Population Structure and Warfarin Resistance in the Brown Rat, *Rattus norvegicus* in the English Countryside

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by

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Declaration

I hereby declare that no part of this thesis has been previously submitted to this or other University as part of the requirements for a higher degree. The content of this thesis is the result of my own work unless otherwise acknowledged in the text or by reference.

The work was conducted in the Department of Biology, University of Leicester during the period July 2004 to November 2007.

Signed.....

Haniza Hanim Mohd. Zain, November 2007.

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Abstract

The brown rat, *Rattus norvegicus* is a relatively recent addition to the English fauna. However it is currently one of the most important vertebrate pest species. Anticoagulant rodenticides are the major control method used for the last 50 years. Widespread use of the rodenticide has led to evolution of resistance leading to problems controlling brown rat populations. One thus far ignored aspect of resistance biology is the population structure of the brown rat. In this research mitochondrial DNA and microsatellite DNA were used to elucidate the population structure of the brown rat at different scales in the English countryside. I also report on the type and the distribution of different warfarin resistant mutations in the English rural population.

Mitochondrial DNA data suggests a structured population at county level and the English brown rat population as a whole appears not to be at equilibrium. The relative lack of diversity in the mitochondrial DNA sequences examined can be explained by a founder effect and a subsequent spatial expansion. However, the microsatellite data shows much more mixing of populations at county level, even more clearly at the farm level. I discuss two possible explanations for this discrepancy; small effective population size of mitochondrial genomes and the greater movement of males compared to female rats.

Through mutation analysis we found 3 types of mutations in the exon 3 region of the *VKORC1* gene among our brown rat populations. One mutation type is almost ubiquitous being found in almost all counties. We also found a geographical pattern to the different mutation distribution. As these mutations are also found in other parts of Europe, we suggest that they have entered England through different ports from different parts of the Continent which would explain the geographical structure of the mutations.

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Chapter 1

INTRODUCTION

1.0 Introduction

1.1 History of the brown rat in Britain

Brown rats (*Rattus norvegicus* Berk.) are one of the most important vertebrate pests in the United Kingdom. They are kleptoparasitic on humans (Buckle & Smith, 1994) causing a diverse range of unwanted effects, such as eating and contaminating food, causing economic losses through building damages and spoilage of crops and also affecting public health (Webster & Mc Donald, 1995). William Faulkner (1962) in his final novel "The Reivers" described the rat as; "*He lives in your house without helping you buy it or build it or repair it or keep the taxes paid; he eats what you eat without helping you raise it or buy it or even haul it into your house*". However, the rat is also important in a positive way, as it has been used as a laboratory model species since the late 18th century (Clause, 1993).

The brown rat is believed to have been a marginal species in the steppe areas of Central Asia before 1700. In the first decade of the 18th century, this species spread to the Western parts of Russia and migrated further to almost all other parts of the world (Di Castri, 1989). During the industrial and agricultural revolutions of the 18th century, the size of big cities including London increased as trade grew dramatically. Commerce and consumer demand expanded enormously. Beginning from 1720s, there were about two thousand ships of all kinds, using the wharves and quays especially from the east. That is when the brown rat is believed to have first arrived in England (London), in 1731, by ship. Not only England, most of the Western Europe was invaded by the brown rat during the first half of 18th century, Denmark being one of the first places, also by ship in 1716.

Figure 1.1 : The brown rat (*Rattus norvegicus*), the most important vertebrate pest in United Kingdom.

Within approximately 50 years after its first arrive in London, this rat has already occupied the whole of England. In fact by 1776, brown rats were seen to be tunnelling under houses in Selkirk in Scotland (Twigg, 1975). The population density became higher especially where food, water and shelter was available. It can eat almost any biological substance and only needs about 25mm in diameter to squeeze through (Lund, 1994). This species can enter houses even through lavatory u-bends and is also a good swimmer, it can swim for 72km non-stop (Taylor and Quy, 1978). For sometime, the brown rats existed together with the earlier introduced roof rat (*Rattus rattus*), but the aggressive nature of the brown rat has lead to a decrease in the *Rattus rattus* population (Barnett, 1955).

Although some introductions are beneficial, e.g. fruits, many others have been criticized, this includes the brown rat and other invasive species. This species is a reservoir of pathogens, which is known to carry over 70 diseases such as Trichia, Typhus, Leptospirosis, Cowpox, Cryptosporidoisis, Viral Hemorhagic Fever (VHF), the Plague and many more (Simpson, 2002). This rat also carries bacteria in its saliva that can infect those bitten and cause a sickness known as Rat Bite Fever.

Brown rats also cause billions of dollars per year in the destruction of crops and food stores. Greaves (1978) estimated 94% of the farms in Hampshire were rat infested in 1979-1980, as one of the worst areas in the country, the others varying from 21% to 44%. Rat infestation outside of human dwellings has increased in 2001 compared to 1996 although the levels of rat infestation inside dwelling is similar for the same period of time. Damage to stored grain and animal feed was estimated to be £10,000,000 to £20,000,000 per year in the United Kingdom (Sheard, 2001).

1.2 Rat control

Various methods to control the brown rat infestation have been tried, including manual control, by using traps (Greaves, 1994), biological control (e.g. using predators such as birds) and chemical control (poisoned baits). A total of 37 eradication programmes have been recorded in Europe with rat (*Rattus sp.*) being the most common target species (67%) followed by rabbit (Genovesi, 2005) to prevent the impacts they cause to biological diversity, economy and human well being (Simberloff 2002).

The need for control of this species and other pest populations has lead to much research on many different aspects of pest biology, and has involved both ecological and genetic studies. Early work (Middleton, 1954) concentrated on the ecology of the rats' population. The late 1970s was the beginning of the use of many advanced tools to obtain data on the population biology of brown rats, especially on their movement patterns on farms using radio-transmitters. Taylor and Quy (1978) found that some rats covered large distances, and the average home range of male rats was found to be 660 metres. Knowledge of rat movements patterns on the farm lead to an efficient control program (Fenn *et al.*, 1987) where a single bait point, placed at the centre of rat activty gave a high level of management.

The usage and effect of chemical rodenticides have also been one of the major areas of research (Buckle and Smith, 1994). Chemical rodenticides, are still the first line of defence against rat infestations on most farms throughout the world. It is believed that the first rodenticide used was as early as in 1500 B.C. in Europe (Freeman, 1954). Most commercial rodenticides used are as poisoned baits, although there are also other forms such as poisonous gas and liquids. The first generation of commercial rodenticide used included warfarin, coumatetralyl, and chlorophacinone. Warfarin, the subject of this current study, was first proposed and used in United Kingdom by O'Connor in 1948. It was happily accepted by rats when included into bait, slow acting, and the sign of toxicity were delayed. Due to its delay in acting, the rat did not learn the effects of eating the bait and would repeatedly eat the bait for several days until the toxicity was enough to cause mortality.

Warfarin is a derivative of 4-hydroxycoumarin and anticoagulant compounds which inhibit blood coagulation by repression of the vitamin K reductase reaction (VKOR) (Pelz *et al.*, 2005). However, widespread use of Warfarin and other first generation rodenticides (chlorophacinone and coumatetralyl) has led to the evolution of resistance (Heiberg, 2002). As early as in 1958, the first report of resistance emerged in Scottish lowlands and soon after that (1960) in Welsh-English border (Drummond, 1970). In addition, the extensive use of the rodenticides also presents a high risk to some non-target animals (Mason & Littin, 2003) and there are serious concerns about the humaneness of chemical control of rodents. Brakes & Smith (2003) demonstrated that routine rat control by using rodenticide reduced the size of non-target small mammal populations in their study site. About 48% of non-target small mammals fed on rodenticide from bait boxes and the population declined significantly.

In the 1970s, new chemicals, known as 'second generation' anticoagulants (e.g. bromadiolone, difenacoum), were developed that could control warfarin-resistant rats . The most potent second-generation anti-coagulants (brodifacoum, flocoumafen) may only be used inside buildings to minimise exposure of non-target animals. Rats in some places have developed resistance to some of the new chemicals as well as to warfarin and older compounds, with the main focus of resistance being in central southern England (Kerins *et al.*, 2001).

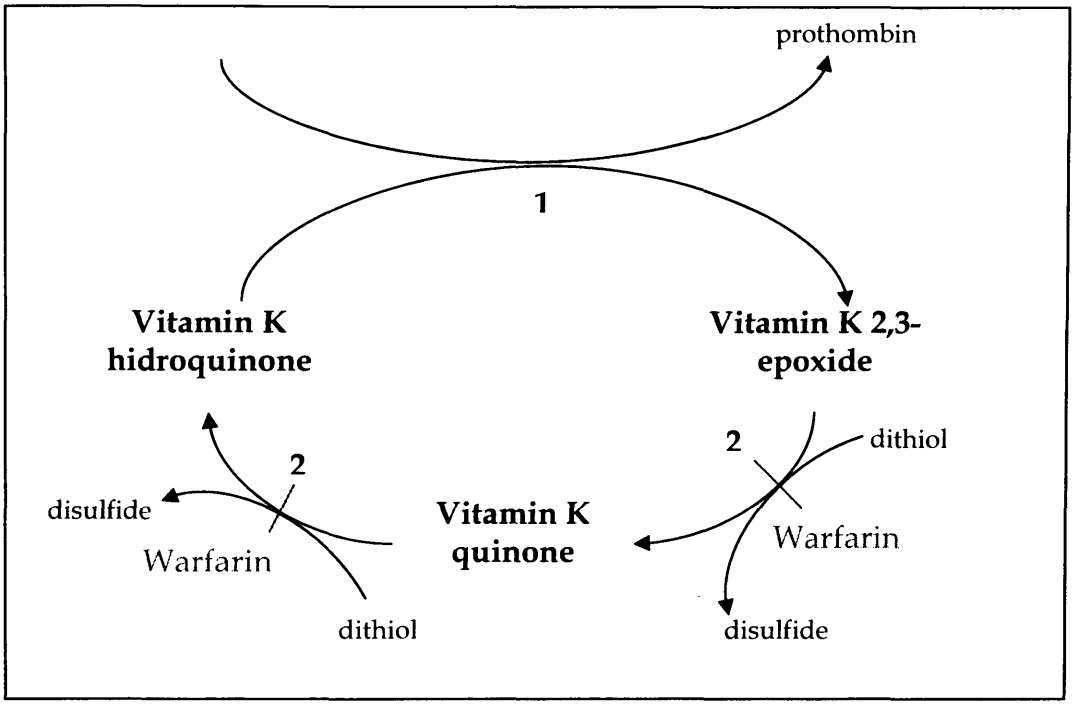
Non-chemical approaches (Smith, 1994), such as the use of repellents and reproductive inhibitors as well as environmental manipulation have been tested (Lambert, 2003) as an alternative to rodenticide use. However, the use of repellents is not convincing (Meehan, 1984) and both biological and chemical reproductive inhibitors have

produced poor outcomes. Lambert (2003) however found that by making the farm environment less suitable for rats, good results were achieved. Non-chemical approaches to rat control in tropical countries have also been practised and achieved good results. In Indonesia, a 4- year village-scale experiment where 'Ecologically-Based Rodent Management' was practised has resulted in a paradigm for large scale rodent management (Singleton *et al.*, 2004). Another example is the use of the Barn Owl as a biological control in the oil palm plantations in Malaysia to control *Rattus tiomanicus* (Wood & Feeb, 2003). In a 500ha area of oil palm, damage fell below the 5% threshold.

1.3 Warfarin resistance

As mentioned above, the extensive use of the anticoagulant rodenticides has led to resistance evolution. The highly effective anticoagulant, Warfarin, is a victim of its own success. Warfarin is known to reduce the ability of blood to coagulate by binding to the enzyme vitamin K 2,3-epoxide reductase (VKOR) and interrupting the reduction of vitamin K quinone to vitamin K epoxide (Fig. 1.2) in the vitamin K cycle. This means there is no substrate for the formation of prothrombin (Thijssen, 1995). This cause the susceptible rats to die due to internal bleeding (Bishop & Hartley, 1976). Rare mutants, which are resistant to Warfarin, will increase in frequency until the pesticide used is no longer effective. The resistance is also inheritable (Thijssen, 1995).

Figure 1.2. The cyclic metabolism of vitamin K. The carboxylation reaction (1), converts vitamin K hydroquinone to vitamin K 2, 3-epoxide. Vitamin K 2, 3-epoxide is reduced back to the quinone and then to vitamin K hydroquinone by vitamin K epoxide reductase (2). The reductase activity (2) is dithiol dependent and inhibited by anticoagulants such as warfarin.



Due to the small number of examples and lack of advanced technology, Crow (1957) made no conclusion about the number of loci responsible for resistance. Resistance appeared due to many loci or only one major locus. Later on during the 1960s, as a result of increase in knowledge, improvements in assays and wide demand, resistance to pesticides was found to be due to allelic variants at just one or two loci. Warfarin resistance was first determined by a single autosomal dominant gene (*Rw*) through breeding experiments on brown rats and house mice from areas in Wales (Greaves & Ayres, 1967). It was found on

chromosome 1 for the brown rat and chromosome 7 for the house mice. A Scottish type of resistance in rats was identified later (Greaves & Ayres, 1976), similar to the Welsh type of resistance, also associated with the *Rw* (Warfarin resistance) locus. However, the biochemical mechanisms of the two resistances are different. MacNicoll (1985) reported that the enzyme responsible in the cycling of vitamin K, i.e epoxide reductase, is altered in the Welsh type of resistance until the enzyme is less sensitive to Warfarin. However, in Scottish type of resistance, epoxide reductase is still sensitive to Warfarin, as in the susceptible rats, but it has a reversible binding to the pesticide, unlike the binding found in the susceptible rats.

In 2004, Rost identified *VKORC1* (codes for the first protein subunit of the VKOR complex) as the gene responsible for warfarin resistance and also combined deficiency of vitamin K-dependent clotting factors in humans. Later on, Pelz *et al.* (2005) reported eight different *VKORC1* mutations in warfarin-resistant rodents tested, i.e. 286 out of 428 German rats were resistant and carried Tyr139Cys mutation (Tyrosine is replaced by Cysteine at position 139, denoted by Tyr139Cys), two were Ser56Pro mutants, six English resistant rats had Leu120Gln, two resistant Scottish rats were Leu128Gln mutants and two Welsh resistant rats were Tyr139Ser. Others were rats from Denmark (Tyr139Cys), Belgium (Tyr139Phe) and France (Tyr139Phe and Arg35Pro). Six mice were found to carry Leu128Ser.

1.4 Spread of Warfarin resistance

Raymond *et al.,* (1991) reported that the mutations derived from independent origins and the number of independent origins of resistance-associated mutations is crucial in measuring the relative importance of mutation rate and migration in the spread of individual resistance alleles. Previously, Roush and Mackenzie (1987) suggested that the evolution of resistance is affected by 4 factors; allele frequency, dominance, the relative fitness of being resistant and the pest population structure itself. The fourth factor, population structure is often overlooked but needs to be understood. The subdivision of population into smaller interbreeding units, is important because it affects gene flow from area to area.

1.5 Population structure and gene flow

Endler (1977) described gene flow as the proportion of newly immigrant genes moving into another population. In animals, gene flow comprises migration of gametes through movements of adults, sometimes only movements of males whilst the females remain in the original group (Lowe *et al.,* 2004). Gene flow is an important aspect of abundance and spread of a species and to maintain the genetic connectivity. Without gene flow, populations will diverge and differentiate over time. Gene flow depends on historical processes i.e. bottle necks or colonization, two intrinsic biological factors i.e. mode of reproduction and vagility of the species, and two extrinsic factors, i.e. physical barriers and environment selection (Lowe *et al.,* 2004). Constant gene flow would help us to predict spatial genetic structure among populations but reduction in gene flow can create high genetic divergence between isolated populations (Latta &

Mitton, 1997). Gene flow between populations can be increased in three ways: expansion in favourable conditions by increasing the population size, succesful dispersal into recently occupied areas after a population collapse and mass emigration when shortage of food resource develops. In the case of resistance, gene flow has two effects. Firstly, the greater the gene flow between areas the more likely resistance genes are to spread. Secondly, if resistance genes spread into areas where pesticides are not used, resistance genes will be diluted by susceptible individuals (Wool & Noiman, 1983).

Gene flow can be estimated by direct or indirect methods (Slatkin, 1985). Direct methods require tracking the dispersal movements of individuals. In contrast, indirect methods involve analyzing genetic materials of adult populations, calculating population structure, population size and pattern of dispersal by analysing the genetic variation obtained. Time consuming and prone to bias techniques such as mark and recapture have limited the direct method being extensively used. It is also impossible to trace the movements of individuals over long distance. More over, dispersal does not necessarily reveal the movement of genes if reproduction does not occur effectively in the new location (Whitlock & McCauley, 1999). These problems have lead to the increasing use of indirect methods to estimate gene flow.

Gene flow can be explained by examining the genetic variation between populations and calculating genetic structure according to theoretical models (Lowe *et al.*, 2004). Wright (1931) introduced the Island Model (Fig. 1.3.a) which assumes that a species is divided into infinite populations or islands (in terms of number and size) and each population with the same number of individuals, receives and gives migrants to each

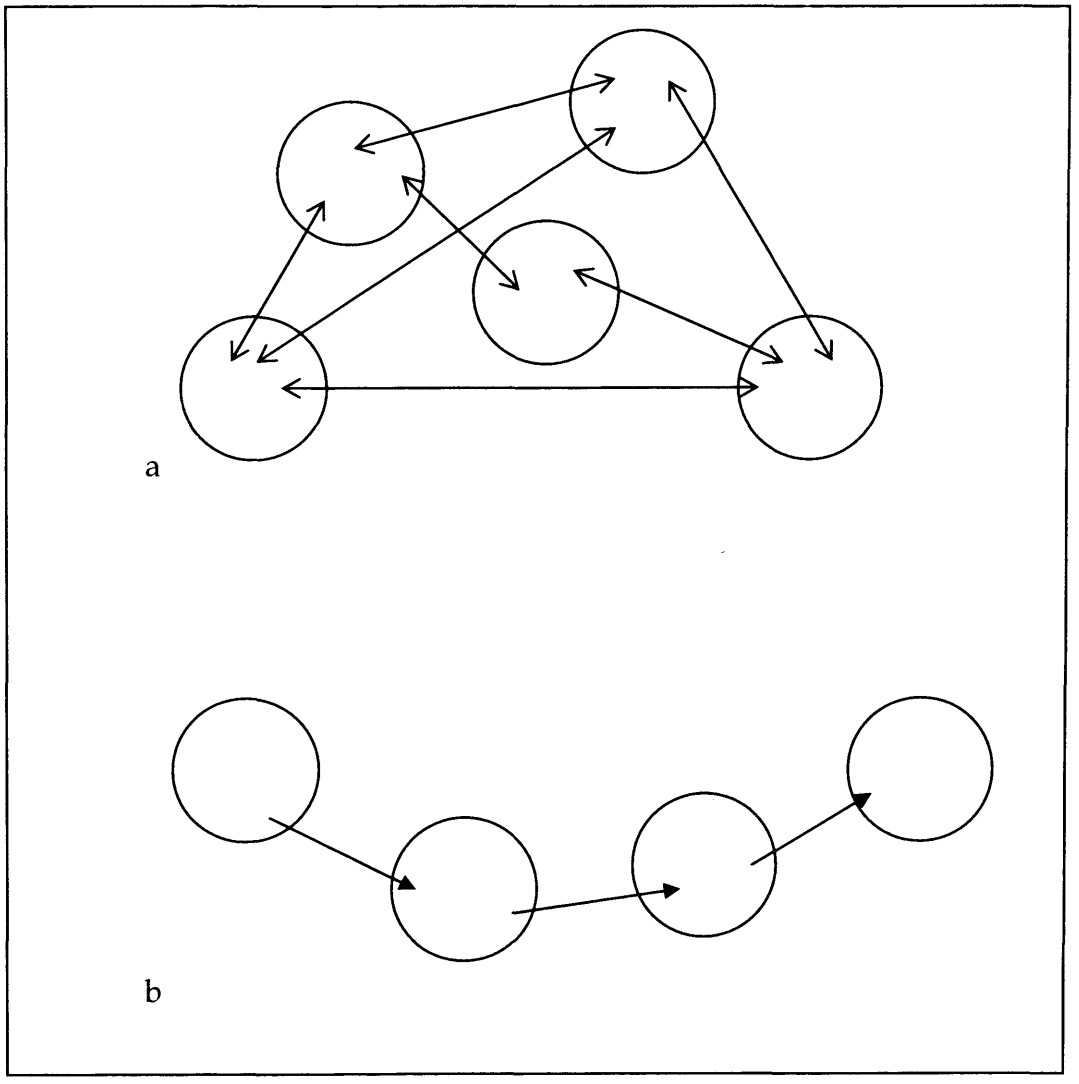
population at the same rate (Whitlock & McCauley, 1999). The population in this model is also assumed to be unspecified in terms of location. "The Stepping Stone Model" (Kimura, 1953) assumes that gene flow only occurs between neighboring populations. Gene flow from non-neighboring populations can also happens but need intermediate populations as "stepping stones" (Fig 1.3.b).

Wright (1951) built up the basic answer for any kind of approaches to gene flow questions. He predicted a simple relationship between the number of migrants entering a population per generation and the genetic variance among population F_{ST} :

$$F_{ST} \approx 1/(4Nm + 1),$$

where Nm is the number of individuals exchanged between populations per generation. From this equation, F_{ST} = 0.2 when Nm =1 , denote that only one migrant per generation, which is considered to be the critical limit above which populations will not be different from one another genetically (Beebee & Rowe, 2004). The Nm value associate to a mean value of F_{ST} (0.2), used as a criterion to classify whether there is high gene flow (above this value) or low gene flow (below this value) (Lowe *et al.*, 2004). F_{ST} is an excellent measure of the genetic differentiation among populations, and also to measure inbreeding coefficient, or heterozygote deficit that is due to population subdivision.

Figure 1.3. a) The island model (Wright, 1931) where migrants move between populations of equal size and randomly and b) the stepping stone model by Kimura (1953), where gene flow only occurs between neighboring populations and gene flow between 'non-neighboring' populations must use intermediate 'stepping stones' populations.



Molecular tools are now commonly used to obtain valuable information on genetic aspects (indirect methods) such as genetic variation and genetic distance of populations for many plant and animals species. They can also be useful to solve problems relating to how gene flow affects group structure and reproduction. Knowledge of population genetic structure of the brown rat (*Rattus norvegicus*) is limited but with the use of molecular tools such as protein and DNA markers, more information can be collected.

1.6 Genetic markers

Protein markers were among the most cost-effective methods and first popular tools used to study genetic variation in natural populations. One example of a protein marker isozymes, first described by Hunter and Markert (1957), as "*different variants of the same enzyme having identical functions and present in the same individual*". They are produced by different genes and therefore represent different loci (Markert & Moller, 1959). Isozymes can be separated partly on the basis of net charge and size; the larger the size of the isozyme, the slower it will migrate on the gel. The presence of the isozyme will be indicated by a stained band on the gel. Quantities and the positions of the bands will be scored to compare individuals in the population. Each stained band is representing an allele at a particular locus and therefore we can compare the amount of genetic variations between populations. Much isozyme research has been done to study population structure. For example, among 14 populations of Siamese fighting fish, *Betta splendens,* Meejui *et al.* (2005) found seven polymorphic loci. In rodents, Rogers and Engstrom (1992) found high

variability in the genetic structure in spiny mice (*Liomys pictus*). A study on ten populations of wood lemming, *Myopus schisticolor* (Fedorov *et al.*, 1995) using 20 isozyme loci showed that two were highly polymorphic (Idh-1 and Pgi-1). However, only Idh-1 locus confirmed significant differences within and between populations. Lanzaro *et al.*, (1995) found that variability pattern in isozymes was different from microsatellites in their *Anopheles gambie* samples. All eleven microsatellite DNA loci were polymorphic, compared to only 40% of 20 enzyme-coding gene loci. Although in some studies, isozymes do not show very high levels of polymorphism their typical codominance and extensive application make them suitable for estimating diversity. Advantages of using isozymes are:

a) They provide the basis for monitoring the genetic variation at specific structural gene loci.
b) Variation is usually expressed codominantly so that heterozygous and homozygous genotypes can be distinguished accurately.
c) It is easy to perform using animal and plant tissue.
d) It allows rapid population monitoring.

Another alternative method in screening polymorphism is the usage of DNA markers such as mitochondrial and microsatellite DNA. These DNA methods are becoming increasingly important especially in population studies for many reasons:

a) Rapid evolution of hypervariability.
b) The material used can potentially be sampled non-invasively from free living populations.

c) The primers developed for a particular species have been shown to be applicable across related taxa in animals.

The animal mitochondrial genome (mtDNA) is a small circular molecule (15 -20kb) (Boore, 1999), except for three Cnidarian Classes which have linear mtDNAs. Since the size is small, mitochondrial genomes only carry a small fraction of the genes needed for mitochondrial function. Unlike nuclear DNA, there is usually no change in mtDNA from parent to offspring due to meiosis and the mutation rate of mtDNA is higher and easier to measure. Avise *et al.* (1979) showed for the first time that heterogeneity in mtDNA sequences can be used to estimate relatedness between individuals and populations. However it can only be used to measure female-mediated gene flow as mtDNAs are inherited through the maternal parent. Since then, much research have been done using mtDNA to study animal population genetic structure, such as in Atlantic Walrus (Andersen *et al.,* 1998), African Savannah Elephant (Nyakaana *et al.,* 2002) an also in fish (Gold & Richardson, 1998; Consuegra & de Leaniz, 2007).

In rodents, mtDNA sequences have been used previously in population studies. For example, Good *et al.* (1997) suggested that the endangered giant kanggaroo rat, *Dipodomys ingens*, population fluctuated over time and the populations have not been isolated from one another. From 95 individuals that he had tested, 50 mtDNA haplotypes with 54 nucleotide differences were found along the 293 base pair of the mtDNA control region sequenced. In another study, Hingston *et al.,* (2005) analysed the variation in the non-coding hypervariable region (HVR1) of the mtDNA control region in 93 individuals of *R. rattus* in Southern Madagascar to elucidate the population structure. 13 haplotypes were

found and from his statistical analysis, he concluded that the majority of the variation was assigned to diversity within populations (86%). He also compared his samples' mtDNA sequences with data available outside Madagascar to locate the origin of the introduced species.

Microsatellite DNA is another molecular marker extensively used in molecular ecology. Also known as short tandem repeats (STRs) or simple sequence repeats (SSRs), they consist of 1 to 4 nucleotides repeated a variable number of times, ranging from 8 to 100 times. They are relatively abundant and randomly distributed throughout the nuclear DNA and mtDNA in animals. Microsatellites tend to be present in non-coding regions of the genome with no function (Tautz & Renz, 1984). Microsatellites are hyper variable and the mutation rates are high compared to other DNA markers and are often the marker of choice (Parker *et al.*, 1998) for estimating gene flow. In rodents, microsatellites had been used in medical research (Mironov et al., 1995, Bell & Jurka, 1997 and Roy & Liehr, 1999) and also in studying population structure. Ehrich *et al.*, (2001) used four microsatellite loci to compare the genetic population structure between two lemming species: collared lemming (*Dicrostonyx groenlandicus*) and brown lemming (*Lemmus trimuconatus*) from a fragmented landscape the Central Canadian Arctic. Higher genetic differentiation (Fst: 0.124) was found in brown lemming than in the collared lemming (Fst: 0.047). They suggested that this was due to the differences in dispersal rates and average effective population size for the two species. Hinten et al., (2003) found low levels of genetic variation in all 14 island *Rattus fuscipes greyii* population compared to the mainland population using six microsatellite loci. In addition, they also detected population substructuring within populations where sampling was conducted over a broader geographical area.

Variation in DNA sequences between individuals within a population can also be assessed indirectly through electrophoresis, by comparing the size and number of DNA fragments appearing on a gel (Lessa & Applebaum, 1993). Restriction endonucleases, enzymes derived from bacteria, cut DNA at particular parts of a sequence. The enzyme was first isolated by Meselson and Yuan in 1968 (Beebee & Rowe, 2004) and by 2003 about 3392 restriction endonucelases had been identified and registered on the restriction enzymes database REBASE (Roberts & Macelis, 2001). Differences in DNA sequence recognised by the restriction endonuclease will be cut during the incubation period. The restriction fragments are then separated according to length (basepairs) by electrophoresis. This will result in different fragment sizes appearing on the electrophoresis gel that indicates the differences among individuals in a population. The variations in fragments resulting from this method is called Restriction Fragment Length Polymorphism (RFLP). In rodents, a number of studies have used RFLPs mainly in finding genetic variation. Ittig & Gardenal (2002) found 20 different haplotypes in five populations of *Calomys musculinus*, the natural reservoir of the virus producing Argentine hemorrhagic fever, with only two haplotypes shared by all populations. Klebs *et al.*, (2003) designed a screening assay based on polymerase chain reactions (PCR) and RFLPs to distinguish transgenic mice defined by certain MHC backgrounds. He suggested that combining the PCR with RFLP method is simple, cost-effective and specific compared to conventional methods such as back crossing, which need at least 12 generations to get the particular mouse strain.

Another new established molecular method to detect changes or differences in genomic DNA is the tetra-primer Amplification Refractory

Mutation System (ARMS) - PCR. This method employs two primer pairs (outer and inner primers) to amplify two different alleles of a single nucleotide polymorphism in a single PCR reaction. Both inner primers of the tetra-primer (ARMS)-PCR method covers a deliberate mismatch at position -2 from the -3 terminus. This technique was first developed by Ye *et al.*, (2001) based on certain principles of the tetraprimer PCR method (Ye *et al.*, 1992) and the Amplification Refractory Mutation System (Newton *et al.*, 1989). The obvious differences between tetra-primer (ARMS)-PCR with tetra-primer PCR are that the length of inner primer used in tetra-primer (ARMS)-PCR can be up to 28 basepairs long (up to 15 in tetraprimer PCR) and the inner/outer primer ratio is 10 whilst 1 in the latter. Pelz *et al.*, (2005) used this assay to screen Tyr139Cys in their brown rats as mentioned earlier.

1.7 Thesis outline

The need for knowledge of the brown rat genetic population structure has encouraged me to carry out this research in the hope that the outcomes will contribute to the management of this species. The overall aim of the research is to understand the genetic structure of the brown rat population at different scales in the English countryside. The focus will be on three different aspects, large scale population structure, smaller scale population structure and the spread of warfarin resistance among the studied populations.

To be more specific, the story of the brown rat population structure begins in Chapter 3 looking at the county level. For this purpose, three molecular markers were used, i.e., isozymes, mtDNA and microsatellites

DNA. Several issues are been addressed. What distances do rats spread? Is the English brown rat population homogenous or broken up into discrete populations? How important is gene flow between these populations?

Chapter 4 is about the brown rat population structure at smaller scale, which focus on the farm level, using microsatellites DNA. Is there any gene flow between farm populations? How different are the populations?

In Chapter 5, I have studied the spread of warfarin resistance in the brown rat among the studied populations, using two molecular techniques: Amplification Refractory Mutation System (ARMS)-PCR and Restriction Fragment Length Polymorphisms (RFLPs). I also compared the resistant brown rat populations between the areas with resistance (Berkshire and Yorkshire), where population size is high and stable, with areas with low resistance, i.e. Leicestershire, where population size is often reduced to very low levels by chemical control are discussed.

Chapter 2

MATERIALS AND METHODS

SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
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2.0 Material and Methods

2.1 Brown rat samples

A total of 185 rats (*Rattus norvegicus*) from 14 rat populations were used in this study (Table 2.1). The majority of the samples were frozen livers and tails stored at -20^{0}C, provided by an extensive trapping program carried out by the Central Science Laboratory, York. Rats were also trapped in several farms in Leicestershire and Yorkshire throughout 2005 and stored at -20^{o}C. Some samples were also provided as extracted DNA by G. Butcher at the Babraham Institute, Cambridge.

Table 2.1: Number of rat individuals (N) from different Farm/Site used in this study.

Farm / Site	National Grid Ref.	N
Berkshire		
Beale Wildlife Park, Pangbourne, Reading	SU612792	3
Berkshire College, Burchetts Green	SU822833	3
Gidley Farm	SU465761	3
Goring Heath Farm, Goring Heath, Nr Reading	SU652798	7
Hadley Farm, Lambourne Woodlands, Newbury	SU303765	4
Henwick Manor Farm	SU497686	1
Lambders Farm, Beenham, Reading	SU610700	1
Milk Hill Farm, Hampstead Norris, Newbury	SU525773	1
South Fawley Farm, Wantage	SU390802	1
Upper Farm Farmborough, Wantage	SU438820	1

Cambridge		
Little Shelford	TL454514	1
Little Walden	TL545415	2
Fulbourn	TL515565	5
Wilobe Farm, Pidley, Huntingdon, Cambridgeshire	TL335786	5
Babraham	TL515505	7
Swavesey	TL365685	1
Dorset		
Bradford Down Farm, Dorchester	SY645908	3
St Mary Estate, Sturminster Newton	ST790160	4
Gloucestershire		
Anchorage Farm, Fretherne	SO734098	3
Hampshire		
Willow Farm, West Of Andover, Hampshire	SU365455	9
Kent		
Woodcote Farm, Maidstone	TQ817553	3
Tvs Livestock, Hollingbourne	TQ843551	4
Leicestershire		
Hill Farm, Launde, Leicester	SK793038	5
Oxey Farm, Leicester	SK777035	16
Hall Farm, Loddington , Leicester	SK793021	14
Middlesex		
Orchard Grove Allotments, Kenton, Harrow	TQ190890	3

Oxfordshire		
Manor Farm, Harwell	SU494890	4
Poultry Unit, Crowmarsh Gifford	SU611886	3
Winterbrook Farm, Blewberry	SU536858	3
Shropshire		
Knock In Egg Farm, Near Oswestry	SJ300300	1
Knocked Egg Farm, Ford Site Near Shrewsbury	SJ408138	3
Oliver Price & Son, Cilcewydd	SJ229039	2
Sussex		
The Oak Poultry Farm, Ditchling Common, Hassocks	TQ332172	4
Mr Griffriths Jeffreys, Farm, Horstel Keynes	TQ379278	8
Wiltshire		
Freeth Farm, Compton Bassett, Near Calne	SU024726	2
Old Park Farm	ST995600	6
Maer Farm, Fifield Bavart	SU017251	4
Burdens Ball Farm, Wilton	SU115325	2
Pewsey Hill Farm	SU164576	4
Worcestershire		
Grange Farm, Little Comberton, Near Pershore	SO966430	5
Yorkshire		
TBS 10	SE67707284	2
TBS 11	SE68097322	2
TBS 2	SE67907252	6

TBS 3	SE66857272	2
TBS 4	SE66837261	4
TBS 5	SE66887228	2
TBS 7	SE68107224	5
TBS 8	SE68257239	1
Total		**185**

2.2 Sample preparation.

Rats kidneys and livers were used for isozymes work. We extracted DNA from livers and tails.

2.2.1 Samples preparation for isozymes work

Livers and kidneys are known to be commonly used in isozymes study and successful results have been obtained *(Amori et al., 2001, Kim et al., 1996)*. 1ml of extraction buffer with 30ug of each samples were load in a watch glass on a bucket of ice to homogenize. Once the sample is homogenised, several filter wicks (7mm x 3mm) were placed and left in the homogenate to absorb the extract. The paper wicks and the homogenate were stored in -20°C prior to use.

2.2.2 DNA extraction

Total genomic DNA was extracted from either 20 μg of liver or 1.5 cm from the tip of tails using the Wizard SV Genomic DNA Purification

System (Promega) as per instructions. DNA samples were stored in -20^{0}C until use.

2.3 Isozyme work

Homogenates were subjected to electrophoresis on 12% starch gel (Sigma) at 240V for 4 hours. Each gel was sliced horizontally and stained for Esterase (EST), Phosphoglucomutase (PGM) and Isocitrate dehydrogenase (IDH) in a 37^{o}C incubator . Electrophoresis buffers used for each enzyme are as Table 2.2.

Table 2.2: Electrophoresis buffer used for each enzyme.

Enzyme	E.C. Number	Buffer
Esterase	3.1.1.1	Lithium Borate (LiBO3)
Phosphoglucomutase	5.4.2.2	Tris Citrate pH 7.0
Isocitrate dehydrogenase	1.1.1.42	Lithium Borate (LiBO3)

2.4 Mitochondrial DNA (mtDNA)

A 425 base pair region of the Hyper Variable Region 1 (HVR1) segment within the mtDNA control region was PCR amplified for each individual rat. PCRs were carried out as per Hingston *et al.,* (2005) with slight modifications. Primers used were L283 (5′-TACACTGGTCTTGTAAACC -3′) and H16498 (5′-CCTGAAGTAGGAACCAGATG-3′). A 20µl reaction was used in which 2µl genomic DNA was added to the reaction mixtures containing 10µl PCR reaction mix (YorkBio), 1µl (10uM) of each primer

and 6µl of H_2O. PCR was carried out on a T1 Thermocycler (Biometra, Goettingen, Germany). The PCR condition was as follows: 30 cycles of denaturation at 94°C for 45s, annealing at 50°C for 45s, elongation at 72°C for 1 min, and a final extension at 72°C for 30 mins.

PCR products were then cleaned with the YorkBio PCR Cleanup kit to remove any unincorporated nucleotides and primers that can interfere with the sequencing process. Cleaned PCR products were sequenced by the John Innes Genome laboratory in Norwich using an ABI3700 capillary sequencer.

2.5 Microsatellite DNA

Six primers that are highly variable for brown rats were used (Table 2.3) and one primer of each locus was labelled with one of the fluorescent dyes, PET, 6FAM or VIC. The Polymerase Chain Reaction (PCR) was carried out in a total volume of 10ul, containing 5ul of PCR reaction mix (YorkBio), 0.5ul of DNA, 0.5ul of each primer and 3.5ul of ddH_2O. PCR was run on a T1 Thermocycler (Biometra, Goettingen, Germany) using the following conditions: one cycle of denaturation at 94°C for 5 mins, 35 cycles of denaturation (94°C for 30s), annealing (50°C for 30s) and elongation (72°C for 30s). Finally, one cycle of extension at 72°C for 30s. PCR products were analysed by Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, on an ABI3770 for fragment analysis.

Table 2.3: Primers used in this study.

Primer	Dye	Primer sequences (5′ – 3′)	Repeat motif	Size Range (bp)
D17Rat65	VIC (red)	TGCCAGTCTTTTCAATGTGG GGGTAAGGACAAGGTTCCTGA	(GT)	220-460
D3Mit13	FAM (blue)	TCCTCTTAGTAAAATTGCACGC TCAGCCCTTCTCCTGTCTA	(CA)	83-113
D5Rat95	PET (green)	GGAACCTGCACAATCATGTG CCATCTACTCCAGTCCTTGGTT	(GT)	100-163
D12Rat43	PET (green)	TCCCACAAGTTCTCTGTGCA CTCACTATGGCCTGGACCTT	(CA)	245-324
D14Rat1	VIC (red)	CAGTCCCTGGGTTTTCACAT CTCCAAGACACAAAACGATCA	(CA)	128-164
D8Mgh7	FAM (blue)	TGAAGAGATTTTACTGGGTAGCTCC TGGACCAGGCAAGTTCTCTT	(CT/GT /GA)	198-238

2.6 Mutation screening

Four published mutations (Pelz *et al.*, 2005) within the exon 3 (Tyr139Cys, Leu120Gln, Leu128Gln and Tyr139Ser) were screened among the samples (Table 2.4). Amplification Refractory Mutation System ((ARMS– PCR), Pelz *et al.*, 2005) was used to screen Tyr139Cys mutation whilst the other mutations were screened using Restriction Fragment Length Polymorphisms (RFLPs).

Table 2.4: Mutation screened in this study.

Mutation	Wild type (bp)	Mutant (bp)
Tyr139Cys	123	101
Leu120Gln	330	195
		135
Leu128Gln	330	170
		160
Tyr139Ser	160	110

2.6.1 ARMS - PCR

ARMS - PCR (Ye *et al.* 2001) for Tyr139Cys screening was carried out as per Pelz *et al.* (2005) using 2pmol each of the outer primers F: 5'-ATCCTGAGTTCCCTGGTGTCTGTCGCTG-3' and R: 5'-TCAGGGCTTTTTGACCTTGTGTTCTGGC-3' and 10pmol each of the inner primers F: 5'-TGATTTCTGCATTGTTTGCATCACCACATG-3' and R: 5'-CAACATCAGGCCCGCATTGATGGAAT-3'. PCR buffer stock solution was prepared using 100ul of 10x PCR buffer (invitrogen), 2 ul of each dNTP (100mM), 30ul of $MgCl_2$ (invitrogen) and 762ul of distilled water. 31.25 ul of reaction was used, containing 25ul PCR buffer (from the PCR buffer stock solution), 0.25ul Taq polymerase (invitrogen), 1ul of each primer, 1ul of 5M Betaine and 1ul of DNA. The PCR condition was as follows: 95^{0}C for 3 mins, 32 cycles of denaturation at 95^{o}C for 20s, annealing at 62^{o}C for 20s, elongation at 70^{o}C for 10 s, and a final extension at 70^{o}C for 3 mins.

The PCR products were visualised on a 3.5% Ultra High Resolution (MELFORD) Agarose gel. The electrophoresis was carried out for 1 hr and

45 mins at 110 volts. The outer primer pair produces a control band of 163 base pairs, the inner primer pair gives a band of 123 for the wild type and 101 base pairs for the mutant, respectively (Pelz *et al.* 2005).

2.6.2 Restriction Fragment Length Polymorphisms (RFLPs)

PCR was carried out to amplify the 330 base pair region of exon 3 for each individual of rats. Primer used (Pelz *et al.* 2005) were 5'-CATTGGGGAGGTGTTACAGAG-3' (forward primer) and 5'-GATACACTTGGGCAAGGCTC-3' (reverse primer). 20ul of PCR reactions were performed with 0.25ul of Taq polymerase, 1ul of 200umol dNTPs mix, 1.5ul of $MgCl_2$, 5ul of 10xPCR buffer, 1ul of each primer, 2ul DNA and 8.25 distilled water for 45s at 940C, 30 cycles of denaturation at 94°C for 45s, annealing at 50°C for 45s, elongation at 72°C for 1 min, and a final extension at 72°C for 5 mins. The PCR products were visualised on a 1% agarose gel for 40 mins at 110 volts.

Three different restriction endonucleases were used for the 3 different mutations. Digestion of the PCR products by StuI for mutation Leu120Gln results in fragments of 195 and 135 base pairs (mutant) and 330 base pairs for the wild type (Rost *et al.*,2004). Mutation Leu128Gln produced by Bsr1 with fragments of 170 and 160 base pairs in mutants and 330 base pairs in wild type. Mutation Tyr139Ser can be identified by MnlI digestion with a fragment of 110 base pairs and 160 base pairs for the wild type (Pelz *et al.*, 2005).

10ul reaction (Table 2.5) containing buffer, enzyme and PCR product was used and incubated in a heat block for 2 to 4 hrs. At the end

of incubation, the digested PCR products were visualised on 3.5% Ultra High Resolution Agarose gel for 1 hr and 45 mins at 100 volts.

Table 2.5: Restriction enzyme digestion reactions

Mutation	Restriction enzyme (ul)	Buffer 10x	BSA	PCR product	Incubation temperature
Leu120Gln	StuI (0.1ul)	1ul	-	8.9ul	30°C
Leu128Gln	BsrI (0.2ul)	1ul	-	8.8ul	65°C
Tyr139Ser	MnlI (0.2ul)	1ul	0.2ul	8.6ul	30°C

2.7 Data Analysis

MtDNA sequences were aligned using CLC Free Workbench version 2.0 computer software, to distinguish haplotypes among rat individuals within populations. The phylogenetic relationships among the haplotypes were then analysed using two methods. First, a minimum spanning network based on a matrix of the observed nucleotide differences was calculated using the program ARLEQUIN 3.01 (Excoffier et al. 2005). Second, the genetic distance between haplotypes was calculated assuming a Tamura & Nei model of sequence evolution. These distances were used to construct a neighbour-joining tree using the computer program SPLITSTREE (Huson 1998). 1000 bootstrap replicates were calculated to estimate the support for each node in both the minimum spanning network and the neighbour-joining tree.

The geographic distribution of genetic variation was estimated using Analysis of Molecular Variance (AMOVA) performed by ARLEQUIN 3.01. Gene diversity (h) and nucleotide diversity (π) of the

various populations and their respective standard deviations were calculated using ARLEQUIN 3.01. Nei (1987) described haplotype diversity (h) as the probability that two randomly chosen individuals have different haplotypes, and nucleotide diversity (π) as the average of pairwise nucleotide difference between individuals within samples. ARLEQUIN 3.01 was also used to perform mismatch distribution analysis. Mismatch distributions are histograms showing the pattern of nucleotide site differences between pairs of individuals in a sample. They can be used to test hypotheses about the history of population size and subdivision (if selective neutrality is assumed) or about selection (if a constant population size is assumed). The distribution is usually unimodal in samples drawn from populations having passed through a recent demographic expansion (Rogers & Harpending 1992).

Geographical distances were calculated as the distance from the central national grid reference of one population area to the central national grid reference of the other. This was compared with Φ_{st}, an analogue of Wright's F_{ST} statistic (Wright 1951), using a Mantel test carried out with the computer program GenAlEx version 6.0 (Peakall & Smouse 2006).

For microsatellite DNA, Genemapper (Applied Biosystem version 4) was used to determine the number and size of microsatellite alleles in each locus per population. Observed and expected levels of heterozygosity and a Mantel test (Mantel, 1967) to test the relationship between geographical distance and the level of genetic differentiation between populations (F_{ST}) were calculated using the program GenAlEx version 6.0 (Peakall & Smouse 2006). STRUCTURE (version 2.01) was used to determine the most likely number (K) of population units. A burn-in period of 1,000,000 of steps followed by another 1,000,000 steps was

chosen. Minitab 14 (Minitab Inc., Pennsylvania) were used to test the relationship between F_{ST}, geographical distance and resistance level.

The genetic population structure was characterised from allele frequencies by estimates of F_{ST} calculated in AMOVA also using GenALeX. The estimates were tested for significant difference from zero, by means of permutation test exchanging genotypes between populations (1,000 000 replicates).

Spearman's Rank Correlation was used to assess if there is any relationship between Warfarin usage and prevalence of mutants within study areas.

SPECIAL NOTE

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Chapter 3

LARGE SCALE POPULATION STRUCTURE OF THE BROWN RATS

3.0 Large Scale Population Structure of the Brown Rats

3.1 Introduction

This chapter will elucidate the brown rat population structure at the macro level. It is important to understand the genetic variation in the brown rat in England as a valuable tool for future control strategies.

Control of the brown rat is important for health and environmental reasons. Eradication of an invasive species is also a powerful tool for conservation in an insular ecosystem. Many threatened species have recovered after invasive species were controlled (Graham & Veitch, 2002; Pascal *et al.*, 2005). However, eradication operations involve large economic and ecological costs and some of them have failed (Thorsen *et al.*, 2000). Although the use of the anticoagulant rodenticides have been the mainstay of rat control, control of the species is still an issue. Resistance to warfarin, the first generation of anticoagulant rodenticide has been reported across England and detection of resistance to the second generation has been noted as well (Drummond, 1970, Gill *et al.*, 1993 and Gill *et al.*, 1994). One major problem contributing to the failure is the lack of knowledge about the genetic population structure of the the brown rat. Analyzing the genetic population and interpreting it in terms of gene flow will provide a useful approach to help control this species.

3.1.1 Rat population structure

Rat population dynamics are influenced by local environment conditions, whether food and shelter is available, the rate of predation, birth rate and rates of immigration and emigration.

The brown rat is associated with farm buildings in rural areas. Permanent populations occur in many farm buildings where the environment is favourable and breeding occurs throughout the year. Abundant food in the farm was though to be the main cause of this condition (Lund, 1994). However, recent studies (Husni, personal communication) reported that rat abundances among farms is not mainly related to food, but due to the management practices and surrounding environment.

Wild brown rats live in colonies with one or a few males sharing with one to six females in a small burrow system in which they may raise their young together. Their social organization and mating system depends on population density; at low density, males defend their territory from intruders and the mating system is polygynous, on the contrary, at high densities, males do not defend their territory and the mating system is promiscuous.

Data on reproductive rates provides help in understanding population changes, dispersal and mortality rates (Davies, 1953). Normally, the gestation period for females is 20 to 23 days with litter sizes about 7 to 9 and a peak in breeding from March - April and in September. Davies (1953) estimated that a single female farm rat weaned about 14 young per year. Males have a larger body size (mean body weight = 307g)

than females (mean body weight=233g). It has also been reported that males grow more rapidly than females (Calhoun, 1962 and McGuire *et al.*, 2006). Bishop and Hartley (1976) in their study showed that weight increases linearly between the ages of 2 and 7 months. However, growth slows and stabilizes when rats weigh more than 300g.

Taylor (1978) found that rats living near to a food source rarely moved more than 30 m from their home sites and their home range is much greater at the arable sites (Lambert, 2003). The home range of male rats has been reported as greater than that of females: 660m for males compared with 340 for females (Taylor, 1978).

In this study, I used three different methods, isozyme electrophoresis, mitochondrial DNA sequencing and microsatellite DNA analysis to elucidate the population structure of the brown rat in England.

3.2 Materials and methods

3.2.1 Isozymes

A total of 23 brown rat individuals were used in this study. 8 rat individuals from 3 different farms in Yorkshire and 15 from three different farms in Leicestershire. Three enzyme systems were tested, i.e; Esterase (Est), Isocitrate dehydrogenase (IDH) and Phosphoglucomutase (PGM). Electrophoresis was conducted on 12% starch gel (Sigma) at 240V for 4 hours in 4^0C. Gels were sliced and stained for each isozyme respectively.

The stain recipe for each isozyme are as follows:

Esterase

0.1M Tris/HCl pH7.5	50ml
α-Napthyl acetate solution	3ml
Fast Blue BB salt	50mg

Isocitrate dehydrogenase (IDH)

0.1M Tris/HCl pH8.0	50ml
Isocitric Acid	100mg
NADP	10mg
MTT	15mg
PMS	3mg

Phosphoglucomutase (PGM)

0.1M Tris/HCl pH7.5	50ml
Glucose-1-phosphate, sodium salt	100mg
NADP	10mg
MTT	15mg
PMS	3mg
ATP	25mg
Glucose-6-phosphate dehydrogenase	20-40µl

3.2.2 Mitochondrial DNA (mtDNA)

As described in Chapter 2, the Hyper Variable Region 1 (HVR1) segment within the mtDNA control region was PCR amplified for each of 185 individual of rat. Primers used were as per Hingston *et al.*, (2005), i.e; L283

(5'-TACACTGGTCTTGTAAACC -3') and H16498 (5'-CCTGAAGTAGGAACCAGATG-3'). A 20μl reaction was used in which 2μl genomic DNA was added to the reaction mixtures containing 10μl PCR reaction mix (YorkBio), 1μl (10uM) of each primer and 6μl of H_2O. PCR was carried out on a T1 Thermocycler (Biometra, Goettingen, Germany). The PCR condition was as follows: 30 cycles of denaturation at 94°C for 45s, annealing at 50°C for 45s, elongation at 72°C for 1 min, and a final extension at 72°C for 30 mins. The cleaned PCR products were sent to John Innes Genome laboratory in Norwich for sequencing. Sequences were then aligned by using CLC Workbench Version 2 software package.

Geographical distances were calculated as the distance from the central national grid reference of one population area to the central national grid reference of the other. This was compared with Φ_{st}, an analogue of Wright's F_{st} statistic (Wright 1951), using a mantel test carried out with the computer program GenAlEx6 (Peakall & Smouse, 2006).

3.2.3 Microsatellite DNA

A total of 185 rat individuals was used in this part. 6 primers were used(D17Rat65, D3Mit13, D5Rat95, D12Rat43, D14Rat1 and D8Mgh7) and one primer of each locus was labelled with one of the fluorescent dyes, PET, 6FAM or VIC, as described in Chapter 2. The PCR was run individually for each primer. PCR products of D17Rat65, D3Mit13, D5Rat95 and products of D12Rat43, D14Rat1 and D8Mgh7 were pooled together respectively. PCR products were run as 2 batches on a Applied Biosystems 3730 capillary sequencer fragment analysis. Results were

analysed by statistical program; GeneMapper (Applied Biosystem version 4). Another 2 statistical analysis programmes were used to analyse the population structure, i.e; STRUCTURE (version 2.1) to determine the most likely number (K) of population units and Mantel test (Mantel, 1967) to test the relationship between geographical distance and the level of genetic differentiation between populations (F_{ST}).

3.3 Results

3.3.1 Isozymes

3.3.1.1 Phosphoglucomutase (PGM, E.C.5.4.2.2)

The results showed that two (2) bands were identified on the gel stained for Phosphoglucomutase -1 (PGM -1) and a single band of PGM-2 anodal to the origin (Figure 3.1), no polymorphisms was observed from all rat individuals tested.

Figure 3.1 Leicestershire brown rats PGM band pattern observed following electrophoresis on the Lithium borate buffer system.

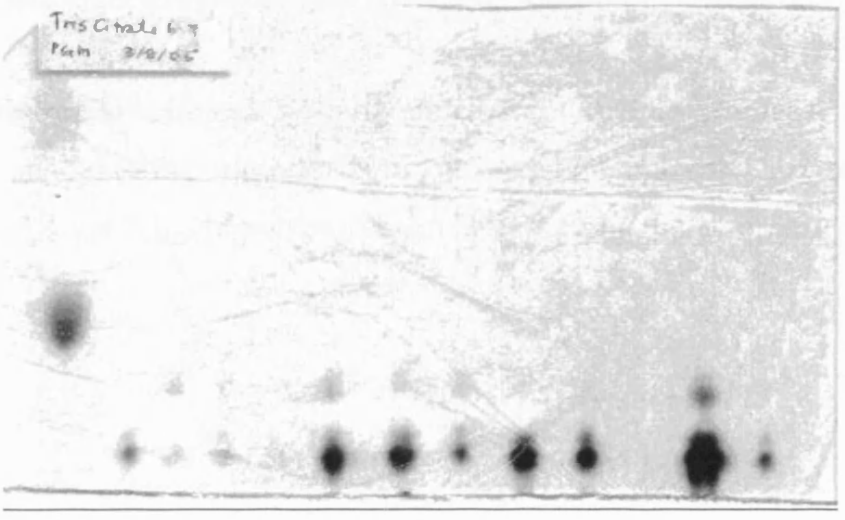
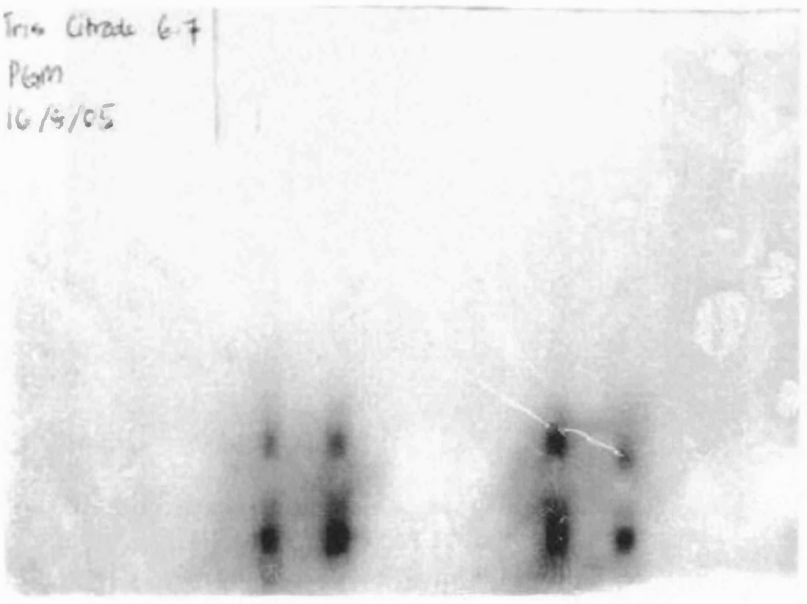


Figure 3.2 Yorkshire brown rats PGM band pattern observed following electrophoresis on the Lithium borate buffer system



As in Leicestershire rats samples 2 PGM-1 bands and a single PGM-2 band (Figure 3.2) appeared anodal from the origin and no polymorphism detected in the Yorkshire brown rat samples.

3.3.1.2 Isocitrate dehydrogenase (IDH, E.C. 1.1.1.42)

The electrophoresis and staining process resulted in 3 intense IDH-2 bands, anodal to the origin for both Leicestershire and Yorkshire samples (Figure 3.3 and 3.4). No polymorphism noted among the samples.

Figure 3.3 Leicestershire brown rats IDH band pattern observed following electrophoresis on the Tris Citrate pH6.9 buffer system.

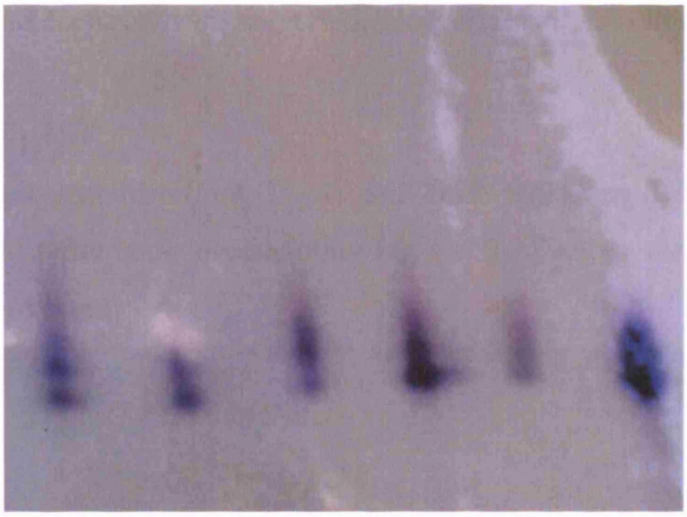
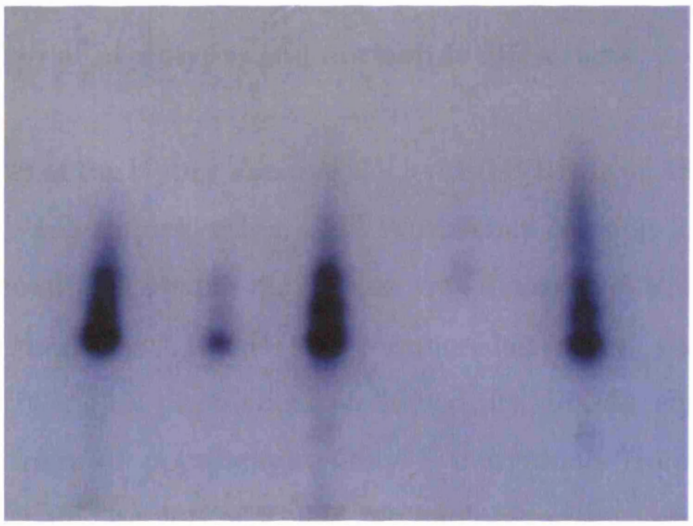


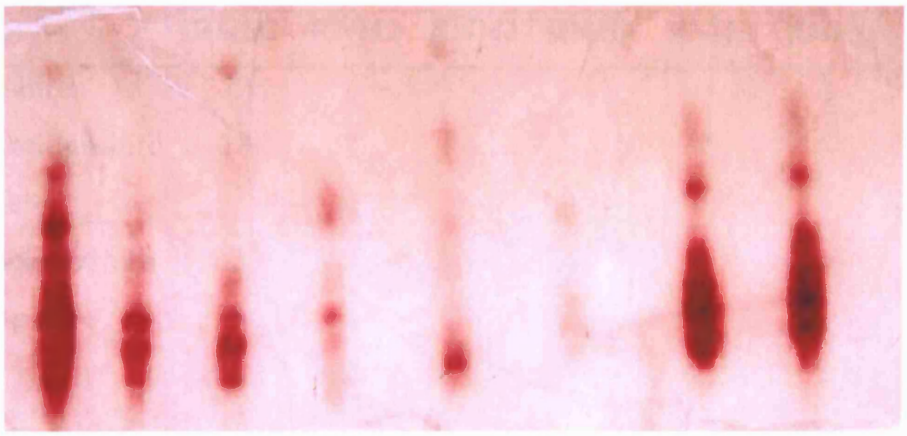
Figure 3.4 Yorkshire brown rats IDH band pattern observed following electrophoresis on the Tris Citrate pH6.9 buffer system



3.3.1.3 Esterase (Est 3.1.1.1)

A total of 4 zones of staining was identified on the gel. However, staining of zone 4 was not sufficient to score. Only 3 esterase loci (zones 1, 2 and 3) are considered to have been screened. No variation was detected among samples.

Figure 3.5 Leicestershire (lane 1 – 4) and Yorkshire (lane 5-8) brown rats Esterase band pattern observed following electrophoresis on the Lithium borate buffer system



3.3.2 Mitochondrial DNA (mtDNA)

3.3.2.1 Number of haplotypes and nucleotide differences

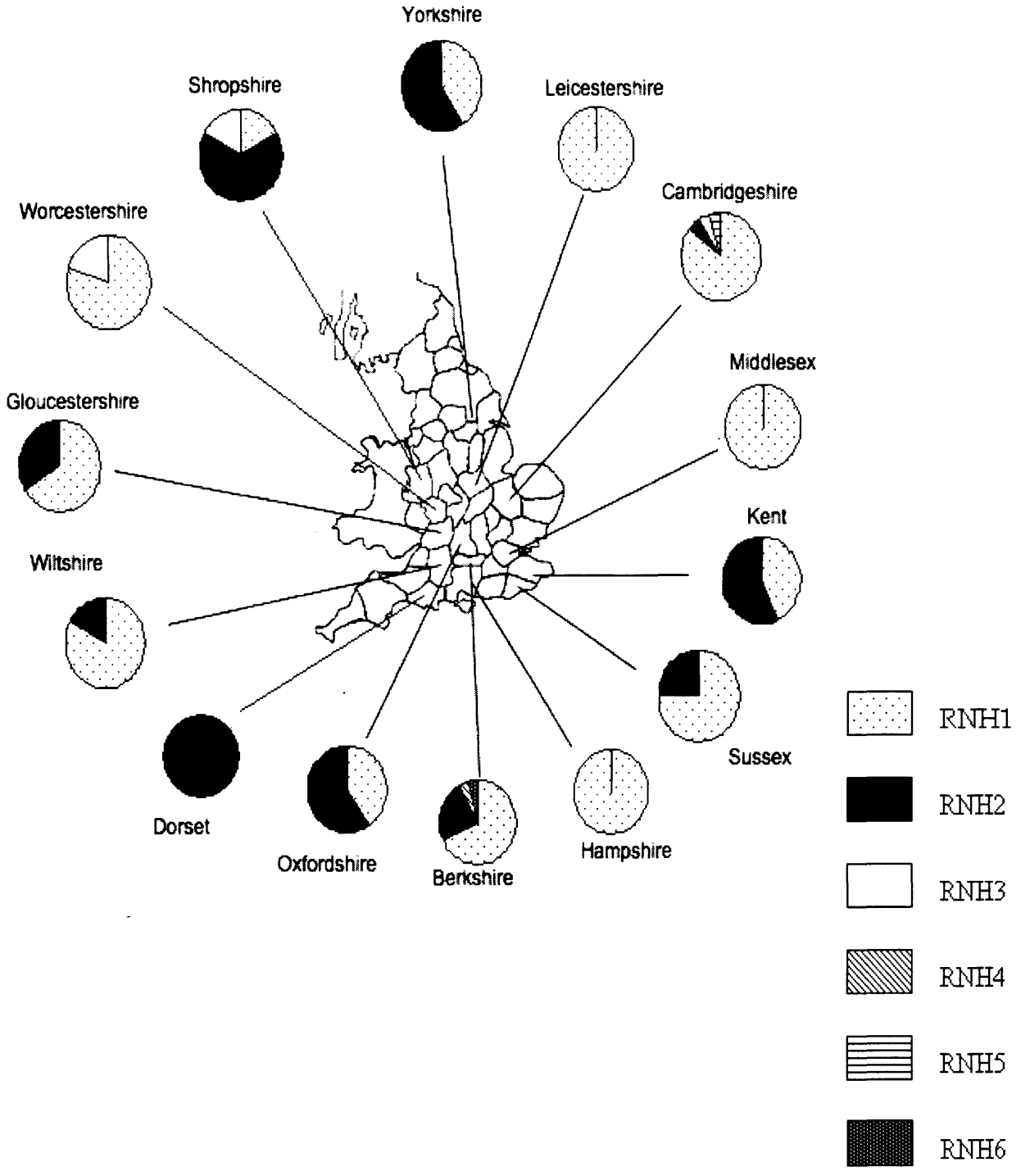
The sequences of the Hyper Variable Region 1 (HVR1) from 185 individual brown rats were aligned using CLC Workbench Version 2.0 software package. A total of 6 unique haplotypes was discovered, RNH1 – RNH6 (Table 3.1). Haplotypes RNH1, the common haplotype, shared by 130 individuals from 13 populations followed by RNH2 shared by 49 individuals from 10 populations. Only 3 individuals from 3 different populations shared haplotype RNH3. No haplotype was present in all our 14 populations. Haplotypes RNH1, RNH2, and RNH3 were found in more

than one population. 3 populations have more than 2 haplotypes; Berkshire (4 haplotypes), Cambridgeshire (4 haplotypes) and Shropshire (3 haplotypes). 3 private haplotypes were found, i.e; RNH4 (Berkshire), RNH5 (Cambridgeshire) and RNH6 (Berkshire). Figure 3.6 shows the distribution of haplotypes among our sampled populations.

Table 3.1 Number of haplotypes and individuals in each haplotypes among study areas.

Area	Haplotypes						Total
	RNH1	RNH2	RNH3	RNH4	RNH5	RNH6	(N)
Berkshire	17	6	-	1	-	1	25
Cambridgeshire	18	1	1	-	1	-	21
Dorset	-	7	-	-	-	-	7
Gloucestershire	2	1	-	-	-	-	3
Hampshire	9	-	-	-	-	-	9
Kent	3	4	-	-	-	-	7
Leicestershire	35	-	-	-	-	-	35
Middlesex	3	-	-	-	-	-	3
Oxfordshire	4	6	-	-	-	-	10
Shropshire	1	4	1	-	-	-	6
Sussex	9	3	-	-	-	-	12
Wiltshire	15	3	-	-	-	-	18
Worcestershire	4	-	1	-	-	-	5
Yorkshire	10	14	-	-	-	-	24
Total (N)	130	49	3	1	1	1	185

Figure 3.6 Map showing the origin of our samples and the proportion of each haplotype found at that site.



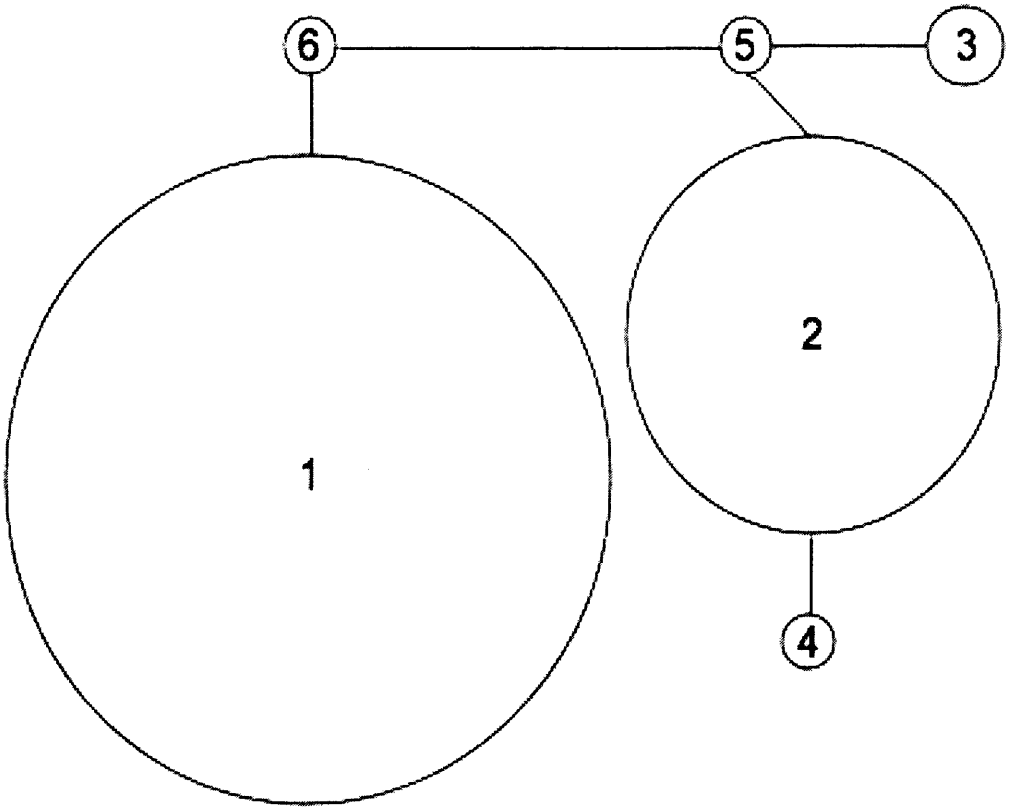
The 6 haplotypes can be distinguished by 10 nucleotide differences (Table 3.2) along the 425 base pairs of the HVR1. All the 10 variable nucleotide positions are transitions.

Table 3.2 The nucleotide differences of the 6 haplotypes (RNH1-RNH6) in a sample of 185 *Rattus norvegicus* individuals from 14 different sampling areas in England. The top row is the position of the variable nucleotides within the 425 bp sequence.

Position	***95***	***97***	***157***	***204***	***244***	***246***	***260***	***265***	***276***	***313***	***n***
Haplotype											
RNH1	T	T	C	T	C	T	T	G	A	G	130
RNH2	.	C	.	C	.	C	C	A	G	A	49
RNH3	.	C	T	C	T	C	.	A	G	A	3
RNH4	C	C	.	C	.	C	C	A	G	A	1
RNH5	.	C	.	C	.	C	.	A	G	A	1
RNH6	.	.	.	.	.	.	.	.	.	A	1

Figure 3.7 shows the relationship between each of the haplotypes along with its relative proportion in our sample. Only RNH6 differed by one nucleotide from the most common haplotype RNH1. The results also showed that there are 3 groups of haplotypes: first group consists of RNH1 and RNH6, second group consists of RNH2, RNH4 and RNH5 and RNH3 as the third group.

Figure 3.7 Minimum spanning network of the phylogenetic relationships between the 6 mtDNA haplotypes found. The area of the circles represents the frequency of the haplotypes in the entire population.



3.3.2.2 Haplotype and nucleotide diversity

The haplotypic (*h*) and nucleotide diversity (π) indices are given in Table 3.3. The genetic diversity was low in all populations. No genetic diversity was found in 4 populations; i.e, Leicestershire, Dorset, Hampshire and Middlesex where only 1 haplotype was found in each population.

Table 3.3 Haplotypic (h) and nucleotide (π) diversity in each population.

Area	*n*	$h \pm SD$	$\pi \pm SD$
Total	185	0.44 ± 0.03	0.0069 ± 0.0040
Berkshire	25	0.49 ± 0.09	0.0120 ± 0.0072
Cambridgeshire	21	0.27 ± 0.12	0.0070 ± 0.0040
Dorset	7	0.00 ± 0.00	0.0000 ± 0.0000
Gloucestershire	3	0.67 ± 0.31	0.0179 ± 0.0150
Hampshire	9	0.00 ± 0.00	0.0000 ± 0.0000
Kent	7	0.57 ± 0.12	0.0153 ± 0.0100
Leicestershire	35	0.00 ± 0.00	0.0000 ± 0.0000
Middlesex	3	0.00 ± 0.00	0.0000 ± 0.0000
Oxfordshire	10	0.53 ± 0.09	0.0143 ± 0.0089
Shropshire	6	0.60 ± 0.21	0.0123 ± 0.0085
Sussex	12	0.41 ± 0.13	0.0110 ± 0.0070
Wiltshire	18	0.29 ± 0.12	0.0079 ± 0.0052
Worcestershire	5	0.40 ± 0.23	0.0123 ± 0.0089
Yorkshire	24	0.51 ± 0.04	0.0136 ± 0.0080

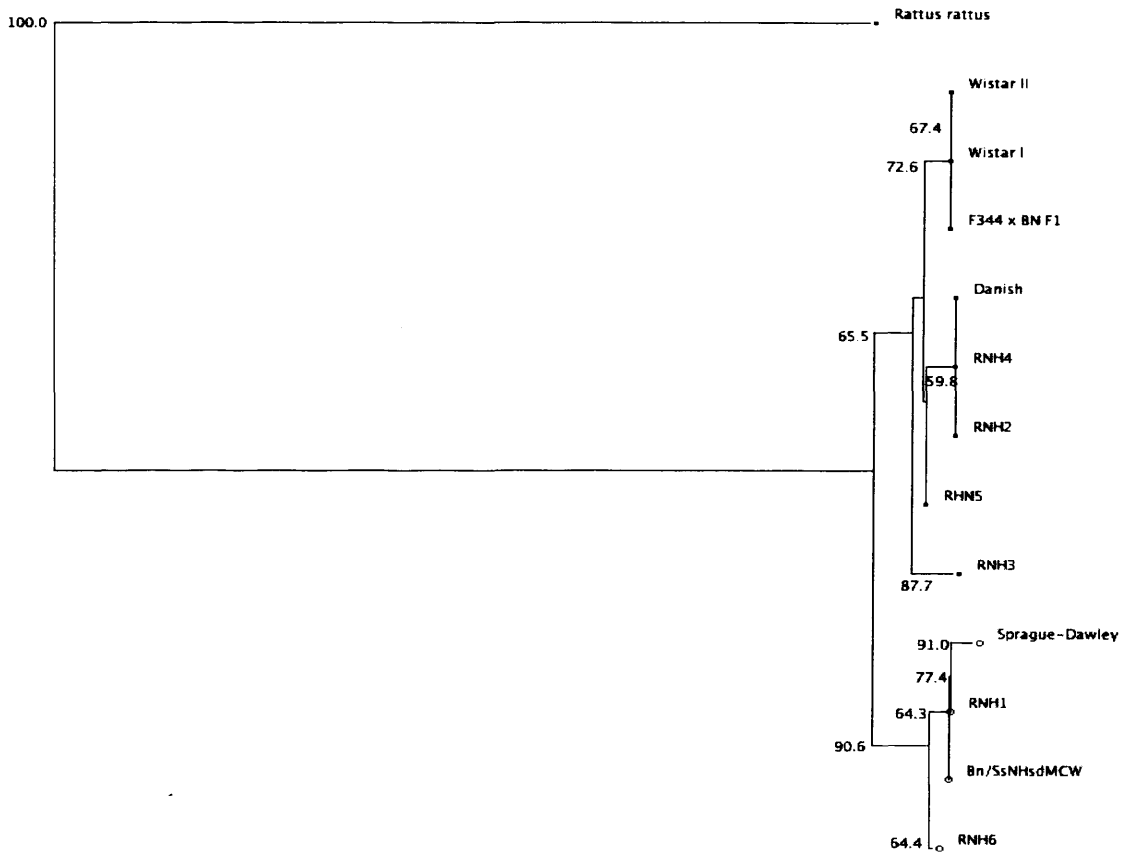
From Analysis of Molecular Variance, (AMOVA, $p < 0.001$) 33% of the total variance can be assigned to among population diversity while 67% can be attributed to diversity within populations (Table 3.4).

Table 3.4 Analysis of molecular variance

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Population	13	98.420	0.510	33
Within Population	171	172.856	1.017	67
Total	184	271.276	1.527	

The phylogenetic relationships between all available *R. norvegicus* mtDNA sequences from the Genbank were analysed by a neighbour-joining tree (Figure 3.8). These include sequences from several lab strains, a wild caught brown rat from Denmark and as an outgroup, a *Rattus rattus* sequence. Relative to the sample in this study, the Wistar strain has a deletion at position 77, the *R. rattus* and Sprague-Dawley strain have an insertion at position 305. The *R. rattus* sample also has an insertion at position 266. From the results, it showed that rats with haplotype RNH1 and RNH6 have the closer link to the common lab strain Spraque Dawley, while rats with the other haplotypes are closer to the Danish wild caught brown rat.

Figure 3.8 Neighbour-joining tree calculated with Tamura & Nei (1993) distances for the 6 mtDNA haplotypes of English brown rats (RNH1-6), several strains of lab rats (Wistar - Accession numbers:MIRNXX, RNMITDLO, Sprague-Dawley - MIRNDNC, BN/SsNHsdMCW - AY172581, F344 X BN F1 - AY769440), a Danish wild caught brown rat - RNO428514 and the closely related black rat *Rattus rattus* - DQ009794. The percentage bootstrap support (1000 replicates) are shown for nodes with greater than 50% support.



A Mantel test found no relationship between Φ_{st} and the geographical distance between the populations (r = 0.171, n = 91, p = 0.120, Figure 3.9). To examine if variance increased over geographical distance the residuals from a linear regression of Φ_{st} against geographical distance were plotted against geographical distance (Hutchison & Templeton 1999). A Mantel test found no significant relationship (r = 0.0001, n = 105, p = 0.482).

Figure 3.9 Scatterplot of Φ_{st} estimates against geographical distance separating each pairwise combination of populations.

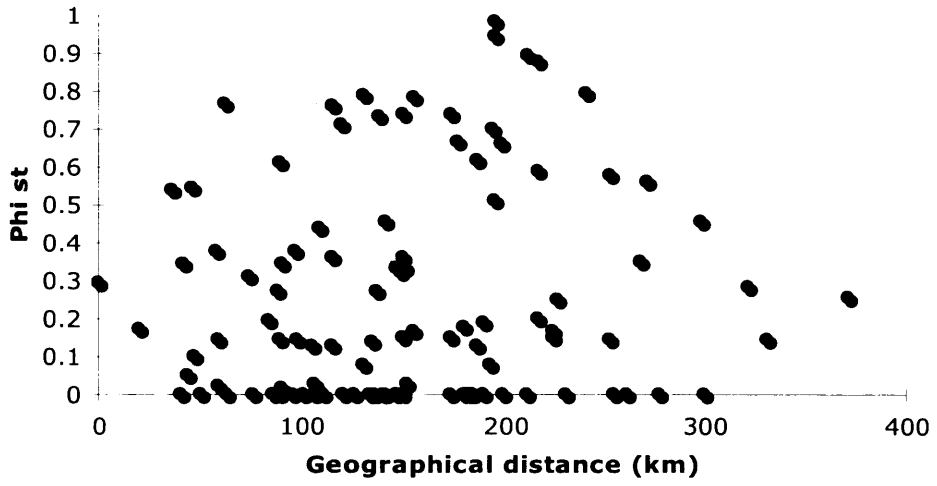
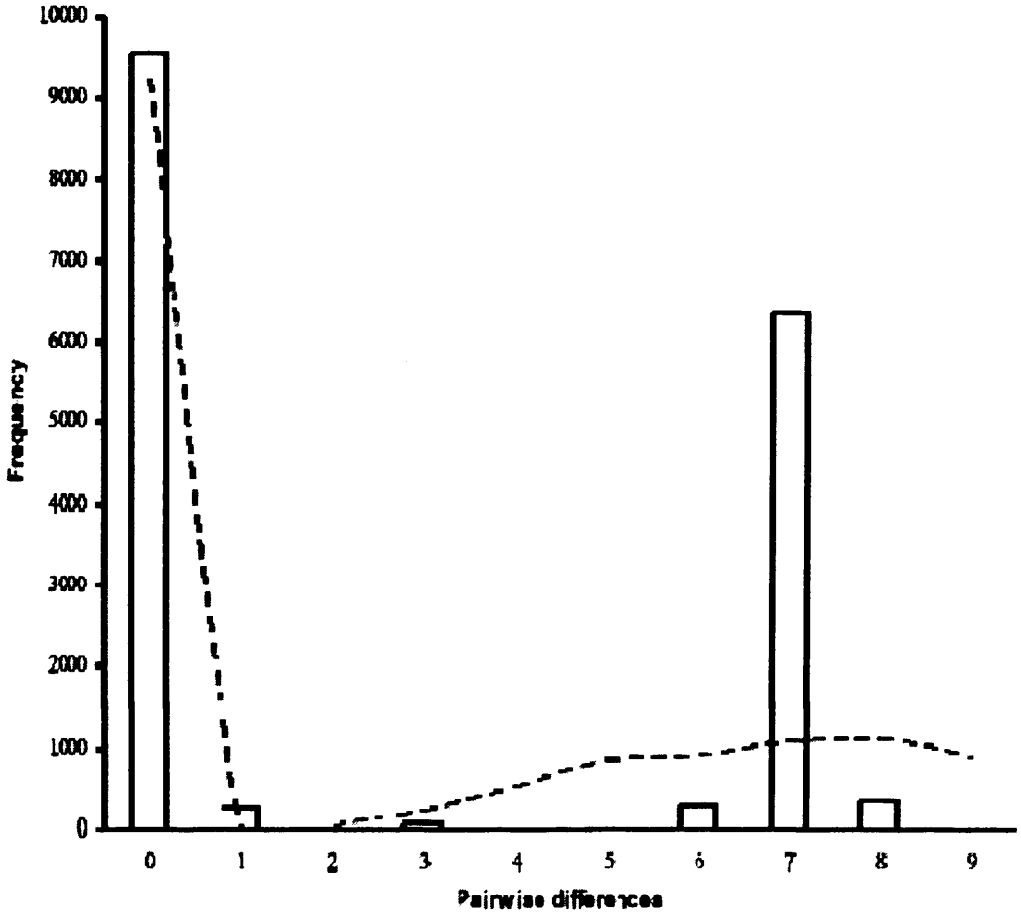


Figure 3.10 shows the observed number of differences between pairs of haplotypes. This distribution is not significantly different from the

spatial expansion model (SSD = 0.1023, Bootstrap replicates = 1000, p = 0.133)

Figure 3.10 Mismatch distribution of pairwise sequence differences in mtDNA from *R. norvegicus* (black bars). The dotted line represents the expected results from a spatial expansion model.



3.3.3 Microsatellite DNA

3.3.3.1 Number of allele and allele frequencies

A total of 81 alleles was detected across 6 microsatellites loci tested in all 185 of the brown rat individuals. The results showed that all of the loci were polymorphic and the number of alleles ranges from 10 (D5) to 18(D12). The mean number of alleles detected at each locus was 13.5.

Allele frequencies of each microsatellite loci in brown rat individuals tested were as in Table 3.5. In microsatellite locus D5, D14 and D8, allele frequency was dominated by certain alleles. Locus D5 is dominated by allele size 142bp, locus D14 is dominated by allele size 152bp and locus D8 is dominated by allele size 214bp. There are also a few private alleles, allele size 158bp in locus D5 (4.8% in Cambridgeshire), alleles size 78bp (33.3% in Hampshire), 80bp and 82bp (5.6% and 2.8% respectively in Wiltshire) in locus D3.

Table 3.3 Locus, Allele sizes and Allele Frequencies for rats individual in 14 populations.

Locus	Allele	Allele Frequency													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
D17	200	0.000	0.000	0.214	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.083	0.000	0.052
	202	0.000	0.000	0.071	0.167	0.111	0.063	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.121
	204	0.460	0.786	0.286	0.167	0.278	0.375	0.500	0.333	0.100	0.750	0.545	0.583	0.000	0.517
	222	0.020	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.034
	226	0.060	0.024	0.286	0.000	0.111	0.063	0.014	0.500	0.300	0.083	0.000	0.167	0.000	0.034
	228	0.100	0.143	0.071	0.000	0.000	0.000	0.171	0.000	0.200	0.000	0.091	0.000	0.000	0.034
	380	0.060	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.050	0.000	0.000	0.028	0.000	0.000
	382	0.000	0.000	0.000	0.000	0.222	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	384	0.120	0.000	0.071	0.333	0.056	0.125	0.057	0.000	0.050	0.083	0.045	0.083	0.200	0.017
	386	0.100	0.024	0.000	0.333	0.222	0.125	0.114	0.000	0.250	0.083	0.045	0.056	0.300	0.155
	396	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.050	0.000	0.091	0.000	0.000	0.000
	398	0.080	0.000	0.000	0.000	0.000	0.125	0.057	0.000	0.000	0.000	0.091	0.000	0.500	0.017
	400	0.000	0.024	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017
D3	76	0.000	0.071	0.000	0.167	0.000	0.000	0.086	0.000	0.000	0.083	0.000	0.111	0.200	0.259
	78	0.000	0.000	0.000	0.000	0.333	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	80	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000
	82	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.000
	84	0.000	0.000	0.071	0.167	0.111	0.000	0.014	0.000	0.000	0.083	0.000	0.000	0.000	0.000
	86	0.220	0.333	0.286	0.000	0.167	0.000	0.329	0.167	0.100	0.250	0.091	0.389	0.400	0.155
	88	0.060	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.100	0.000	0.000	0.000	0.100	0.086
	90	0.040	0.024	0.000	0.000	0.333	0.063	0.057	0.000	0.000	0.000	0.000	0.028	0.100	0.069
	92	0.100	0.071	0.071	0.000	0.000	0.125	0.171	0.500	0.400	0.000	0.000	0.000	0.000	0.069
	94	0.260	0.167	0.000	0.333	0.000	0.188	0.157	0.000	0.150	0.250	0.227	0.194	0.200	0.259
	96	0.080	0.119	0.071	0.000	0.000	0.063	0.171	0.000	0.000	0.250	0.136	0.056	0.000	0.034
	98	0.060	0.048	0.357	0.167	0.056	0.250	0.000	0.000	0.200	0.083	0.091	0.000	0.000	0.017
	100	0.160	0.167	0.071	0.167	0.000	0.000	0.000	0.333	0.050	0.000	0.227	0.083	0.000	0.034
	102	0.020	0.000	0.071	0.000	0.000	0.313	0.000	0.000	0.000	0.000	0.227	0.056	0.000	0.017

Locus	Alelle	Alelle Frequency													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
D5	98	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.083	0.000	0.000	0.000	0.000
	102	0.340	0.214	0.143	0.167	0.556	0.750	0.386	0.000	0.600	0.250	0.136	0.417	0.600	0.172
	122	0.060	0.000	0.357	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.034
	140	0.000	0.024	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	142	0.300	0.405	0.071	0.167	0.444	0.250	0.443	0.833	0.400	0.417	0.591	0.167	0.400	0.517
	146	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.028	0.000	0.000
	152	0.280	0.071	0.286	0.667	0.000	0.000	0.043	0.000	0.000	0.167	0.227	0.361	0.000	0.241
	154	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.028	0.000	0.034
	156	0.000	0.238	0.143	0.000	0.000	0.000	0.000	0.167	0.000	0.083	0.000	0.000	0.000	0.000
	158	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D12	242	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.150	0.000	0.000	0.000	0.000	0.000
	244	0.060	0.071	0.357	0.333	0.000	0.250	0.100	0.167	0.150	0.083	0.091	0.417	0.200	0.172
	246	0.160	0.048	0.071	0.000	0.056	0.000	0.071	0.000	0.050	0.000	0.045	0.111	0.000	0.034
	254	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.150	0.083	0.000	0.000	0.000	0.000
	256	0.040	0.262	0.357	0.167	0.167	0.125	0.043	0.167	0.100	0.417	0.045	0.056	0.000	0.172
	258	0.160	0.071	0.000	0.000	0.222	0.063	0.043	0.000	0.100	0.000	0.136	0.083	0.100	0.086
	260	0.020	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	262	0.000	0.024	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	264	0.020	0.000	0.000	0.000	0.000	0.188	0.000	0.000	0.100	0.000	0.000	0.028	0.000	0.000
	308	0.140	0.119	0.000	0.000	0.056	0.000	0.114	0.000	0.100	0.083	0.000	0.028	0.000	0.034
	312	0.100	0.000	0.000	0.000	0.056	0.063	0.086	0.000	0.000	0.000	0.091	0.028	0.000	0.017
	314	0.120	0.167	0.000	0.167	0.389	0.125	0.443	0.500	0.100	0.083	0.273	0.056	0.300	0.121
	316	0.040	0.024	0.000	0.167	0.000	0.063	0.043	0.000	0.000	0.000	0.136	0.083	0.200	0.086
	318	0.020	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.167	0.000	0.083	0.000	0.000
	320	0.020	0.000	0.143	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.086
	322	0.060	0.119	0.071	0.167	0.000	0.063	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.190
	324	0.020	0.024	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.083	0.091	0.000	0.000	0.000
	326	0.000	0.024	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.028	0.200	0.000

Locus	Alelle	Alelle frequency													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
D14	**140**	0.080	0.048	0.000	0.000	0.167	0.125	0.029	0.000	0.100	0.000	0.000	0.028	0.000	0.086
	142	0.100	0.000	0.000	0.333	0.333	0.250	0.143	0.333	0.050	0.167	0.455	0.250	0.400	0.103
	144	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.045	0.000	0.000	0.017
	146	0.060	0.000	0.000	0.000	0.000	0.000	0.014	0.333	0.100	0.000	0.000	0.000	0.000	0.086
	148	0.140	0.000	0.000	0.000	0.000	0.125	0.171	0.000	0.100	0.000	0.136	0.028	0.000	0.034
	150	0.080	0.119	0.214	0.000	0.056	0.063	0.114	0.000	0.250	0.250	0.227	0.028	0.000	0.172
	152	0.100	0.357	0.214	0.333	0.111	0.125	0.100	0.333	0.200	0.083	0.045	0.306	0.300	0.172
	154	0.180	0.381	0.214	0.333	0.167	0.188	0.157	0.000	0.100	0.083	0.000	0.194	0.200	0.207
	156	0.240	0.071	0.143	0.000	0.167	0.125	0.157	0.000	0.100	0.250	0.045	0.167	0.000	0.000
	158	0.000	0.024	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.017
	160	0.020	0.000	0.214	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.100	0.103
	164	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.045	0.000	0.000	0.000
D8	**196**	0.020	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	198	0.000	0.048	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.052
	200	0.000	0.071	0.000	0.000	0.000	0.000	0.057	0.000	0.150	0.000	0.000	0.000	0.000	0.000
	202	0.080	0.024	0.071	0.000	0.000	0.125	0.029	0.000	0.000	0.167	0.000	0.000	0.000	0.034
	204	0.080	0.024	0.000	0.000	0.000	0.000	0.114	0.000	0.000	0.000	0.182	0.028	0.200	0.086
	206	0.140	0.071	0.214	0.333	0.056	0.000	0.086	0.333	0.100	0.167	0.091	0.194	0.000	0.155
	208	0.140	0.095	0.000	0.000	0.000	0.188	0.157	0.167	0.200	0.083	0.000	0.139	0.200	0.138
	210	0.200	0.000	0.000	0.500	0.556	0.125	0.114	0.167	0.100	0.333	0.091	0.250	0.200	0.138
	212	0.100	0.024	0.429	0.000	0.111	0.188	0.086	0.167	0.200	0.083	0.136	0.250	0.200	0.052
	214	0.100	0.071	0.143	0.167	0.167	0.125	0.171	0.167	0.050	0.167	0.273	0.028	0.100	0.086
	216	0.080	0.310	0.143	0.000	0.000	0.000	0.086	0.000	0.100	0.000	0.045	0.028	0.000	0.086
	218	0.020	0.190	0.000	0.000	0.111	0.063	0.000	0.000	0.000	0.000	0.136	0.028	0.100	0.103
	220	0.040	0.071	0.000	0.000	0.000	0.188	0.071	0.000	0.100	0.000	0.000	0.028	0.000	0.052
	222	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.028	0.000	0.017

Note: Numbers on the top row represent the county; 1: Berkshire, 2: Cambridge, 3: Dorset, 4: Gloucestershire, 5: Hampshire, 6: Kent, 7: Leicestershire, 8: Middlesex, 9: Oxfordshire, 10: Shropshire, 11: Sussex, 12: Wiltshire, 13: Worcestershire, 14: Yorkshire.

Table 3.3. Name of County, Locus, Observed Heterozygosity (Ho) and Expected Heterozygosity (He)

		Locus						Heterozygosity (Observed/Expected)					
County	N	D17	D3	D5	D12	D14	D8	D17	D3	D5	D12	D14	D8
Berkshire	25	8	9	5	15	9	11	0.32/0.74	0.76/0.83	0.44/0.71	0.84/0.90	0.68/0.85	0.60/0.87
Cambridge	21	5	8	6	12	6	11	0.38/0.36	0.85/0.80	0.76/0.72	0.76/0.85	0.62/0.70	0.71/0.83
Dorset	7	6	7	5	5	5	5	0.71/0.77	0.71/0.76	0.85/0.74	0.43/0.71	0.57/0.80	1.00/0.72
Gloucestershire	3	4	5	3	5	3	3	1.00/0.72	1.00/0.77	0.67/0.50	1.00/0.77	0.66/0.66	1.00/0.611
Hampshire	9	6	5	2	7	6	5	0.77/0.80	0.77/0.73	0.44/0.50	0.66/0.76	0.66/0.80	0.55/0.63
Kent	8	8	6	2	9	7	7	0.75/0.80	0.87/0.78	0.50/0.37	0.62/0.85	0.87/0.83	0.62/0.84
Leicestershire	35	9	8	6	10	11	12	0.31/0.70	0.74/0.80	0.60/0.64	0.71/0.76	0.83/0.87	0.77/0.88
Middlesex	3	3	3	2	4	3	5	0.67/0.61	1.00/0.61	0.33/0.27	0.66/0.66	1.00/0.66	0.66/0.77
Oxfordshire	10	7	6	2	9	8	8	0.50/0.79	0.70/0.75	0.40/0.48	0.50/0.88	0.70/0.84	0.70/0.85
Shropshire	6	4	6	5	7	6	6	0.33/0.41	0.83/0.79	0.50/0.72	0.50/0.76	0.66/0.80	0.50/0.79
Sussex	11	7	6	4	10	7	8	0.45/0.66	0.72/0.81	0.27/0.58	0.72/0.85	0.72/0.71	0.63/0.83
Wiltshire	18	6	9	5	11	7	10	0.22/0.61	0.72/0.78	0.66/0.66	0.83/0.78	0.66/0.77	0.61/0.81
Worcester	5	3	5	2	5	4	6	0.60/0.62	1.00/0.74	0.00/0.48	0.80/0.78	0.40/0.70	0.20/0.82
Yorkshire	29	10	10	5	10	10	10	0.31/0.68	0.76/0.82	0.34/0.64	0.72/0.86	0.58/0.86	0.82/0.90
Total	190	86	93	54	119	92	107						

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Degree : PhD.

Year: 2008

3.3.3.2 Observed and expected heterozygosity

The observed heterozygosities in the brown rat populations in this study range from 0.00 to 1.00. The highest observed heterozygosity was observed in locus D17 from Gloucestershire population, D3 from Gloucestershire, Middlesex and Worcester population, Locus D12 from Gloucestershire population, Locus D14 from Middlesex population and locus D8 from Dorset and Gloucestershire populations.

Only 22.6% of the observed heterozygosity (Ho) is higher than expected heterozygosity (He), 1.2% Ho is the same as He (locus D5 from Wiltshire population) and 76.2% of Ho is lower than He. A substantial deficit of heterozygotes was observed for locus D17 in Berkshire, Leicestershire, Wiltshire and Yorkshire population, D5 locus in Sussex and Worcestershire population, and D8 locus in Worcestershire population (Table 3.6). Absence of heterozygosity was observed for locus D5 in Worcester population.

3.3.3.3 Analysis of population structure

The statistical software package, STRUCTURE version 2.01 (Pritchard *et al.*, 2000) was used in this study to determine the most likely number of population units (K). This method can accurately cluster individuals into their appropriate populations, even using a modest number of loci. However, the accuracy of the assignment also depends on a number of factors, including the number of individuals, the number of loci, the

amount of admixture and the extent of allele-frequency differences among populations.

In our study, rat individuals were assigned to populations which is assumed to be K population (where K is unknown). The program was run 4 times for each value of K from 1 to 8 (number of populations). The run with the highest likelihood was retained for each K value. After some preliminary tests of the convergence time needed for the Monte-Carlo Markov Chain, a burn-in period of 1,000,000 of steps followed by another 1,000,000 steps was chosen. The proportion of membership of each population in each cluster was calculated when the number of population units K was estimated (Figure 3.11)

The clustering method showed that rat individuals in our study are divided into 4 populations (K=4) (Figure 3.12). Individuals collected from Dorset and Wiltshire as Population 1, Leicestershire as population 2, individuals from Yorkshire and Cambridgeshire as population 3 and Gloucestershire, Hampshire, Middlesex, Oxfordshire and Worcestershire were categorised as population 4. However, individuals from Berkshire, Kent, Shropshire and Sussex cannot be categorised as any named population because each of the county consists of completely mixed set of individuals (Figure 3.12).

3.3.3.4 Analysis of molecular variance (AMOVA)

When rat individuals were assumed as a single population in each county, AMOVA ($p<0.0001$) of the 14 populations showed that 8% of the total variance can be allocated to among population diversity, while 92% to

within population diversity (Table 3.7). However, AMOVA ($p<0.0001$) of the K=4 populations through clustering method showed that 11% is assigned to variation among populations and 89% to within population (Table 3.8).

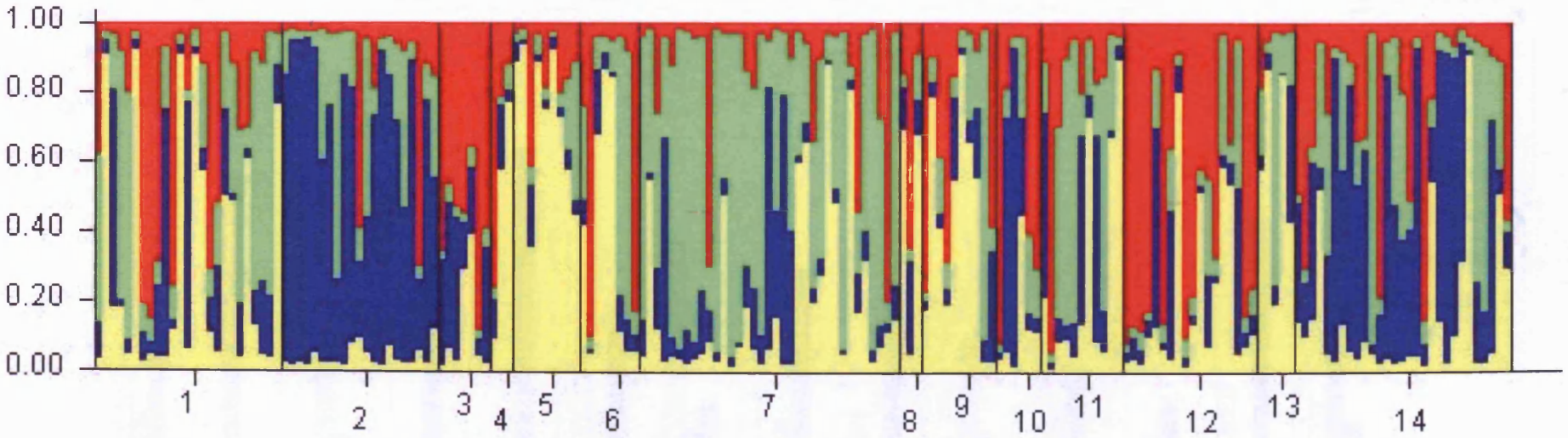
Table 3.7 Analysis of molecular variance for brown rats from 14 counties.

Source of Variation	df	Sum of Squares	Variance Components	Percentage of Variance
Among Population	13	156.399	0.501	8%
Within Population	171	969.439	5.669	92%
Total	184	1125.838	6.170	

Table 3.8 Analysis of molecular variance for brown rats samples from 4 populations.

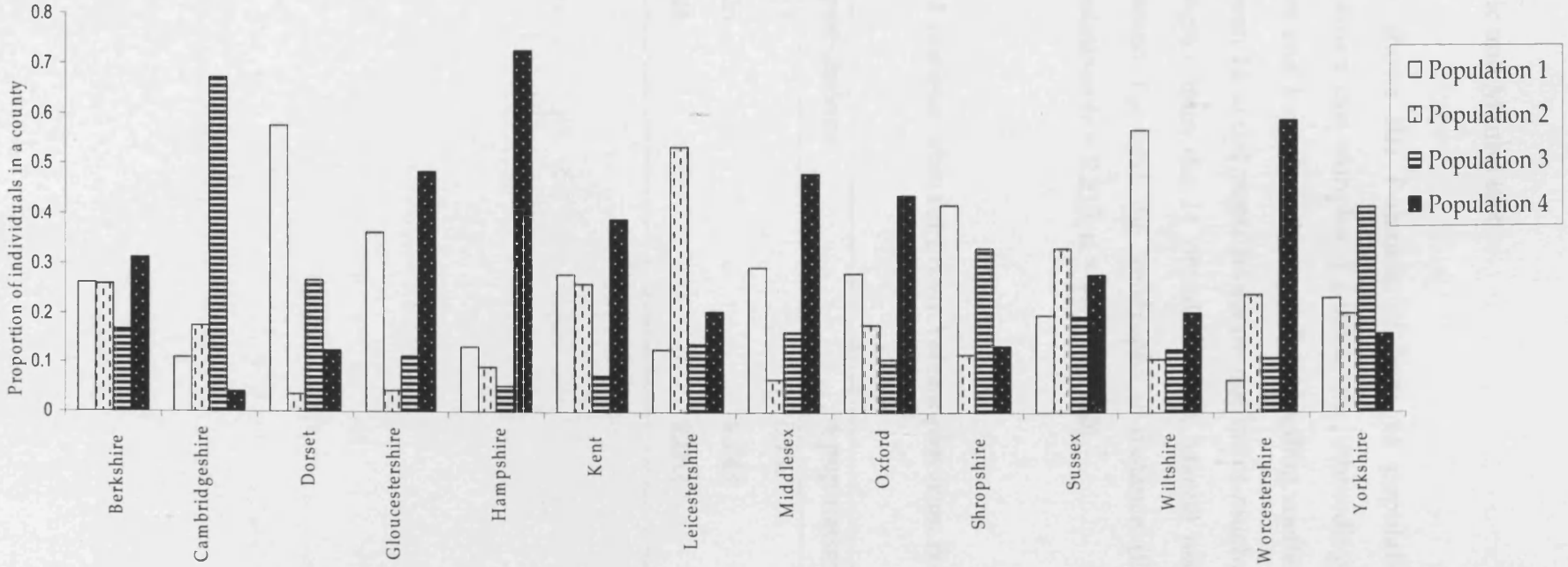
Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Population	3	112.109	0.691	11%
Within Population	171	1013.728	5.601	89%
Total	184	1125.838	6.292	

Figure 3.11 Bar plot of K4 STUCTURE analysis for brown rats in England.



Note : Numbers on X axis represent the county; 1: Berkshire, 2: Cambridge, 3: Dorset, 4: Gloucestershire, 5: Hampshire, 6: Kent, 7: Leicestershire, 8: Middlesex, 9: Oxfordshire, 10: Shropshire, 11: Sussex, 12: Wiltshire, 13: Worcestershire, 14: Yorkshire

Figure 3.12 Brown rat proportion comparison for each county analysed by STRUCTURE (ver 2.01)



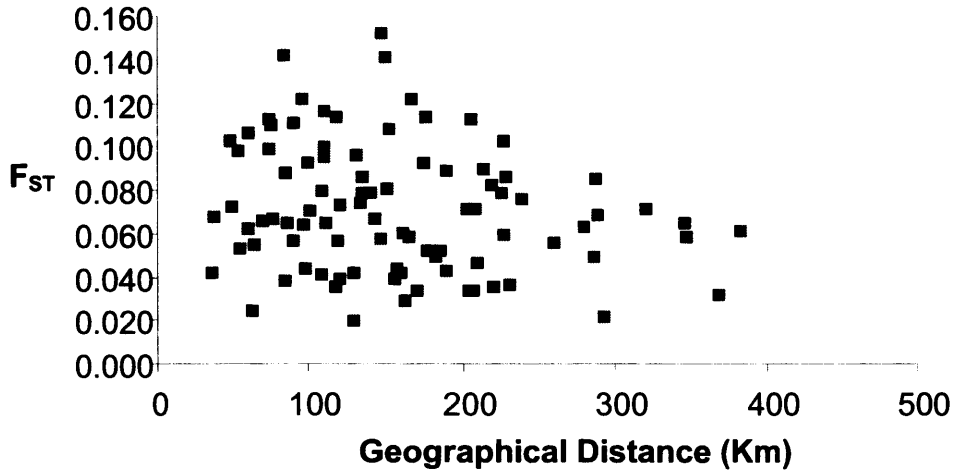
3.3.3.5 F-statistic and Mantel test

Table 3.9 below shows the F-statistic of both 14 populations and 4 populations of brown rats samples. F_{IS} value, the inbreeding coefficient within population and F_{IT} value, the overall inbreeding coefficient (Lowe *et al.*, 2004) between 14 and 4 populations do not differ much. F_{ST} from 4 populations is higher than the 14 populations. A Mantel test found no relationship between F_{ST} and the geographical distance (Figure 3.13) between the populations ($r = 0.215$, $n = 91$, $p = 0.150$)

Table 3.9 Mean F-statistic table for brown rat samples from both 14 and 4 populations.

	14 populations	**4 populations**
F_{IS}	0.117	0.197
F_{IT}	0.226	0.243
F_{ST}	0.124	0.062

Figure 3.13 Scatterplot of F_{ST} estimates against geographical distance separating each pairwise combination of populations.



3.4 Discussion

3.4.1. Isozymes

Although level of genetic polymorphism in our brown rats cannot be quantified as a result of this study, what can be said is that, it is lower than in all rodent species which have been studied by protein electrophoresis.

Mammals usually have three independent Phosphoglucomutase (PGM) loci (Harris & Hopkinson, 1976). Result from our study revealed only 2 loci of PGM, i.e.; PGM-1 and PGM-2 which also agree with findings from Filippucci *et al.,* (2002) where two (PGM-1 and PGM-2) have been scored in several *Apodemus* species; *Apodemus agrarius, A. peninsulae, A. flavicollis, A. sylvaticus, A. alpicola, A. uralensis, A.* cf. *hyrcanicus, A.hermonensis, A. m. mystacinus* and *A. m. epimelas.* However, no variation

were detected in our samples compared to a study done by Koga *et al.*, (1972) where variation was found in his outbred (both wild and laboratory) populations of *R. norvegicus* for PGM. No polymorphism was detected in the inbred strains (Adams *et al.*, 1984) for the same enzyme.

It is possible that the brown rats throughout England are not polymorphic at the 3 isozyme loci. However, another reason might be that the levels of isozymes activity from the frozen samples that have been collected several years previously have been reduced and not suitable for isozyme analysis (Murphy *et al.*, 1996).

3.4.2. Mitochondrial DNA (mtDNA)

A total of 6 haplotypes with 10 different nucleotide positions within the Hyper Variable Region 1 was detected from 185 rat individuals. The haplotype and nucleotide diversity is quite low in this study, however it is similar to what has been found in the black rat, *Rattus rattus* (Hingston *et al.*, 2005). The results from AMOVA also shows that variation can mainly be found within (67%) rather than between populations (33%). These findings demonstrate a low level of variability, when compared to studies done on other *Rattus* species. 30 haplotypes were found in 383 individuals of *R. fuscipes gryii* from 14 island and 2 mainland population in Australia (Hinten *ef al.* 2003). The percentage of variation between populations is also very high (92%). Their results showed that gene flow between islands did not occur. Another study on *R. exulans* identified 94 haplotypes in 132 individuals from 18 populations (Matisoo-Smith *et al.*, 1998).

The distribution of pairwise haplotype differences (Figure 3.10) matches those expected under a spatial expansion model (Rogers & Harpending, 1992), that is where the range of a population is initially restricted to a very small area and then the range of the population increases. The resulting population then subdivides. Low nucleotide diversity found in a widespread species is often attributed to a slow range expansion following a small population size (founder/ bottleneck effects) (Joseph et al. 2002). Bottlenecks often generate ragged distribution with peaks at large values (Rogers & Harpending 1992), as demonstrated by the data in Figure 3.10. Results in this study suggesting a recent bottle neck and subsequent range expansion fits in with the invasion history of the brown rat to Britain.

If, as seems likely from our data, the English brown rat population has undergone a recent (on an evolutionary scale) expansion, it is unlikely to be yet at equilibrium. This would make relating F_{ST} values to gene flow and drift by the Wright (1931) equation $F_{ST} \cong 1/(4Nm + 1)$ inappropriate when natural populations are not at equilibrium (McCauley 1993). Regional equilibrium can be tested for by comparing F_{ST} to geographical distance between regions (Hutchison & Templeton 1999). If the population has reached equilibrium there will be a linear relationship between F_{ST} and geographical distance. Our results (Figure 3.9) show there is no relationship between Φ_{ST} (analogous to F_{ST}) and geographical distance. Our results most closely resemble Hutchison & Templeton's case III, where the population is fragmented into small, isolated populations and drift becomes more important than gene flow. This allows allele frequencies in each population to drift independently relative to geographical distance and random sampling of gametes creates a large degree of variance between the plotted points which are also independent

of geographic distance (Hutchison & Templeton 1999). We found no significant correlation between the residual of Φ_{ST} (a measure of the degree of variance) and geographical distance, indicating that our data does indeed fit the case III model. This model and our data suggest that the English rural rat population is not yet at equilibrium and that gene flow is less important than drift in explaining the genetic structure found.

3.4.3 Microsatellite DNA

The mean number of alleles detected in this study was 13.5. As a comparison this result displayed higher mean number of alleles compared to a study on the same species in Denmark, which is 4.8 (Heiburg, 2002). Result in our study also shows higher mean number of alleles than results from Calmet *et al.*, (2001) on the same species in France, which is 12.4. Another study done by Vazquez-Domiguez *et al.* (2001) on *R. fuscipes* and *R. leucopus* have mean number of alleles were 11.1 and 7.1 respectively. The average number of alleles observed at each locus is a good indicator of genetic variability, inbreeding and population bottlenecks (Grzybowski and Prusak, 2004).

The number of alleles in this study ranges from 10 to 18 and this result is also higher than results from Heiburg (2002) which range between 2 to 6 alleles. Number of alleles ranges from 7 to 17 in *R. fuscipes* and 1 to 14 in *R. leucopus* (Vazquez-Domiguez *et al.* 2001). The range of alleles number in Calmet *et al.*, (2001) work is quite large, i.e; 7 to 21. They used 2 loci used in our study; D3 and D8. 11 and 14 allele were detected for the respective loci in their study compared to 14 alleles in each respective loci were identified in our study.

76.2% of the Ho in our study were lower than the He and some of the Ho showed significant deficit in this study. Loew *et al.*, (2005) suggested that decrease in heterozygosity indicate that non random mating and genetic drift within population might have occurred. It is also clear that the F_{IT} value when population assumed to be 4 or 14 is positive (+ ve). The major contribution, F_{IS} is also + ve. This positive value also suggests reduction of heterozygosity (Lowe *et al.* 2004) and could be due to selection or non random mating (Cramer *et al.*, 1988). Although rat individuals in this study were collected from different geographic regions and at different times, the reduction in heterozygosity was most likely due to inbreeding, non random mating and genetic drift. There is also a probability that the population have small effective sizes or high consanguinity within demes, or both (Cramer *et al.*, 1988). If the effective population size is small, this is expected to lead to changes in the genetic structure of a population because of random drift and hence, distinct population differentiation (Surridge *et al.*, 1999). Moreover, the high percentage of variation within population through AMOVA (Table 3.3.7 and 3.3.8) and the reduced levels of heterozygosity may reflect a non random mating with significant amounts of inbreeding and consanguinity.

Percentage of variation among populations by AMOVA in microsatellite DNA study is less (11%) than what have been shown in mtDNA (33%). The result is as expected since mt DNA only measures female-mediated geneflow. Whereas, microsatellite DNA measures both paternal and maternal. It has been reported that male rats move further than the females. In one experiment using radio telemetry, a rat moved 3.3km in one night (Taylor & Quy, 1978). Another study revealed that

males moved a mean distance of 660m and females 340m. Rats do move often when changing home sites, which males did every 7 days compared to female every 14 days (Taylor, 1978).

The lack of agreement between microsatellite and mitochondrial data could also be due to genetic drift effects of a recent population bottleneck (Haavie *et al.*, 2000). Reduced effective population size arising through bottlenecks would increase the rate of genetic drift, thereby increasing the rate of differentiation at polymorphic loci (Hedrick, 1999). Moreover, the frequency of inbreeding would increase in a small population thereby leading to heterozygous deficiency. Whereas nuclear DNA has sexual, diploid inheritance, mtDNA is haploid and is inherited asexually, predominantly, if not completely, through the maternal line (Avise, 1994). This makes the effective population size of the mitochondrial genome four times smaller than that for the nuclear genome. Therefore, in a finite population, genetic drift will be a stronger force in mtDNA evolution compared to nuclear DNA evolution (Avise, 1994). Accordingly, the rate of differentiation due to genetic drift is expected to be particularly rapid at the mitochondrial genome when the overall population size is small; i.e. during a bottleneck.

However, results from microsatellite DNA agree with the results from mtDNA where there is no relationship between F_{ST} and geographical distance. Again, this resemble Hutchison & Templeton's case III; the population is fragmented into isolated populations. Our study also indicates that low gene flow occurs between populations and this explain that drift is more important than gene flow.

SPECIAL NOTE

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Chapter 4

FINE SCALE POPULATION STRUCTURE OF THE BROWN RATS

4.0 Fine Scale Population Structure of the Brown Rats

4.1 Introduction

Dispersal is an essential process with a number of consequences for populations. It affects population stability, controls the pattern of gene flow (Johnson & Gaines, 1990) and therefore influences the genetic make-up of a population (Beebee & Rowe, 2004). High levels of effective dispersal or gene flow can result in homogeneity of alleles among populations and may promote plasticity of phenotypes, particularly in fitness traits (Via & Lande, 1985).

Dispersal parameters are notoriously difficult to determine in the field, especially for small organisms (Schweizer *et al.*, 2007). Measuring dispersal/migration through mark and recapture method is one well-used method but very time consuming, expensive and not suitable for long distance dispersal. Futhermore, it does not necessarily reflect the movement of genes, unless it is an effective dispersal where the migrant is successful in reproducing in its new population.

The difficulty of using direct measures of dispersal/migration has lead to the use of indirect measures, using genetic data. Genetic based studies can provide dispersal estimates, and are easier and quicker to perform compared to the direct methods (Berry *et al.*, 2004).

Wright (1951) developed the basic method to calculate the number of migrants entering a population per generation; which gives reasonable estimates of dispersal rate using the assumptions of the island model, $F_{ST} \approx$

$1/(4Nm + 1)$ where Nm is the number of individuals exchanged between populations per generation. In cases where the spatial scale is small, migration rate is relatively high, sample sizes and numbers of loci need to be large (Whitlock and McCauley 1999). These assumptions do not always true at large scale populations if we expect no selection or mutation occurs and if each population persists indefinitely and has reached equilibrium between migration and drift. Strong selection can change the pattern of genetic differentiation if the migration rate is small.

Studies have shown that genetic techniques answer dispersal issues within fine scale population of a variety of vertebrate taxa. For example, 7 microsatellite loci were found to be highly polymorphic with allele numbers ranging from 12 to 42 in 668 túngara frogs in Panama (Lampert *et al.,* 2003). The frogs were originated from 17 different sites, where the distance between sites ranges from 260 m to 11.8 km. Genetic differences were significantly correlated with geographical distances as shown by Mantel test. They suggested that although túngara frogs seem to be quite mobile on a small scale of several hundred metres, there was a high level of genetic population differentiation at a geographical scale of several kilometres. A river (The Charges River) provided a major gene-flow barrier but migration still occurred, perhaps by unintentional human transport. Another example is a study on the common vole (*Microtus arvalis*) by Schweizer *et al.* (2007). They combined direct and indirect measures of dispersal to evaluate genetic consequences of dispersal for patchy populations conducted in northwestern Switzerland. 440 voles were trapped at 6 different sites over 3 time periods with distances between sites of 330m to 2.5km. Field data (direct measures) showed no direct evidence for dispersal, although no dispersal barriers between sites. Moreover, genotypic data (indirect measures) from 321 captured voles

revealed high levels of genetic diversity even between the closest populations at the 10 microsatellite loci analysed, with the number of alleles ranging between 7 to 37 per locus. Despite significant genetic structure, assignment analyses showed a relatively high proportion of individuals as being immigrants into the population where they were captured. They suggested low and sex dependent effective dispersal rate due to only a few immigrants reproducing successfully in the new populations can explain the high genetic differentiation.

Genetic data are also frequently used to understand similarities between populations, using genetic distance. Genetic distance values typically vary between 0, which means the two populations are identical and 1, where they are completely different. There are many genetic distance measures, but one of the most popular and widely used is Nei's genetic distance (D) (Nei, 1972). This method is based on an Infinite Alleles Models where D can be described as;

$$D = -\ln J_{xy} / \sqrt{J_x J_y}$$

Jxy is the probability that an allele drawn from population x is the same as that from population y, Jx is the probability that 2 alleles drawn from population x are the same and Jy is the probability that 2 alleles drawn from population y are the same. Nei's genetic distance (D) measures the accumulated number of gene substitutions per locus. If the rate of gene substitution per unit time is constant, then it is linearly related to evolutionary time and geographical distance (Lowe *et al.*, 2004).

In general, brown rats do not move great distances (Meehan, 1984). In one experiment using radio telemetry, one male rat moved 3.3km at

speeds of 0.5 - 1.1 km/hr in a single night (Taylor & Quy, 1978). It is believed that brown rats tend to move from the fields into the farm building in early winter and move back to the field during spring (Huson & Rennison, 1981). Bishop and Hartley (1976) showed that more males than females are involved in movements into and out of the fields. Lambert (2003) showed that the size of home range of rats on Yorkshire farms varied from 19.5 m^2 to 14,571 m^2 for males and from 38.5m^2 to 1,695 m^2 in females.

Brown rats in the United Kingdom are predominantly commensal in both rural and urban areas. In the field, Middleton (1954) and Brodie (1981) found that arable field margins become more infested with brown rat in late summer and early autumn when crops are ripening. However, rats populations on farms tend to fluctuate throughout the year depending on availability of food. They usually form burrows outdoors for nesting, which are generally 65-90mm in diameter and 0.5m deep. When burrowing, they tend to select sites that are near to water and a food source. In urban areas, the presence of brown rats is correlated with the presence of water and vegetation (Traweger *et al.*, 2006).

In the wild, brown rats live in colonies with up to 6 females living in a small burrow system. One or a small number of males are associated with the group. Brown rats breed continuously in an unchanging environment with good food source, with about 30% females pregnant throughout the year (Leslie *et al.*, 1952). However, in less favourable habitats, breeding only occur mainly in summer and autumn. Husni (personal communication, 2007) found that more fecund males moved into the farm during spring accompanied by non-breeding females.

Mating system however depends on the population density. If population density is low, the males will defend the territory from intruders and the mating system is polygynous, however, in high density population, the mating system is promiscuous.

The gestation period for females is 20-23 days and the litter size is about 7 to 9 young (Meehan, 1984). The growth of the progeny depends on maternal food intake. Restriction of maternal food intake, by as little as 25% from normal food intake, both during gestation and lactation will reduce the growth of the progeny. The number of young will also affect growth rate, the fewer in the litter, the heavier the young animals at 14 days which also tend to grow more rapidly.

In this chapter, I will look at the genetic differentiation at the farm level using a highly informative polymorphic marker: microsatellite DNA. Much has been learned about the biology of brown rats in the wild; however, no study has ever looked at gene flow between farms. An understanding of this aspect of the brown rats population biology will be crucial when trying to provide a scientific basis for pesticide use and control programmes in general.

4.2 Materials and methods

A total of 156 rats from 10 different regions was sampled. Within each region, local populations were between 0.1 to 55 kilometres apart. Because of the brown rat wild status, some localities (farms) have low sample sizes. Only region with more than one farm and farms with more than 2 rat samples were used for further analysis.

4.2.1 Microsatellite DNA

As in Chapter 3, 6 primers were used (D17Rat65, D3Mit13, D5Rat95, D12Rat43, D14Rat1 and D8Mgh7). The PCR was run individually for each primer. PCR products of D17Rat65, D3Mit13, D5Rat95 and products of D12Rat43, D14Rat1 and D8Mgh7 were pooled together respectively. PCR products were run as 2 batches on a Applied Biosystems 3730 capillary sequencer fragment analysis. Results were analysed by statistical program; GeneMapper (Applied Biosystems version 4).

Genetic differentiation (F_{ST}) was estimated from microsatellite DNA fragment sizes in GenAlEx computer software. A Mantel test (Mantel, 1967) was used to test the relationship between geographical distance and the level of genetic differentiation between populations (F_{ST}). Possible associations between mean F_{ST} and geographical distance and level of resistance in each region were investigated by regression analysis (Minitab 14).

4.3 Results

I will only show the statistical analysis of the localities, since number of alleles, allele frequency and heterozygosity have already been shown and discussed in Chapter 3.

4.3.1 Analysis of molecular variance (AMOVA)

AMOVA of 156 samples from 35 localities were analysed using GenAlex in 2 different ways, AMOVA for the whole localities (farm) in England and AMOVA for localities in each region.

AMOVA ($p<0.001$) of the whole farms in England showed that 13% of genetic variation is found between farms and 87% within farms (Table 4.3.1).

Table 4.1 Analysis of Molecular Variance (AMOVA) at farm level in the whole of England

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P value
Among Population	34	303.626	0.804	13%	0.001
Within Population	121	652.554	5.393	87%	
Total	155	956.179	6.197		

Tables 4.2 to 4.11 below show the AMOVA at farm level in each county. Berkshire, Cambridgeshire, Sussex and Wiltshire showed significantly ($p<0.05$) low percentage of variation between farms (2-20%). Only Shropshire showed 31% variation to between farms and 69% within farms but it is not significant.

Table 4.2 Berkshire

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Population	4	33.762	0.561	8%	0.027
Within Population	15	94.238	6.283	92%	
Total	19	128.000	6.843		

Table 4.3 Cambridgeshire

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Population	3	25.112	0.974	20%	0.001
Within Population	15	59.414	3.961	80%	
Total	18	84.526	4.935		

Table 4.4 Dorset

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	1	6.202	0.229	4%	0.324
Within Pops	5	27.083	5.417	96%	
Total	6	33.286	5.646		

Table 4.5 Kent

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	1	6.417	0.319	6%	0.187
Within Pops	6	31.333	5.222	94%	
Total	7	37.750	5.541		

Table 4.6 Leicestershire

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	2	12.889	0.094	2%	0.180
Within Pops	32	174.225	5.445	98%	
Total	34	187.114	5.538		

Table 4.7 Oxfordshire

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	2	13.850	0.226	4%	0.229
Within Pops	7	43.250	6.179	96%	
Total	9	57.100	6.405		

Table 4.8 Shropshire

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	1	10.100	2.194	31%	0.106
Within Pops	3	14.500	4.833	69%	
Total	4	24.600	7.028		

Table 4.9 Sussex

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	1	9.198	0.718	11%	0.025
Within Pops	9	49.893	5.544	89%	
Total	10	59.091	6.261		

Table 4.10 Wiltshire

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	4	29.500	0.730	13%	0.009
Within Pops	13	63.167	4.859	87%	
Total	17	92.667	5.589		

Table 4.11 Yorkshire

Source of Variation	df	Sum Of Squares	Variance Components.	Percentage of Variation	P values
Among Pops	6	37.941	0.113	2%	0.270
Within Pops	16	95.450	5.966	98%	
Total	22	133.391	6.079		

4.3.2 F_{ST} and Mantel tests

Tables 4.12 to 4.17 below show the F_{ST} values of brown rat samples of local population in each county. F_{ST} values between farms in Leicestershire are considerably lower than other farms in other counties. The highest F_{ST} value is 0.367, between C4 and C6 in Cambridgeshire. Mantel test found no significant relationships between F_{ST} and the geographical distance for all the populations (Figure 4.1 - 4.6). Figure 4.7 showed the relationship between combinations of F_{ST} for all counties with geographical distance between farms in each county.

Table 4.12 Pairwise population F_{ST} values between local populations of *Rattus norvegicus* in Berkshire.

B1	B2	B3	B4	B5	
-					**B1**
0.121	-				**B2**
0.132	0.154	-			**B3**
0.100	0.103	0.172	-		**B4**
0.100	0.112	0.138	0.120	-	**B5**

Table 4.13 Pairwise population F_{ST} values between local populations of *Rattus norvegicus* in Cambridgeshire.

C1	C2	C3	C4	C5	C6	
-						**C1**
0.112	-					**C2**
0.115	0.050	-				**C3**
0.149	0.197	0.172	-			**C4**
0.202	0.166	0.193	0.188	-		**C5**
0.270	0.222	0.238	0.367	0.289	-	**C6**

Table 4.14 Pairwise population F_{ST} values between local populations of *Rattus norvegicus* in Leicestershire.

L1	L2	L3	
-			**L1**
0.042	-		**L2**
0.027	0.043	-	**L3**

Table 4.15 Pairwise population F_{ST} values between local populations of *Rattus norvegicus* in Oxfordshire.

O1	O2	O3	
0.000			**O1**
0.116	0.000		**O2**
0.100	0.125	0.000	**O3**

Table 4.16 Pairwise population F_{ST} values between local populations of *Rattus norvegicus* in Wiltshire.

W1	W2	W3	W4	W5	
-					**W1**
0.098	-				**W2**
0.180	0.159	-			**W3**
0.179	0.144	0.139	-		**W4**
0.100	0.128	0.106	0.140	-	**W5**

Table 4.17 Pairwise population F_{ST} values between local populations of *Rattus norvegicus* in Yorkshire.

Y1	Y2	Y3	Y4	Y5	Y6	Y7	
-							**Y1**
0.149	-						**Y2**
0.153	0.161	-					**Y3**
0.168	0.161	0.122	-				**Y4**
0.137	0.149	0.068	0.105	-			**Y5**
0.182	0.155	0.158	0.156	0.126	-		**Y6**
0.141	0.143	0.084	0.107	0.086	0.092	-	**Y7**

Figure 4.1 Scatterplot of F_{ST} estimates against geographical distance separating each pairwise combination of populations in Berkshire (r= 0.005, n=10, p=0.430).

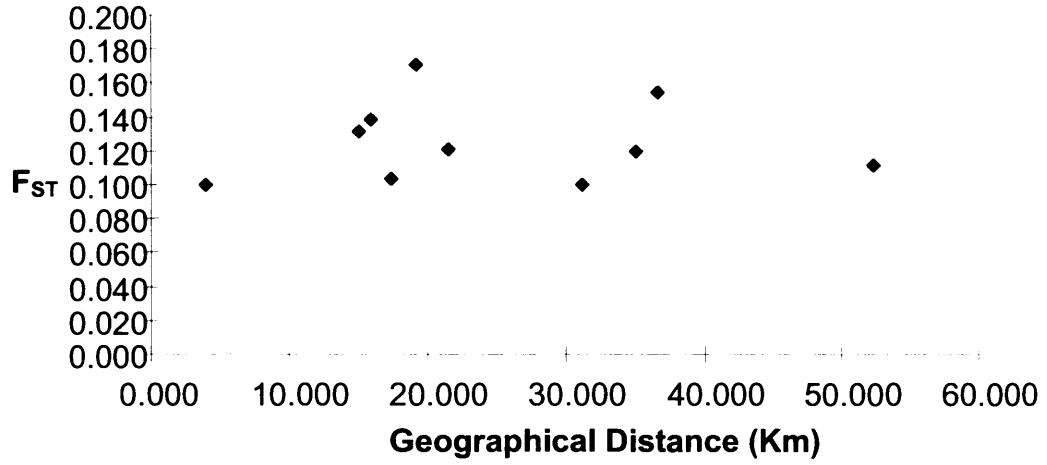

Figure 4.2 Scatterplot of F_{ST} estimates against geographical distance separating each pairwise combination of populations in Cambridgeshire (r=0.078, n=15, p=0.420).

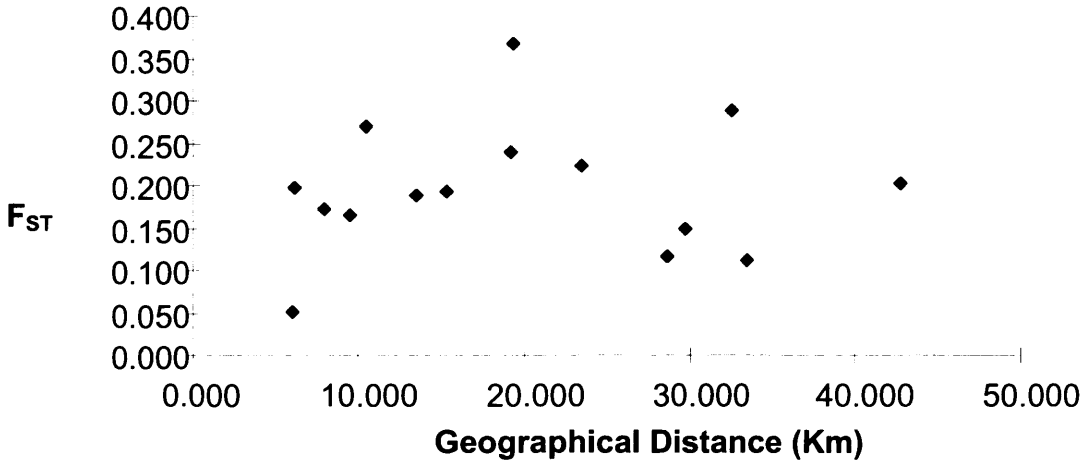

Figure 4.3 Scatterplot of F_{ST} estimates against geographical distance separating each pairwise combination of populations in Leicestershire (r= -0.999, n=3, p=0.160).

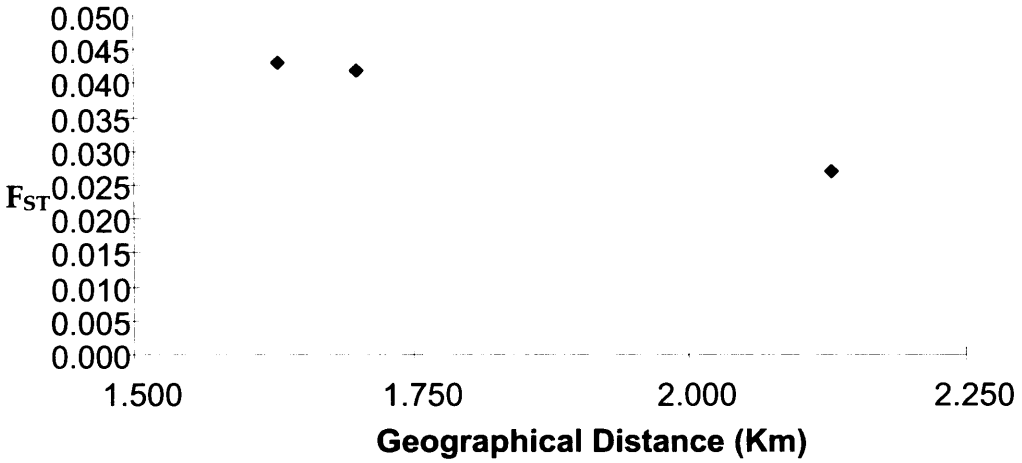

Figure 4.4 Scatterplot of F_{ST} estimates against geographical distance separating each pairwise combination of populations in Oxfordshire (r=0.623, n=3, p=0.330).

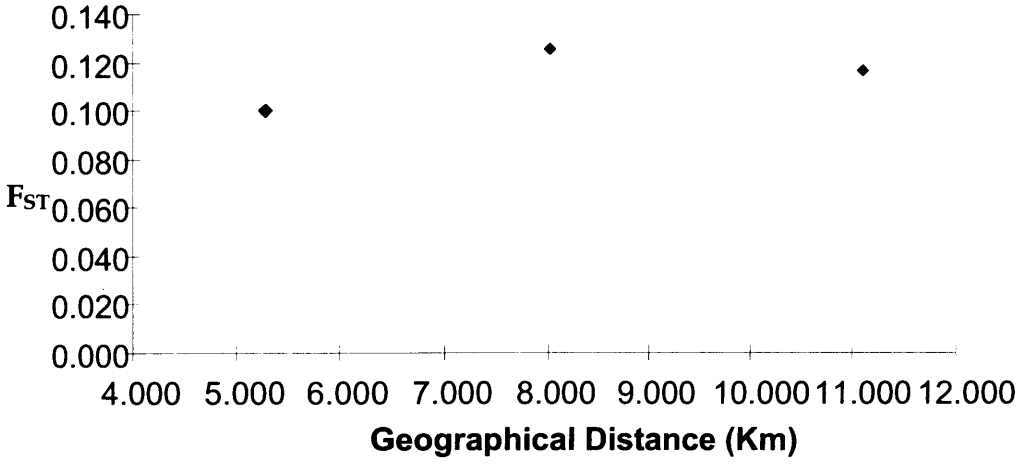

Figure 4.5 Scatterplot of F_{ST} estimates against geographical distance separating each pairwise combination of populations in Wiltshire (r=0.530, n=10, p=0.120).

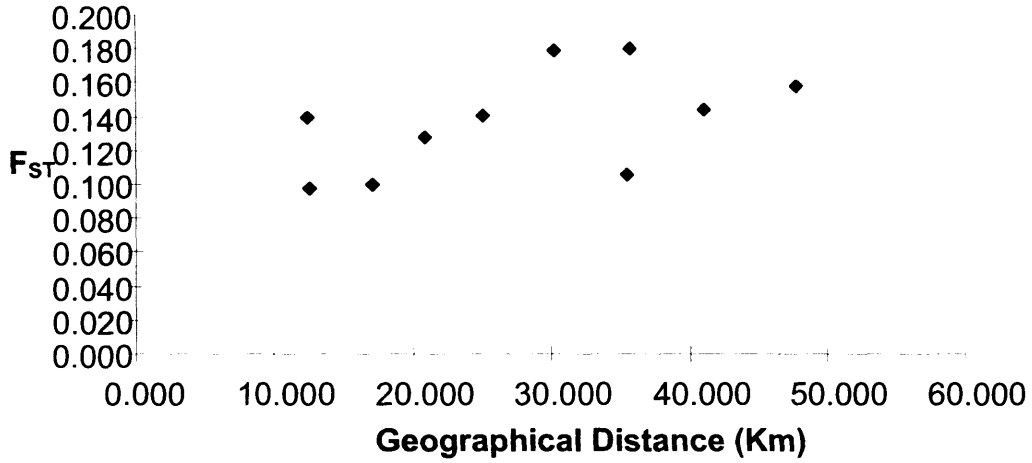

Figure 4.6 Scatterplot of F_{ST} estimates against geographical distance separating each pairwise combination of populations in Yorkshire (r=0.018, n=21, p=0.480).

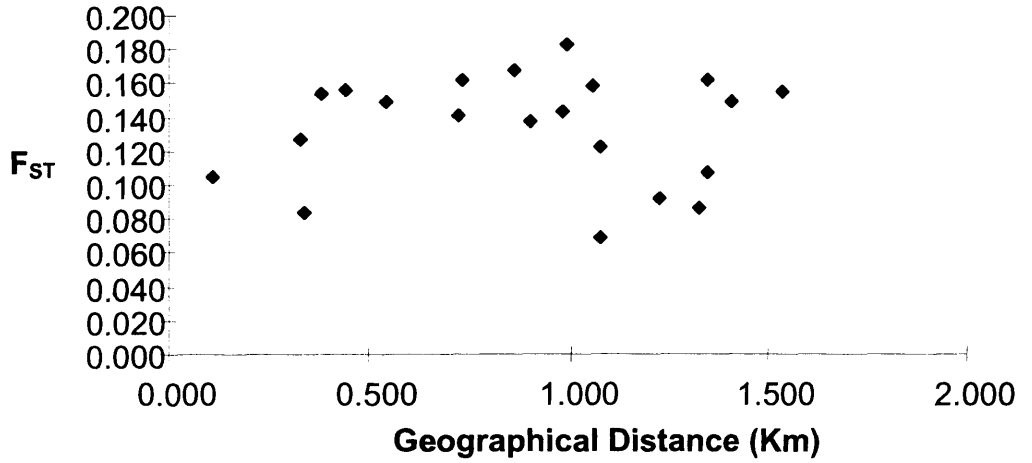

Figure 4.7 Scatterplot of mean F_{ST} estimates against mean geographical distance (km) for all farms. This figure is a metagraph of figure 4.1 - 4.6.

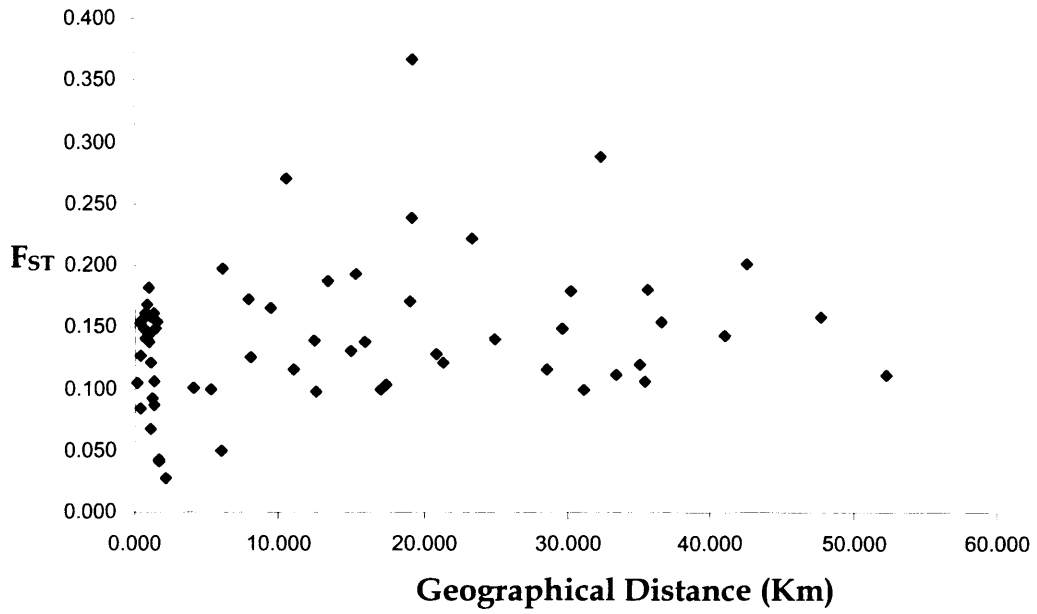

Table 4.18. Mean F_{ST}, geographical distance between farms and the resistance level for each county.

County	Mean F_{ST}	Mean Geographical Distance (Km)	Resistance Level * (%)
Berkshire	0.125	24.781	88
Cambridgeshire	0.195	19.830	61.9
Leicestershire	0.037	1.820	45.7
Oxfordshire	0.113	8.120	80
Wiltshire	0.137	27.796	72.2
Yorkshire	0.133	3.740	45.8

Note: * Resistance level was calculated based on the number of resistant rats divided by the total rat numbers in each county (Table 5.3)

Table 4.18 shows the mean F_{ST}, mean geographical distance (Km) and percentage of resistance level in each county. Statistical analysis (using Minitab 14) showed that there was a non-significant regression of mean F_{ST} against mean geographical distance and resistance level (F_{ST}= 0.117 +0.00326 Geographical Distance - 0.00061 Resistance Level, R^2=0, $F_{2,3}$= 0.82, p=0.52).

4.4 Discussion

The goal of this work is to identify the genetic differentiation at fine scale, i.e. farm level. Highly variable microsatellite DNA marker on 156 brown rats samples were used for this purposes.

In this study, analyses of molecular variance at farm level shows that percentage of variation among population is low. I would suggest that the population structure of the brown rats in England is almost homogenous with little variation between studied populations.

The percentage of variation among populations in Leicestershire is comparatively low (2%) although is not significant and this matches the genetic differentiation value (F_{ST}) saying that dispersal of brown rats and gene flows might have occurred between farms. We can suggest that gene flow among these farms has been more regular than other farms in other counties. Although a Mantel test showed a negative correlation between F_{ST} and geographical distance (r= -0.999), which means that the further the distance, the more similarities between the populations, this is not significant (p= 0.160).The low F_{ST} value is what one would expect since the distance between these farms is also very small compared to distance

between farms on other counties i.e.; from 1.5 km to 2.5 km only and this suggest regular movements of the brown rats between the farms.

Analyses of molecular variance showed that percentage of variation among populations (farms) were low for every county except for Shropshire (31%) and Cambridgeshire (20%). Although it is not statistically significant ($p>0.05$), Shropshire should be treated with caution since the number of samples is very low ($n=6$) in 2 localities. However, this high percentage of variation among populations is appropriate with the distance between farms in respective counties which is quite high (between 6 to 42 km in Cambridge and 20 km in Shropshire). It is difficult to prove and no evidence to show that brown rats move that far unless transported by human vehicles.

Microsatellite F_{ST} for each county (except for Leicestershire) was between 0.113 to 0.195, suggesting restricted gene flow over a scale up to 52 km. Although statistically non-significant, the positive correlation between F_{ST} and geographical distance suggests that movement by these brown rats is restricted to short distances.

We could have naively expected genetic differentiation between local populations as in general, rats only migrate if unstabilized conditions occur, such as lack of food or extreme climate change (Meehan, 1984). However, rats do not suffer from starvation as there is always a huge amount of food on farms and no extreme climate change that would make them migrate to a new destination. Harbourage is always available, although it often varies in quantity, quality and distance from a food source (Cowan *et al.* 2003). McGuire *et al.*, (2006) found no immigration and emigration from their rat population and suggested that decreases in

their rats population size likely resulted from predation rather than dispersal. However, in this chapter, our microsatellite data showed much more mixing of populations, that there is gene flow between farms, perhaps from movements of male rats. Lidicker (1975) stated that dispersers need not necessarily migrate due to either social or economic reasons, but may have discovered some other better home location. There are also conditions where dispersal of surplus individuals from a population living at or near its carrying capacity may be favoured, when fitness may be improved by leaving home. Overall, our microsatellite data shows almost complete gene flow between farms, suggesting that other forces rather than just starvation-induced migration is at work.

The level of resistance to rodenticide depends on the level of rodenticide used in the population and the numbers of resistant rats within that population. If the level of rodenticide used is low and the number of resistant rats is also low, resistant individuals might be buffered by susceptible rats by random mating. I was interested in seeing if warfarin resistance levels could explain the variation in F_{ST} between counties. There is no association between the two (F_{ST}= 0.117 +0.00326 Geographical Distance - 0.00061 Resistance Level, R^2=0, $F_{2,3}$= 0.82, p=0.52). I will look further into warfarin resistance in the next chapter.

SPECIAL NOTE

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Chapter 5

SPREAD OF WARFARIN MUTATION ACROSS ENGLAND

5.0 Spread of Warfarin Mutation across England

5.1 Introduction

Control of pest rodents, particularly of brown rats relies mainly on the use of rodenticide. Anticoagulant compounds have been used as rodenticide worldwide for more than 50 years (Freeman, 1954). The first anticoagulant compound used to control rat populations was warfarin. However large-scale use of warfarin has lead to the evolution of resistance. The first report on warfarin resistance was in 1958 (Scotland) and resistance was also documented in Japan, Australia and the United States as well as continental Europe (Misenheimer & Suttie, 1990). Resistance has lead to a decreased efficacy in pest control leading to an increase in the amount of anticoagulants used, a switch to other and stronger formulations of the same anticoagulant or the introduction of more potent anticoagulants.

It has been reported that warfarin resistance is based on a single dominant autosomal gene; *Rw* in brown rats and *War* in house mice which can be mapped on chromosome 1 and chromosome 7 respectively (Greaves and Ayres, 1967; Kohn and Pelz, 1999). Warfarin acts by interfering with the coagulation (blood clotting) process. Warfarin targets the Vitamin K 2, 3 epoxide reductase (VKOR) of the vitamin K cycle in the liver. Suppression of the VKOR by anticoagulants such as warfarin inhibits the carboxylation of clotting factors and thus compromises the coagulation process. Mutations in a gene of the VKOR complex, *VKORC1*, are involved in the resistance to anticoagulants in rats (Pelz *et al.*, 2005). These mutations decrease the sensitivity of the protein *VKORC1* to Warfarin, increasing the efficiency of the coagulation process in warfarin-

exposed rats (Pelz *et al.*, 2005). Mutations at *VKORC1* are dominant over the wildtype allele.

Vitamin K deficiency feeding tests conducted by Greaves and Ayres (1976) demonstrated that blood clotting activity declines more slowly in Welsh and Scottish heterozygous rats compared to homozygous individuals. Male rats seem to be more sensitive to anticoagulants than females (Wallace and MacSwinney, 1976) but females retained higher percentage blood coagulation activity (Kohn and Pelz, 1999).

It has been suggested that rodenticide resistance spread in European rat populations from initial focal points, with resistant rats moving into new areas (Pelz *et al.*, 2005). Earlier work noted that resistance spread at about 2 miles a year (Drummond & Wilson, 1968). However, warfarin resistance though widespread is not ubiquitous. What has slowed the spread of resistance? One possible explanation is that gene flow between different rat populations is limited. As previously stated the rate at which resistance is acquired is a function of the resistance allele's frequency, its dominance, the relative fitness of being resistant and the subject of the last 2 chapters: the pest organism's population structure (Roush & Mckenzie, 1987). We have shown that the rural rat population is not highly structured. Its seems likely that males travel widely and that there is complete geneflow between farms. Given this, this chapter looks more closely at the spread of mutant genotypes through the English population. We will look at the geographical structure of the mutation distribution and ask is it affected by warfarin usage. In this chapter, we will also look at the type of mutants found and the distribution of the *VKORC1* exon 3 gene mutations across England using different types of molecular approaches.

5.2 Materials and methods

A total of 185 brown rats has been used to screen for warfarin mutations. Warfarin resistant mutants have been characterised in the literature, all in exon 3 of the *VKORC1* gene (Pelz *et al.*, 2005) (Table 5.1).

5.2.1 Amplification refractory mutation system (ARMS-PCR)

Amplification refractory mutation system (ARMS-PCR) employs two primer pairs to amplify, respectively, the two different alleles of a single nucleotide polymorphism (SNP) in a single PCR reaction. 2pmol each of the outer primer; F: 5′-ATCCTGAGTTCCCTGGTGTCTGTCGCTG-3′ and R: 5′-TCAGGGCTTTTTGACCTTGTGTTCTGGC-3′ and 10pmol each of the inner primers; F: 5′-TGATTTCTGCATTGTTTGCATCACCACATG-3′ and R: 5′-CAACATCAGGCCCGCATTGATGGAAT-3′ were used. The PCR condition was as follows: 95^{0}C for 3 mins, 32 cycles of denaturation at 95^{o}C for 20s, annealing at 62^{o}C for 20s, elongation at 70^{o}C for 10s, and a final extension at 70^{o}C for 3 mins.

The PCR products were quantified on a 3.5% Ultra High Resolution (MELFORD) Agarose gel. The electrophoresis was carried out for 1 hr and 45 mins at 110 volts. The outer primer pair produces a control band of 168 base pairs; the inner primer pair gives a band of 123 base pairs for the wild type and 101 base pairs for the mutant, respectively (Pelz *et al.*, 2005).

5.2.2 Restriction fragment length polymorphisms (RFLPs)

Three mutations (Tyr139Ser, Leu120Gln, Leu128Gln) create novel restriction sites in exon 3, which allow one of the three enzymes (*Stu*I, *Bsr*I, *Mnl*I) to cut the fragment at this point creating fragments of characteristic sizes (see Table 5.1).

Primers used for the PCR reactions were 5'-CATTGGGGAGGTGTTACAGAG-3' (forward primer) and 5'-GATACACTTGGGCAAGGCTC-3' (reverse primer) (Pelz *et al.*, 2005). The PCR condition were as follows; 45s at 94^{0}C, 30 cycles of denaturation at 94^{0}C for 45s, annealing at 50^{0}C for 45s, elongation at 72^{0}C for 1 min, and a final extension at 72^{0}C for 5 mins. The PCR products were quantified on a 1% agarose gel for 40 mins at 110 volts.

Three different restriction endonucleases were used for the 3 different mutations. Digestion of the PCR products was performed using New England Biolab enzymes in 10ul reaction containing 1x buffer 0.5 U to 1.0 U of enzymes and 8.0 to 8.5ul of PCR product. The reaction was incubated at 37^{o}C for enzymes *Stu*I and *Mnl*I, and at 65^{o}C for enzyme *Bsr*I overnight. Digested products were separated by 3.5% Ultra High Resolution Agarose gel in 1X TAE running buffer for 1 hour and 45 mins at 100volts (Table 5.2).

Digestion of the PCR products by *Stu*I for mutation Leu120Gln results in fragments of 195 and 135 base pairs (mutant) and 330 base pairs for the wild type (Rost *et al.*,2004). Mutation Leu128Gln produced by *Bsr*1 with fragments of 170 and 160 base pairs in mutants and 330 base pairs in wild type. Mutation Tyr139Ser can be identified by *Mnl*I digestion with a

fragment of 110 base pairs and 160 base pairs for the wild type (Pelz *et al.*, 2005).

Table 5.1 Characterised mutations in exon 3 of VKORC1. The mutations are labelled as wildtype/position/mutant, so Tyr139Cys is a tyrosine at the 139th amino acid converted to a cysteine in the mutant.

Mutation	Detection method	Wildtype	Mutant
Tyr139Cys	ARMS-PCR	168bp	168bp
		123bp	101bp
Leu120Gln	*Stu* I	330bp	195bp
			135bp
Leu128Gln	*Bsr* I	330bp	170bp
			160bp
Tyr139Ser	*Mnl* I	160bp	110bp

Table 5.2: Restriction enzyme digestion reactions

Mutation	Restriction enzyme (ul)	Buffer 10x	BSA	PCR product	Incubation temperature
Leu120Gln	*Stu*I (0.1ul)	1ul	-	8.9ul	30°C
Leu128Gln	*Bsr*I (0.2ul)	1ul	-	8.8ul	65°C
Tyr139Ser	*Mnl*I (0.2ul)	1ul	0.2ul	8.6ul	30°C

5.2.3 Statistical analysis

Spearman's rank correlation was used to assess relationship between variables; between number of samples and number of counties and between warfarin usage and prevalence of mutants.

5.3 Results

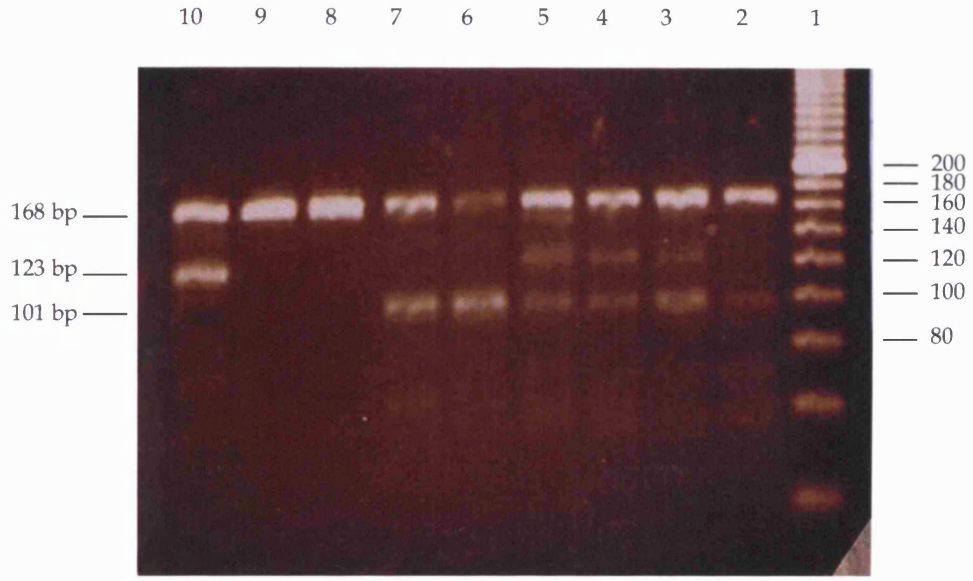
185 brown rats across England were analysed for mutations in the exon 3 of the *VKORC1* gene. 3 mutations were identified: Tyr139Cys, Leu120Gln and Tyr139Ser. A total of 126 mutant rats (68.1%) were found (Table 5.3). The percentage of rodenticide resistant mutants varied from 45% to 100% in different counties (Table 5.3). Figure 5.4 and 5.5 shows the distribution of the various mutations throughout England. This difference was not due to sampling effort (Spearman's rank correlation: r =-0.4, n =14, p =0.155). We have information of warfarin usage in 1989 on farms in 5 large geographical areas of England (Northern, Midlands and western, Eastern, South Western and South Eastern) (Olney *et al.*, 1991). Warfarin usage over this period does not correlate with prevalence of mutants (Spearman's rank correlation: r =-0.2, n =5, p =0.747).

5.3.1 Tyr139Cys mutation

Testing Tyr139Cys mutation by the ARMS-PCR screening assay revealed that 82% of all mutations were Tyr139Cys mutations and the most common mutation found in this study. Figure 5.1 shows the examples of Tyr139Cys mutation fragment on an Agarose gel. Gloucestershire and

Worcestershire has the highest percentage of Tyr139Cys mutants (100%), followed by Sussex (91%) and Berkshire (87.5%) (Table 5.4).

Figure 5.1. Gel image of Agarose gel separation showing examples of Tyr139Cys mutation fragment amplified by ARMS-PCR. Lane 1: Sigma 20bp low ladder. Lane 2, 6 and 7: homozygous mutants. Lane 3, 4 and 5: heterozygous mutants. Lane 8 and 9: control band. Lane 10: wildtype.

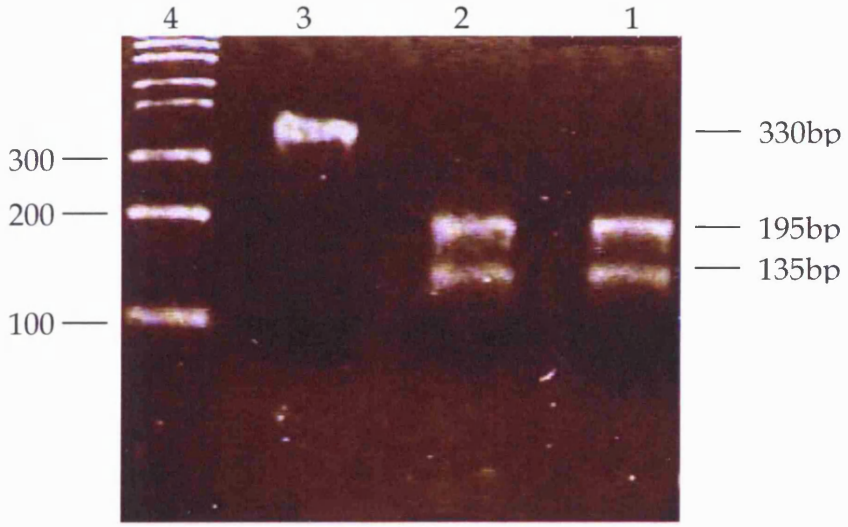


All Tyr139Cys mutant rats (Table 5.4) in Berkshire, Oxfordshire, Wiltshire, Dorset, Gloucestershire, Hampshire, Shropshire, Kent, Leicestershire, Yorkshire and Worcestershire were heterozygous (wildtype/mutant). 50% of mutant rats in Middlesex were homozygous followed by 30.7% of the mutant rats in Cambridge and 20% of mutant rats in Sussex.

5.3.2 Leu120Gln mutation

Leu120Gln mutation was screened by *Stu*I enzyme digestion (Figure 5.2). Only 14% of the brown rats tested were Leu120Gln mutants. Only Berkshire (25%), Sussex (27.3%), Oxford (19%), Wiltshire (11.11%), Gloucestershire (33.3%) and Hampshire (66.67%) have rats with Leu128Gln mutations and all mutants were heterozygous.

Figure 5.2. Gel image of agarose gel separation showing examples of Leu120Gln mutation fragment amplified by PCR and digested by *Stu*I. Lane 1 and 2: Mutants. Lane 3: Wildtype. Lane 4: YorkBio QStep1 Ladder.

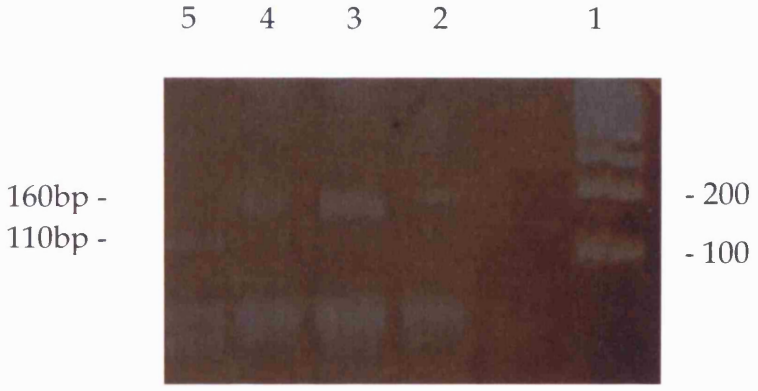


5.3.3 Tyr139Ser mutation

Tyr139Ser mutation was screened by *Mnl*I enzyme digestion (Figure 5.3). This was the rarest of the mutations found, where by only 4% of the rat individuals tested were Tyr139Ser mutants. Only Shropshire (33.33%),

followed by Hampshire (22.22%) and Yorkshire (8.33%) have rats with Tyr139Ser mutation (Table 5.4).

Figure 5.3. Gel image of agarose gel separation showing examples of Leu120Gln mutation fragment amplified by PCR and digested by *Mnl*I. Lane 1: York Bio Q Step 1 ladder. Lane 2, 3 and 4 : Wildtype and Lane 5: Mutant.



5.3.4 Leu128Gln mutation

The Leu128Gln mutation was not found in any of our samples (Table 5.4). To be confident that no error during experiment, we also checked our assay with a positive control obtained from Hans-Joachim Pelz, Munster, Germany.

5.3.5 Compound heterozygous

12 individuals were found compound heterozygous for Tyr139Cys and Leu120Gln, 4 individuals for Tyr139Cys and Tyr139Ser and 1 individual for Leu120Gln and Tyr139Ser (Table 5.3). Only one individual carried 3 mutations (Tyr139Cys/Leu120Gln/Tyr139Ser) which is from Hampshire.

5.3.6 Blood clotting response (BCR) versus molecular based results

A total of 79 rats trapped by Central Science Laboratory Yorkshire had previously undergone blood clotting response (BCR) to test whether they are Warfarin resistant or susceptible. The test was based on changes in blood clotting activity in the rats tested. Blood samples from rats were collected and treated with citrate buffer solution (3.13% tri-sodium citrate) at the ratio 1:9 (citrate:blood). Clotting time were determined and converted to percentage clotting activity (PCA) using separate curves for males and females rats. Rats that are sensitive to the anticoagulant were defined as those obtaining PCA values of 10% or less (Gill *et al.*, 1994; Herberg, 2002).

Out of 79 rats tested for BCR, 4 were found to be susceptible while the other 75 rats were resistant. Results from our ARMS-PCR and enzymes digestion indicated that 10 rats resistant through the BCR test are susceptible. However, we sequenced exon 3 of these 10 BCR positive rats. In six we found no mutants, that is they were mostly likely BCR false positives. Two of the remaining four were Tyr139Phe mutants, which can only be detected by sequencing analysis and the other two were Leu120Gln mutants. Both 2 mutant rats (Tyr139Phe) were from the same

farm in Ken and the other 2 resistant rats that have Leu120Gln were from the same farm in Hampshire. Four rats that were claimed to be susceptible through BCR test had Tyr139Cys mutation by ARMS-PCR (Table 5.5). All the 3 susceptible rats were from the same farm in Berkshire and the rat from Gloucestershire which had Tyr139Cys also had Leu120Gln mutation.

Figure 5.4 Map showing the distribution of pesticide resistant mutations in England

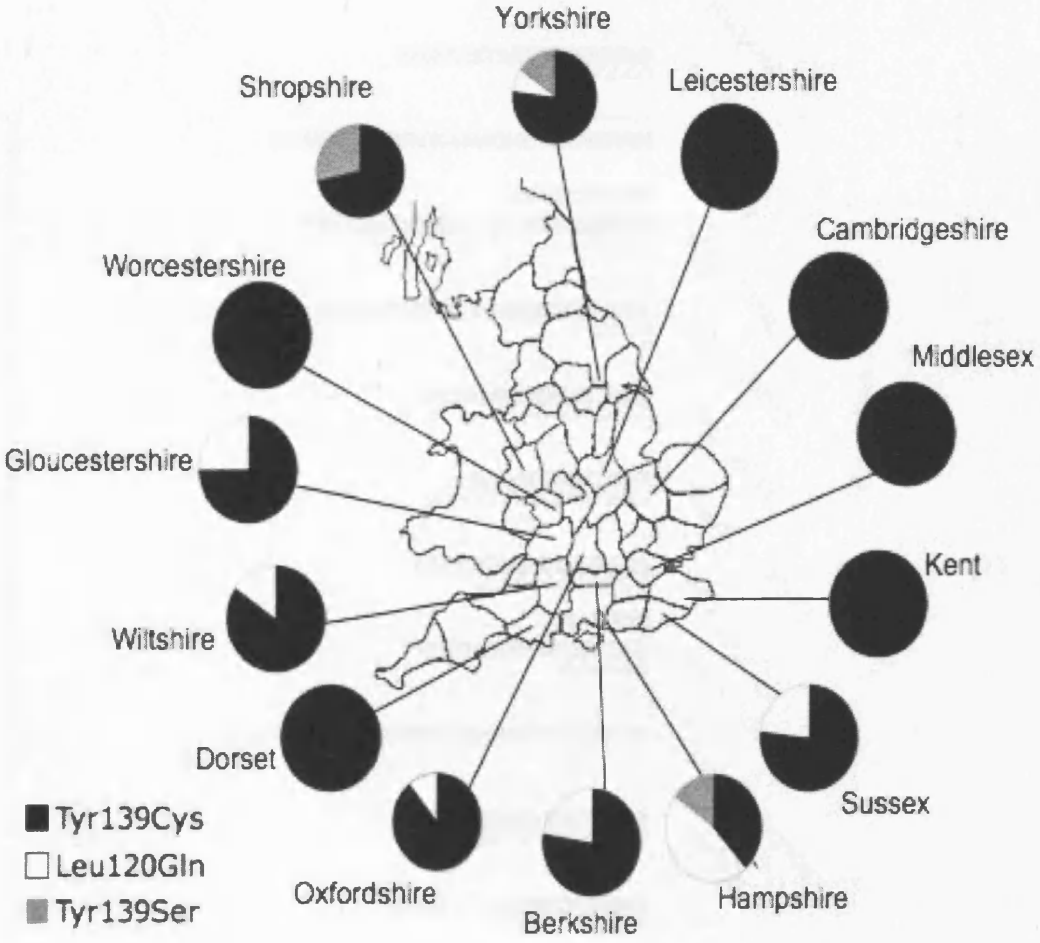


Figure 5.5 Percentage of wildtype and mutant rats versus counties in England

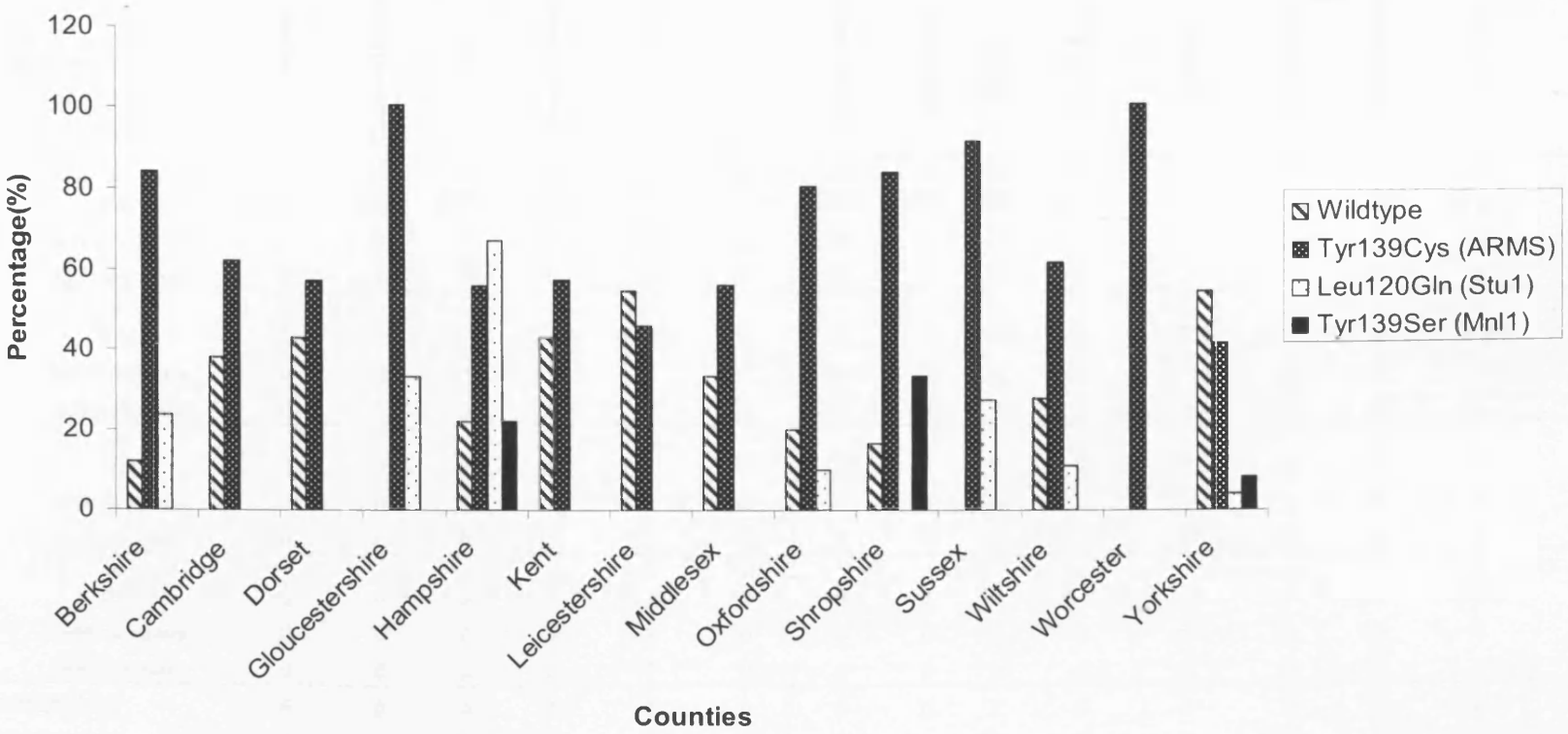


Table 5.3 Number and percentage of mutant rats in each county in England.

Mutants		County														Total (n)
		Berkshire	Cambridgeshire	Dorset	Gloucestershire	Hampshire	Kent	Leicestershire	Middlesex	Oxford	Shropshire	Sussex	Wiltshire	Worcestershire	Yorkshire	
Y139C	n	21	13	4	3	5	4	16	2	8	5	11	11	5	10	
	(%)	(87.5)	(62)	(57.1)	(100)	(55.5)	(57.1)	(45.7)	(67)	(80)	(83.3)	(91.6)	(61.1)	(100)	(41.67)	118
	heterozygous	21	9	4	3	5	4	16	1	8	5	9	11	5	10	111
	homozygous	0	4	0	0	0	0	0	1	0	0	2	0	0	0	7
L120Q	n	6			1	6				1		3	2		1	
	(%)	(25)	0	0	(33.3)	(66.67)	0	0	0	(10)	0	(27.3)	(11.11)	0	(4.16)	20
	heterozygous	6	0	0	1	6	0	0	0	1	0	3	2	0	1	20
	homozygous	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L128Q	n															
	(%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	heterozygous	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	homozygous	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Y139S	n					2					2				2	
	(%)	0	0	0	0	(22.2)	0	0	0	0	(33.33)	0	0	0	(8.33)	6
	heterozygous	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
	homozygous	0	0	0	0	1	0	0	0	0	2	0	0	0	2	5
Y139C/L120Q		5	0	0	1	4	0	0	0	1	0	2	0	0	0	13
Y139C/Y139S		0	0	0	0	0	0	0	0	0	2	0	0	0	2	4
L120Q/Y139S		0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
Tyr139Cys/Leu120Gln/Tyr139Ser		0	0	0	0	1	0	0	0	0	0	0	0	0	0	1

Table 5.4 Number and percentage of mutant rats in each county

	Berkshire	Cambridgeshire	Dorset	Gloucestershire	Hampshire	Kent	Leicestershire	Middlesex	Oxford	Shropshire	Sussex	Wiltshire	Worcestershire	Yorkshire	Total
Number of rat tested (n)	25	21	7	3	9	7	35	3	10	6	12	18	5	24	185
Number of mutant rats (n)	22	13	4	3	9	6	16	3	8	5	12	13	5	11	126
Percentage of mutant rats (%)	88	61.9	57.1	100	100	85.7	45.7	100	80	83.3	100	72.2	100	45.8	68.1

Table 5.5 Mismatch between the results of ARMS-PCR/enzyme digestion tests and BCR tests for warfarin resistance.

County	BCR Test (n)	ARMS-PCR/ Enzyme Test
Berkshire	Susceptible (3)	Resistant
	Resistant (3)	Susceptible
Gloucestershire	Susceptible (1)	Resistant
Hampshire	Resistant (2)	Susceptible
Kent	Resistant (3)	Susceptible
Oxford	Resistant (1)	Susceptible
Wiltshire	Resistant (1)	Susceptible

5.4 Discussion

Our results showed that from the total of 185 tested rats, 118 rats possess the Tyr139Cys mutation, 20 rats are Leu120Gln mutants and 6 are Tyr139Ser mutants. Tyr139Cys was the most prevalent mutation found across England. As in agreement with Pelz *et al.* (2005), codon 139 of the *VKORC1* gene represent a hotspot for mutations in this study as two mutations (Tyr139Cys and Tyr139Ser) were identified. However, only 3 out of Pelz's 4 types of mutations were found in the English rat population.

A subsample of the rats (79) had been previously tested for susceptibility to warfarin by the blood clotting response (BCR) test (MacNicoll *et al.*, 1996; Kerins *et al.*, 2001). Four were found to be susceptible. However each of these possessed the Tyr139Cys mutation (3 from 1 farm in Berkshire and 1 from Gloucester which also contained the Leu120Gln mutation). Pelz *et al* (2005) had previously suggested that such cases were false negatives due to the inaccuracies of the BCR test. More interestingly, 10 of the 75 resistant rats possessed none of the previously described mutations according to our mutation detection. We sequenced exon 3 of these 10 BCR positive rats. In six we found no mutants, that is, they were mostly likely BCR false positives. Two of the remaining four were Leu120Gln mutants, and the other two were Tyr139Phe mutants. The Tyr139Phe mutation can only be detected by direct sequencing. The 2 undetected Leu120Gln mutations represent a small error rate in our mutation detection and does not affect our conclusions about mutation distribution.

We found a geographical pattern to the different mutation distribution (see Figure 5.4). The Leu120Gln mutation is found in the central and southern counties. The Tyr139Cys mutation is the most common and found in the majority in almost all counties, Hampshire being the exception. The Tyr139Cys mutation is reportedly better at ameliorating the effects of warfarin (Pelz *et al.*, 2005) and we suggest rats possessing this mutation are out-competing the Leu120Gln mutants. The Tyr139Cys mutation is almost ubiquitous in Germany and Denmark (Pelz *et al.*, 2005). The change from cysteine to serine is a single codon change (a transversion of guanine to cytosine (Pelz *et al.*, 2005)). It seems likely to us that the Tyr139Ser mutation is a secondary mutation of Tyr 139Cys. The Tyr139Ser is better yet again at controlling the effects of warfarin.

Excepting any other selectional pressure we suggest that it should become more common with continued warfarin usage.

Our results also demonstrate that different mutations exist in the same population. For example, in Berkshire and Hampshire, rats in the same farm carried different types of mutation and some of them are compound heterozygotes.

No Leu128Gln mutation was found during our study. This could be the rarest mutation found within the exon 3 of the *VKORC1* gene. In fact, only 3 Leu128Gln mutant rats were found in England by Pelz *e al*, (2005). 2 rats were Scottish resistant and 1 was Yorkshire wild. In this case we would assume that none of our rats was Scottish resistant since none of the rats carried Leu128Gln mutation.

Although it is clear that the number of resistant mutants in an area will control the efficiency of a pesticide program (Our data showed that there about 45% of tested rats from Leicestershire had Tyr139Cys mutation (Figure 5.1) where there are no problems controlling rats with anticoagulants (Robert Smith - personal communication) compared with 88% of mutant rats in Berkshire where anticoagulants are less useful (Buckle & Smith, 1994)). We found no relationship between the prevalence of warfarin-resistant mutants and use of warfarin in a given area. This is stated with the proviso that we only have data for 1989. It seems likely that current warfarin mutant prevalence is due to warfarin usage in the past. Our rats were collected in the late 1990s, we suggest that 1989's usage is indictive of the selectional pressure on the population. However the idea that warfarin usage should explain pesticide resistance is based

on the assumption that warfarin usage is the only selectional pressure for these mutations.

Resistance to the anticoagulant poison warfarin in the brown rat is commonly thought to incur a cost: resistant rats are prone to vitamin K deficiency, showing reduced growth rates and reduced viability resulting in a lower fitness for resistant rats in a warfarin-free environment (Partridge, 1979). Where use of warfarin is discontinued, the frequency of resistance is likely to decline at a rate determined by the fitness of the Rw^1Rw^2 (heterozygous) and Rw^2Rw^2 (homozygous) genotypes relative to the susceptible (Rw^1Rw^1) (Bishop *et al.*, 1977). However a study carried out in the south of England (Smith *et al.*, 1993) found that in a warfarin-free environment, resistance remained high and contrary to predictions, the vast majority of resistant individuals were significantly heavier than susceptible animals in both populations. Therefore there is a possible selective advantage of warfarin resistance in the absence of poison, a finding with important implications for the dynamics and management of resistance.

SPECIAL NOTE

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Chapter 6

GENERAL DISCUSSION

6.0 General Discussion

The original introduction of anticoagulant rodenticides was a new paradigm for rat control, leaving other methods behind as they were more time consuming and had higher labour costs. However, widespread use of anticoagulant rodenticide has led to an evolution of resistance. The prevalence of resistance to warfarin has led to a decreased efficacy in pest control and a shift towards more powerful formulations of the same anticoagulant or an introduction of more potent anticoagulant compounds. Nevertheless, the problem of resistance will reoccur due the pesticides being imperfect (not 100% fatal) leading to selectional pressure for resistant mutants and reinvasion. It is essential to find an approach that offers a long lasting control strategy. First I will look at what my results tell us about the long-term applicability of pesticide programs. Secondly I will detail non-pesticide programs that have been used elsewhere.

The requirement for knowledge in brown rat population biology is important in preventing the spread of resistance during a control program using rodenticides. The research presented in this thesis was carried out to understand the genetic structure of the brown rat population at different scales in the English countryside. The mtDNA data suggested a structured population at about the county level. The microsatellite data showed much more mixing of populations, with perhaps four weak super-county level populations. I have discussed the possible reasons for this discrepancy, specifically the effective size of mitochondrial populations and the larger dispersal of males compared to female rats. This homogeneity in the rural rat population is arguably the biggest surprise of

my work. From its basic biology I would have predicted that like most small rodents the rat would have settled down to a structured population. Perhaps it is just a matter of time? After all, the mitochondrial population has already been structured, possibly due to the effects of genetic drift on its smaller effective population size. Perhaps with more time, the microsatellite data will show the same results. Or is male dispersal the important trait here? At the current time we cannot say which aspect of mitochondrial/microsatellite biology is causing the observed discrepancy.

This lack of structure is seen even more clearly at the farm level, were very little genetic variation can be assigned. It seems that within a local area there is complete admixing of populations. This result demonstrates that farm by farm usage of a pesticide is next to useless for controlling rat populations on a given farm. Wipe out a rat population on a farm and it can be instantly restocked by immigrants from neighbouring farms. This would mean to keep a farm rat-free would require long-term heavy usage of rodenticides, which is the ideal situation for the evolution of resistance.

I found a geographical structure to the types of *VKORC1* mutations. Although there was a large amount of mixing, some mutations were typical of certain geographical areas. As these mutations are all found in Europe, it is possible that they arose through introductions from the Continent. It may even be that they entered England through different ports from different parts of Europe. This would explain both the geographical structure of the mutations and the fact that no other part of Europe shows such diversity in their mutation type (Pelz *et al.* 2005).

Whatever the specifics of a given pesticide, resistance will always evolve if the pesticide is imperfect, that is, does not kill 100% of all animals that are exposed to it. The few remaining individuals will be by definition resistant, as most importantly will be their offspring. Understanding population structure would allow us to design a pesticide program with the minimal spread of resistant mutants. From this thesis, this seems unlikely to be an attainable goal in England due to the lack of population structure in the rural English rat population. Given that what other methods are available?

Rodenticide is the major approach to control rodent in the whole world. Most rodent control is organised in an ad hoc approach, either at a small scale by local farmers or at a wider geographical level by a government agency. Many other rodent management control strategies have been discussed and practised. For example, the development of agents for fertility control, especially immunocontraceptive vaccines, is another approach that has been practised in China (Shi *et al.*, 2002) and Australia (Chambers *et al.*, 1999). It stimulates an animal's immune system to block fertilisation, implantation or embryo development and has the advantage of being species specific, non-polluting and humane, with little or no undesirable consequences for agricultural production or the environment (Zhang *et al.*, 2003). Another approach, a bioeconomic model which demonstrates how important it is to combine both ecology and economy knowledge in rat control management strategies have been practised in Tanzania. The results showed that strategies with only a few months of control, chosen at the appropriate time of the year, are the most economical, even though they have little effect on rodent population dynamics. Controlling rodents by rodenticide every year just before planting, is the most economically rewarding. The population fluctuations

of the rodents are not affected very much, but the crop is protected during the most sensitive period of planting and, therefore, the harvest is good. Limiting food supply or changing food location in farms so that the environment is less favourable to rats and by destroying its harbourage in farms also another method that can be carried out (Cowan *et al.* 2003).

I was able to tell much about the population structure of the Brown rat. However more samples and more samples collected uniformly over time would have been very useful. However our sample size was the largest ever caught in England. The brown rat is notoriously neophobic. Often traps end up catching other small mammals. Equally the frustrating lack of information on warfarin usage hampered our analysis.

Perhaps future studies could be more local, for example my 3 farms in Leicester, collecting both regular samples of rats and information from farmers on warfarin usage. It would be then quite simple to analyse the change in resistance level, and importantly what mutation was responsible, compared to how much warfarin was used. We could also look at where rats came from when a population was wiped out on a farm.

Time constraints prevented me from looking at my samples for further mutations. There is no reason why mutations may not exist in exon 1 and 2 of *VKORC1*. Once way we could have looked for these unknown mutants would be by Denaturing high-performance liquid chromatography (dHPLC). Denaturing high performance liquid chromatography efficiently identifies unknown polymorphisms by heteroduplex analysis of PCR fragments. Discovery of point mutations by heteroduplex analysis is based on the following principle: if a PCR product contains a mixture of wild-type and mutant DNA, heat

denaturation of the amplified material followed by renaturation will not only allow reannealing of the perfectly matched, fully complementary strands (homoduplexes), but will also allow the formation of heteroduplexes, which have a pair of non-fitting bases (mismatch) at one position. Since a nucleotide mismatch reduces the thermodynamic stability of double stranded DNA, heteroduplexes have a lower melting temperature than homoduplexes. At a certain temperature, homoduplexes are still double-stranded while heteroduplexes are already partially denatured. At that temperature, the two DNA species can be separated by dHPLC because their binding to the dHPLC column differs. The difference in melting temperature between homo- and heteroduplexes is strongly dependent on the nucleotide sequence of the respective DNA fragment (Wulfert *et al.,* 2006). Having said this, Pelz stated that exon 3 seemed to be a hot spot for mutations (Pelz *et al.* 2005) and the mutations I looked at were both easy to assay and the most common in Europe.

Overall, I feel this project has been a partial success in its stated goal of understanding how to use population information to control the spread of pesticide resistance. The population data was collected. The structure was analysed. The mutations were quantified. But the Brown rat had a surprise in store for us with its population homogeneity. A worthy adversary once again. To finish with a quote:

"I wouldn't mind the rat race -- if the rats would lose once in a while."

SPECIAL NOTE

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Chapter 7

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