight into biology and genetic variations of *M. avellanarius* in UK. Both ecological and molecular genetic data strongly congruent in determine the population genetic structure of *M. avellanarius* within and among populations. Through out all of the chapters, reproductive behaviour and dispersal characteristic of reintroduced *M. avellanarius* population was retained as in wild population as an enforced bottleneck could change behaviour of a species.

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