DOCTOR OF PHILOSOPHY

Landscape-scale genetic diversity in Fraxinus excelsior L. : genetic structure within and among populations and the influence of regeneration practices on genetic variation

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Landscape-scale genetic diversity in *Fraxinus excelsior* L.:

Genetic structure within and among populations and the influence of regeneration practices on genetic variation

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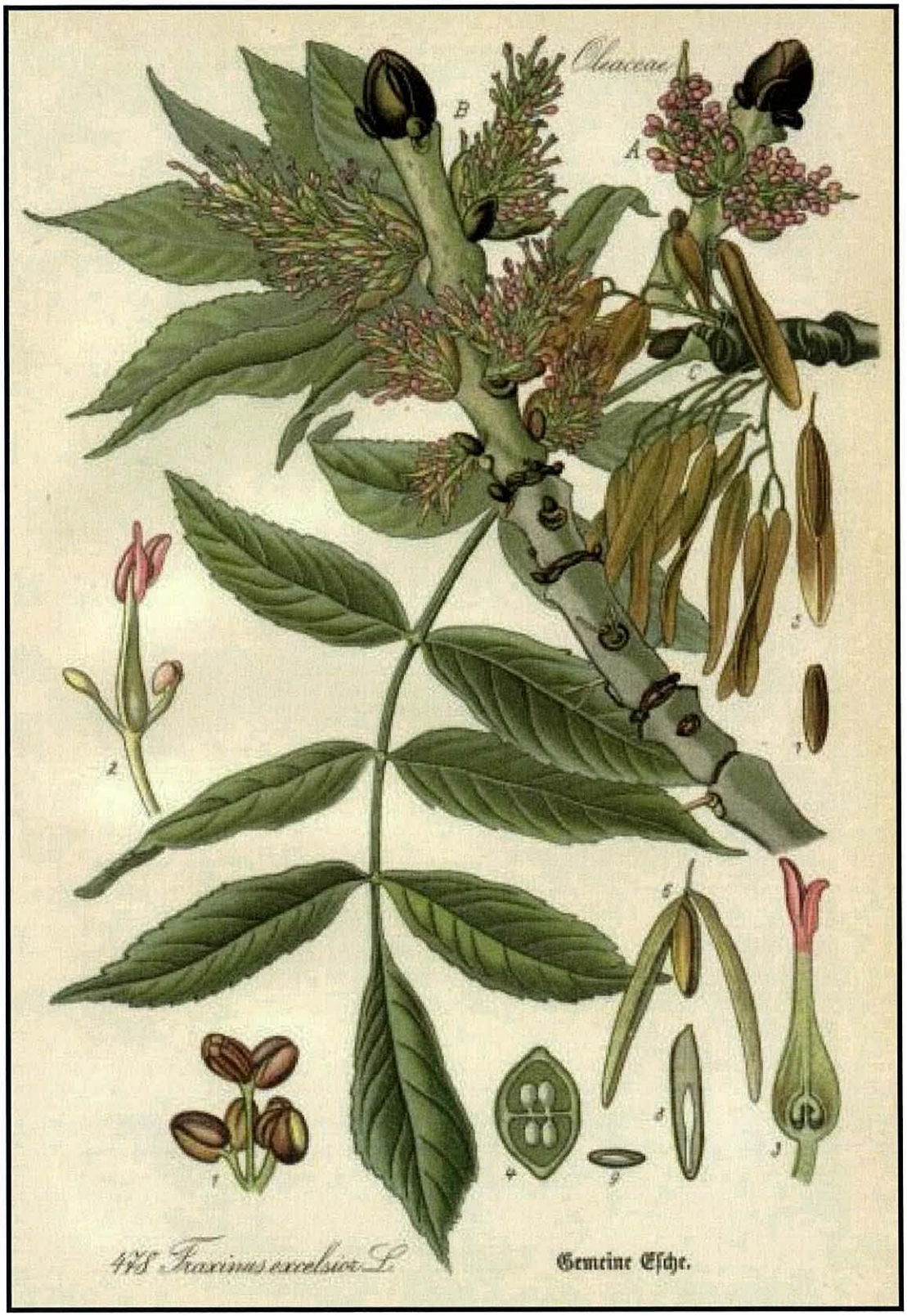
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Abstract

Genetic diversity and structure were investigated in *Fraxinus excelsior*, a common broadleaved tree in Britain. Samples, with geographical coordinates, were collected from six semi-natural populations, three each from south and north Wales. Six microsatellite loci were amplified and genetic parameters were calculated. High levels of polymorphism were found, and expected heterozygosity averaged 0.862 within populations. An excess of homozygotes was found in all populations, as F_{IS} was 0.184 overall. Low levels of differentiation among populations were recorded, with overall F_{ST} estimated at 0.017.

In the most intensively studied stand spatial autocorrelation identified weak small-scale genetic structure. Individuals up to around 700 m showed significant genetic autocorrelation. In another stand, samples were collected from mature trees, seeds, naturally regenerated seedlings, and seedlings grown from seed in a nursery. Comparisons of the genetic diversity among these cohorts showed no differences in allelic richness, heterozygosity, or homozygote excess. Differentiation between these different cohorts was also negligible with F_{ST} values of less than 0.02. Analysis of 200 seeds showed outcrossing rates close to one, with most pollen donors unsampled, and low levels of correlated paternity.

It is concluded that little differentiation exists among populations within the scale studied here, and so forest regenerative material for planting woodlands may be sourced widely. It is also concluded that genetic diversity is maintained equally by natural or artificial regeneration in the present situation where a large number of seed trees are available. The excess of homozygotes found in all populations is similar to that found by other workers and may be partially explained by null alleles. It is also possible that anomalous single-locus patterns may indicate that one or more microsatellite loci are not neutral but are linked to a gene or genes under selection.

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List of abbreviations

Genetics

GSM	Generalised stepwise model
IAM	Infinite alleles model
KAM	K alleles model
SMM	Stepwise mutation model
m	Migration
μ	Mutation
t	Time (number of generations) since population divergence
IBD	Isolation by distance
HWE	Hardy Weinberg Equilibrium
H_E, H_O	Expected heterozygosity (also gene diversity), observed heterzygosity
F_{IT}, F_{IS}, F_{ST} (F_{IR}, F_{RT}, F_{SR})	Wright's F statistics, indicating inbreeding or fixation; subscripts refer to total population, subpopulation, individual inbreeding and regions. F_{IT}, F_{IR} and F_{IS} are measures of homozygote excess in individuals; F_{ST}, F_{RT} and F_{SR} are measures of differentiation between subpopulations or regions.
F (Capf), f (Smallf), θ (theta)	Estimators of Wright's F statistics according to Weir and Cockerham. F is equivalent to F_{IT}, f is equivalent to F_{IS} and θ is equivalent to F_{ST}.
R_{ST}	Equivalent to F_{ST}, based on the SMM, allele sizes taken into account
r	Autocorrelation coefficient
A_O	Number of observed alleles
LOD	Log likelihood score in CERVUS 2.0 (Marshall *et al.*, 1998)
Δ	Delta: difference between LOD scores of two most likely candidate parents in CERVUS
t_s	Single-locus outcrossing
t_m	Multilocus outcrossing
r_p	Correlated paternity

Molecular techniques

bp	Base pairs
cp	Chloroplast
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SSR/ STR	Short sequence repeat/ short tandem repeat (microsatellite)
T_a	Annealing temperature

Other abbreviations

BP	Before present
D	Distance
DBH	Diameter at breast height
N	Number of samples
NGR	National Grid Reference (Ordnance Survey)
p	Probability
R, R_2	Correlation coefficient, coefficient of determination
$\bar{x}$	Mean
σ	Standard deviation

1. Introduction

1.1 Background to the study

Fraxinus excelsior, common ash, is a broadleaved tree species native to Britain. It is widely distributed throughout most of temperate Europe and is one of the most common British broadleaved species. As an important feature of the British countryside, it is widely planted for landscape and amenity purposes. The planting of trees in the British landscape is encouraged for a variety of purposes, but conservation of biodiversity, including genetic diversity, is an important consideration.

Although guidelines are available providing advice on the geographic sourcing of forest reproductive material in Britain (e.g. Herbert *et al.*, 1999), these guidelines are largely based on geoclimatic features. Ecological and genetic factors are not considered and the guidelines are the same for all tree species.

In order to inform policy and provide a scientific background to update guidelines, it is necessary to increase scientific knowledge about genetic diversity within tree species. It is important to understand the geographic scale of differentiation, so that when trying to plant "local" material it is understood whether this should refer to a local watershed or to a broad climatic zone. It is also important to improve understanding of the biological factors underlying the patterns of genetic diversity. The distribution of pollen- and seed-mediated gene flow, the extent of any small-scale genetic structure, and the nature of parentage are all matters of interest.

The distribution of *Fraxinus excelsior* is subject to human influence. There are no woodlands in Britain that have not been disturbed at some point in their history, whether by grazing, harvesting of wood, or planting with desirable species. Areas classified as ancient semi-natural woodland are the least disturbed but have not been immune to all human influence. *Fraxinus excelsior* is planted in many areas but as with all tree species, it has become less common in the increasing areas of urban and agricultural land use. When *Fraxinus excelsior* is planted or encouraged, the effects of choices between natural and artificial regeneration may be considerable but have not yet been well studied, and in particular the genetic effects of such management choices are unknown.

1.2 Aims and objectives of the study

The aim of this study was to use microsatellite markers of *Fraxinus excelsior* to address the following objectives:

(1) to study genetic diversity, genetic structure and geneflow within one intensively studied population;

(2) to quantify genetic diversity within and among a number of populations at different locations within Wales;

(3) to compare genetic diversity in mature trees with genetic diversity in seeds and seedlings, both naturally and artificially regenerated;

(4) to investigate the paternity of seeds collected from known mother trees.

1.3 Structure of the thesis

The structure of the thesis is as follows.

Chapter 2 Literature review

The first section addresses the study of population genetics and genetic variation. A brief history of the use of molecular markers in genetics is included, followed by an introduction to microsatellites and the analysis of genetic data. A discussion of the general nature of genetic diversity in forest trees is followed by a review of molecular genetic studies of broadleaved trees and studies of the genetic effects of regeneration and other management practices. The final section is a detailed introduction to the species and a review of previous studies of genetic diversity in *Fraxinus excelsior*.

Chapter 3 Materials and methods

General descriptions of the choice of field sites, the collection of the samples, and the laboratory analysis are included here. The preliminary analysis of data is also described. More detailed descriptions of the field sites and accounts of the analysis are given in chapters 4-7 where appropriate.

Chapter 4 Bishopston Valley: genetic diversity within a site

The population in Bishopston Valley was the most intensively sampled in this study. Samples of mature trees and natural regeneration were compared, and the small scale genetic structure of the population was investigated using spatial autocorrelation techniques.

Chapter 5 Genetic diversity among sites

Samples were collected from mature trees in six populations at locations at various geographic distances from each other across Wales. Genetic diversity was compared across populations and across microsatellite loci, and genetic differentiation among populations was calculated.

Chapter 6 Genetic diversity and regeneration

From one population, samples were taken from mature trees, natural regeneration, collected seeds and seedlings grown in a nursery from the seeds. Genetic diversity in these different cohorts was compared to determine the genetic effect of the seed collection and nursery treatment.

Chapter 7 Parentage analysis

The sampled seeds were analysed to determine the level of paternity that could be accounted for by the sampled mature trees. The rate of outcrossing was also calculated.

Chapter 8 General discussion

The methods used are reviewed and the limitations discussed. The conclusions from chapters 4-7 are brought together in terms of their contribution to understanding of genetic diversity in *Fraxinus excelsior*. Recommendations for silvicultural practices based on the findings are given, as well as directions for future studies, and the overall conclusions.

2. Literature review

2.1 Population genetics and genetic variation

2.1.1 Genetic variation

One of the defining characteristics of life is variation at all levels, or biodiversity. The Convention on Biological Diversity (1992) acknowledges the importance of diversity amongst ecosystems, diversity amongst species, and diversity within species. It is the intraspecific diversity which is of concern in this study. Intraspecific diversity arises by mutations and is maintained by recombination during sexual reproduction. Mutations are believed to occur at a slow but constant rate, although there is some variation in the mutation rate among different regions of the genome. Dispersal and migration are also key processes as they allow mutations to spread within and among populations.

2.1.2 Genetic markers – history, development, examples

Many genetic mutations are believed to be selectively neutral, meaning that they have no effect on the survival of the individual (Kimura, 1968). In addition, many regions of the nuclear genome are believed to be non-coding and mutations in these regions are also therefore neutral. The accumulation of neutral mutations at a constant rate can be used as a "molecular clock" which allows the time since two species or populations were separated to be determined. If genetic markers can be identified, therefore, which are selectively neutral, much information can be obtained about the history of the species or populations concerned, although the timescale to which this information relates depends upon the mutation rate of the chosen marker.

A number of markers have been used to obtain genetic information. Initially enzymes were used as variation in these near-gene products was found to give useful information (Avise, 1994). However, especially since the rise of the polymerase chain reaction (PCR), it has become increasingly easy to examine DNA directly. Many techniques have been used, including restriction fragment length polymorphisms (RFLPs), where enzymes are used to cut DNA at predetermined recognition sites, amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNA (RAPDs), which use primers to amplify polymorphic regions of DNA (Hartl and Jones, 2000). In

all cases, nucleotide substitutions within the areas recognised by enzymes or primers are identified by changes in the length of the DNA fragments produced. The most complete form of genetic information is the use of sequencing, which may be carried out relatively easily for certain genes.

While nuclear DNA markers such as microsatellites (described below) can give genetic information with very fine resolution, the use of organelle markers, usually chloroplast DNA in plants, can give more large scale information (e.g. Cottrell *et al.*, 2005; Dumolin-Lapegue *et al.*, 1997; Heuertz *et al.*, 2004a). Chloroplast RFLPs or PCR-RFLPs have been found to give useful levels of polymorphism. In several species, chloroplast microsatellites (cpSSRs) have been developed but the level of polymorphism is generally much lower than in nuclear microsatellites. This may be partly due to lower mutation rates in chloroplast DNA, but is also due to its uniparental inheritance and lack of recombination. In most species chloroplasts are maternally inherited (e.g. Morand-Prieur *et al.*, 2002) although in conifers they are paternally inherited (Ziegenhagen *et al.*, 1998).

The focus of this study is on the use of neutral genetic markers to determine genetic diversity and genetic structure within and among populations. It is beyond the scope of this study to discuss (1) adaptive variation, which may be studied using field trials and reciprocal transplant experiments (e.g. Boshier and Stewart, 2005; Cundall *et al.*, 2003) or (2) quantitative genetics where increasing understanding of the genome and gene function allows the study of genes linked to adaptive traits (Gonzalez-Martinez *et al.*, 2006).

Microsatellite markers were chosen for this study because it was felt that these highly polymorphic markers would provide the most information. In addition, a number of markers had already been developed for *Fraxinus excelsior* (Brachet *et al.*, 1999; Lefort *et al.*, 1999) and used successfully by a number of workers (e.g. Bacles *et al.*, 2005; Heuertz *et al.*, 2001; Morand *et al.*, 2002), removing the need for the potentially costly and uncertain development of new markers. The use of chloroplast markers, either RFLPs or chloroplast microsatellites, was rejected as earlier work suggested that little if any polymorphism was likely within Britain (FRAXIGEN, 2005; Heuertz *et al.*, 2004a). The other possibility was the use of dominant markers such as AFLPs, and although this

could provide a large number of loci, it was felt that additional information would be available by using codominant markers.

2.1.3 Development and methods for microsatellites

Microsatellites, also known as simple sequence repeats (SSRs), are regions of DNA where a short (1-6 base pair (bp)) sequence is repeated, usually up to around 70 times (Schlotterer, 1998). They are highly variable in terms of the number of repeats, but the flanking sequences are generally highly conserved. This means that primers can be used to amplify microsatellites in a PCR and their size can then be determined. Microsatellites are evenly distributed throughout eukaryotic genomes and are assumed to be neutral markers. They may be found in nuclear and organelle DNA and, while nuclear microsatellites are biparentally inherited, chloroplast microsatellites in angiosperm species usually show maternal inheritance. The high levels of polymorphism mean that when several loci are used, very fine-scale differences can be observed. In the years since microsatellites have become available, they have become the marker of choice for many studies, especially those involving genetic structure and diversity on a small scale, and those investigating gene flow, parentage and kinship.

The mutation model for microsatellites is a matter for debate, leading to questions about the most appropriate statistics. The original measure of genetic differentiation was Wright's F_{ST}. This is based on allele frequencies, without any information about the size of alleles (Hardy *et al.*, 2003). This is appropriate under an infinite-alleles model (IAM) where any allele has an equal chance of mutating to any other allele, or a K-alleles model (KAM), where any allele has an equal chance of mutating to one of K possible alleles. However, it is believed that mutation at microsatellite loci is closer to either a stepwise mutational model (SMM) (Zhu *et al.*, 2000), where mutations lead to an increase or decrease in allele size of one repeat, or a generalised stepwise model (GSM), where mutations causing changes in allele size occur according to a distribution, with most mutations involving the loss or gain of a single repeat pair, but occasional mutations causing greater losses. These stepwise models mean that the current size of an allele gives information about the historical size of the allele, so that alleles that are closer in length are likely to share a more recent common ancestor. In contrast, with the IAM or KAM no such information is available.

To take advantage of the additional information available from microsatellites if a stepwise model is assumed, the R_{ST} statistic was developed (Slatkin, 1995). This takes allele size into account and may give additional useful information. However, R_{ST} values only have an advantage over F_{ST} values when the mutation rate (μ) is high compared to the migration rate (m) in an island model or compared to $1/t$ in isolated populations (t is the number of generations since population divergence) (Hardy *et al.*, 2003). Although μ is widely believed to be higher in microsatellites than in other genomic regions, it will not usually be this high except in exceptionally isolated populations. F_{ST} remains, therefore, the statistic of choice in many cases (Gaggiotti *et al.*, 1999).

Microsatellites, along with other types of genetic markers, have been used in a wide range of studies on many different types of organism. Genetic structure has been studied using microsatellites in numerous broadleaved tree species. Depending upon the questions addressed, sampling is carried out at different geographical scales and intensities in order to obtain samples from different regions, populations, or within a population. There is also the possibility of addressing questions of gene flow and regeneration by collecting samples from different age cohorts or differently-managed stands of trees. A number of studies are reviewed in section 2.2.4.

2.1.4 Approaches to analysing genetic data

Initially when attempting to analyse genetic data it is helpful to obtain summary statistics, including the number of alleles and allele frequencies. With codominant markers such as allozymes or microsatellites, the number of effective alleles, or allelic richness, and the frequency of different alleles may be compared. Heterozygosity may be defined as the proportion of individuals that are heterozygotes at a given locus. A useful measure is expected heterozygosity (H_E, also sometimes referred to as gene diversity), which is the proportion of individuals in a population that would be heterozygotes under strict Hardy-Weinberg equilibrium. The expected heterozygosity may be calculated for dominant markers such as RAPDs as well as codominant markers, but observed heterozygosity (H_O) may only be calculated for codominant markers. A combination of allelic richness and expected heterozygosity are used to describe the level of genetic diversity.

It is important to assess whether the populations studied are mating randomly, usually by determining whether they are in Hardy-Weinberg equilibrium (HWE). From the genotype frequencies it is possible to calculate expected heterozygosity under HWE and compare this with observed heterozygosity. The difference between expected and observed heterozygosity gives the departure from HWE and this may be defined in terms of Wright's *F*-statistics. Positive *F*-statistics show a deficiency of heterozygotes compared to that predicted under HWE and indicate that mating on the scale studied is not random. It may be that there is population structuring or some form of biparental inbreeding.

A range of *F*-statistics have been used by different workers. The most common are F_{IT}, F_{IS}, and F_{ST}. F_{IT} and F_{IS}, which refer to inbreeding within the total population and inbreeding within a subpopulation are based in their simplest form on Equation 1. F_{ST} refers to differentiation among subpopulations within a total population. Some workers also use the subscript $_R$ to refer to regions which then permits values for statistics such as F_{IR}, F_{RT}, and F_{SR} to be estimated. The interpretation of different values of Wright's *F*-statistics (Wright, 1978) is shown in Table 2.1.

Equation 1: $$F = \frac{H_E - H_O}{H_E}$$

A number of different estimators of Wright's *F* have been used. One common example is that of Weir and Cockerham (1984). Their method is used in programmes including FSTAT 2.9.3 (Goudet, 2001). Alternative terminology has been developed for the different estimators; *F* (Capf), *f* (Smallf) and θ (Theta) may be found in the literature. For all intents and purposes *F* is equivalent to F_{IT}, *f* is equivalent to F_{IS} and θ is equivalent to F_{ST}.

Table 2.1 The interpretation of different values of Wright's *F* statistics (Wright, 1978).

Wright's F	Interpretation	
<0	Excess heterozygosity (e.g. non-assortative mating)	
0	Hardy-Weinberg Equilibrium	
0-0.05	Deficiency of heterozygotes	little differentiation (but not negligible)
0.05-0.15		moderate differentiation
0.15-0.25		great differentiation
>0.25		very great differentiation

It is often important to study the effect of geographic distance or isolation, when trying to assess whether isolation by distance (IBD) is an important factor in the structuring of genetic diversity. This may be studied on a small-scale, individual basis, by comparing the pairwise geographic distance with the pairwise genetic distance between individuals. A number of methods are possible for calculating individual pairwise genetic distances (Lynch and Ritland, 1999), from a simple allele-sharing index to a more complex index based on the SMM and taking into account differences in allele sizes. It is also possible to use F_{ST} as a measure of the genetic difference between two populations. Whether using individuals or populations, it is possible to use a Mantel test to determine the correlation between two matrices of geographic and genetic distance. The use of a Mantel test also allows a large number of permutations with random sampling of the matrices to determine the significance of the correlation.

Studies examining fine-scale spatial genetic structure within populations may use a number of statistical techniques to analyse data. Spatial autocorrelation, one of the more popular, uses a pairwise measure of kinship which is averaged between all pairs of individuals within a distance class. A number of different measures are available, including Moran's *I*, the number of alleles in common (Berg and Hamrick, 1995), and the autocorrelation coefficient, *r* (Smouse and Peakall, 1999). These measures are in many ways similar, and may be interpreted in a similar manner. When *I* or *r* is greater than zero, individuals are more closely related than expected by chance, suggesting that they are genetically related. Frequently the autocorrelation coefficient for the smallest distance class (0-*D* m) is used to indicate the strength of genetic structure. The distance where the *I* or *r* value intercepts the *x* axis indicates the distance above which individuals are no more closely related than expected by chance. Care must be used in interpreting correlograms, however, as a number of factors including the size and number of distance classes and the intensity of sampling can alter both the value for the smallest distance class and the intercept of the *x* axis (Vekemans and Hardy, 2004).

2.2 Genetic diversity in forest trees

2.2.1 The importance of intraspecific diversity

Intraspecific diversity is recognised as one of the constituents of global biological diversity (Convention on Biological Diversity, 1992). It is generally accepted that

diversity within species and populations is desirable because it will improve the ability of a species to adapt to a number of circumstances. For example, in a genetically diverse species subjected to a novel pathogen, some genotypes may be resistant to the pathogen, whereas in a clonal species all individuals are likely to be similarly affected. In the case of a virulent pathogen similar to Dutch elm disease or North American chestnut blight, a diverse species is more likely to include some trees with genes for resistance; these trees may survive and allow the species to recover. Sudden oak death (caused by *Phytophthora ramorum*) is a disease of current concern which has caused massive loss of American *Quercus* spp. in California (DEFRA, 2005).

Diversity may also allow adaptation to changing climatic conditions such as those predicted under global warming. Broadmeadow *et al.* (2005) consider the likely effects of the climate change scenarios given by Hulme *et al.* (2002) in the UK Climate Impacts Programme (UKCIP). They suggest that productivity of timber trees may be significantly affected. In the case of *Fraxinus excelsior*, the direction of change varies among regions and between high and low emissions scenarios. However, Hulme *et al.* (2002) consider that under both scenarios it may be desirable to consider planting trees from areas of Europe that have a warmer, drier climate.

In addition, intraspecific diversity may allow selection for desirable traits of economic importance, whether these relate to the production of timber or the extraction of potentially beneficial compounds.

2.2.2 The conservation of intraspecific diversity

The Convention on Biological Diversity (1992) also provides a commitment for countries to identify, monitor and conserve biological diversity at all levels. In some cases it may be sufficient to set aside reserves for nature conservation, but when species are extensively managed it will also be important to assess the impact of management practices on intraspecific diversity. Many forest trees are widely planted, and the choices made when choosing regenerative material, and when carrying out practices such as thinning, may have an impact on the diversity within these populations.

2.2.3 The nature and causes of genetic diversity in forest trees

The genetic diversity of European tree species is the result of a number of ecological and historical factors. The dominating historical factor is the effects of the last

glaciation (Hosius *et al.*, 2006). It is believed that during the last advance of the glaciers (the Younger Dryas period, until 11,500 radiocarbon years before present (BP)) trees were mainly restricted to isolated refugia, primarily in the Mediterranean peninsulas – Iberia, Italy and the Balkans. When the climate improved, the forests advanced rapidly into northern Europe. This was first documented from extensive pollen core studies across Europe (Birks, 1989) and has more recently been confirmed by chloroplast DNA studies (e.g. Cottrell *et al.*, 2005; Dumolin-Lapegue *et al.*, 1997; Heuertz *et al.*, 2004a). The distribution of European tree species has also been shaped by human deforestation since around 1200 BP (Hosius *et al.*, 2006). However, deforestation since the industrial revolution is likely to have occurred too recently in terms of tree generations to have yet had a great impact on the genetic diversity of populations.

A review by Hamrick *et al.* (1992) found that long-lived woody plants had greater levels of within-species diversity and lower levels of among-population diversity than short-lived and herbaceous plants. An analysis of life history traits (Hamrick and Godt, 1996) suggested that this pattern persisted in woody plants even when compared to non-woody species that shared the same breeding systems, geographic ranges and seed dispersal mechanisms. This may be due to the advantages to seed dispersal offered by tall stature and relatively low population densities.

2.2.4 Molecular genetic studies of broadleaved trees

With the increasing efficiency of methods in molecular biology, the last 10-15 years have seen a huge increase in studies of genetic diversity in all types of organisms. Data collection has become less demanding, in terms of both work and finances. The analysis of larger datasets has also become much easier with the development of faster computers and a wide range of programmes written specifically for the analysis of genetic data. Unsurprisingly, studies in molecular ecology have included a large number of studies on trees. An exhaustive review will not be attempted here. However, the findings of a range of studies of broadleaved temperate trees, on a range of different scales, will be outlined.

2.2.4.1 Large scale (phylogeography)

Large-scale studies of genetic diversity attempt to address questions of phylogeography and the history of species distribution. In order to observe sufficiently large-scale

patterns, highly conserved regions of DNA are required, and the chloroplast genome has been much used. The main markers used are chloroplast microsatellites and chloroplast RFLP or PCR-RFLP.

European studies are linked with the postglacial history of continental vegetation. In a large-scale study of European white oaks, *Quercus* species, cpPCR-RFLP haplotypes were not found to be specific to individual species. The distribution of haplotypes across Europe was not random, with haplotypes being restricted to particular areas, and closely related haplotypes having similar distributions (Petit *et al.*, 2002 and other papers, *For. Ecol. Man.* 156: 1-3)

Quercus ilex and other evergreen oaks belong to a different group of *Quercus* species with a more Mediterranean distribution. A chloroplast study of *Quercus ilex* in Italy showed highest diversity in the south of the country, with particular richness in Sicily, which may have been a refuge during glacial times (Fineschi *et al.*, 2005). In a study of three evergreen *Quercus* species, much complexity was found in the chloroplast haplotypes found in the Balearic Isles, suggesting that these may also have been a glacial refuge (Lopez-de-Heredia *et al.*, 2005).

In a chloroplast DNA study of *Populus nigra* across Europe, similar patterns were found. Distinct chloroplast lineages were found in eastern and western Europe, and greater diversity in the south, near to where the putative refugia would have been located (Cottrell *et al.*, 2005).

Prunus spinosa is a species which was found to have high levels of chloroplast diversity (Mohanty *et al.*, 2000). In a PCR-RFLP study, a greater number of alleles was found in southern than in northern populations, with just seven haplotypes found in seven northern populations but 29 haplotypes in 18 southern populations (Mohanty *et al.*, 2002).

The evidence from these different species supports the southern refugia theory, suggesting that old reservoirs of genetic diversity have persisted in the south, with some haplotype lineages migrating northwards in comparatively recent times. These different lineages tend to have distinct distributions although there is sometimes a zone of overlap.

2.2.4.2 Medium scale (among populations)

For medium scale studies, allozymes and microsatellites are the most popular markers. These studies address questions about how much diversity is present within populations. In microsatellite studies, measures of allelic richness may be used as an indication of genetic diversity. Gene diversity or expected heterozygosity (H_E) is another parameter useful in comparing genetic diversity. Wright's (1978) F statistics are frequently used in studies of this scale, in order to estimate departure from Hardy-Weinberg equilibrium, inbreeding within populations and differentiation among populations.

Sorbus torminalis was studied in 20 isolated populations in Poland (Bednorz *et al.*, 2006). Isozymes (11 polymorphic loci) showed high diversity, with expected heterozygosity (H_E) ranging from 0.193–0.473 within populations and averaging 0.373 over all populations. F_{ST} (among-population differentiation) was high with an overall value of 0.167 and a Mantel test showed strong significant correlation between genetic and geographic distances ($R = 0.410$, $p < 0.000$).

The Japanese species *Betula maximowicziana* was found to have relatively low diversity, based on 11 microsatellite loci. Mean allelic richness was 2.8 and H_E was calculated at 0.361. Estimates of F_{IS} did not differ significantly from zero, while estimates of F_{ST} were significant at all loci, with a mean value of 0.062 (Tsuda and Ide, 2005).

In South Korea, population structure of *Quercus acutissima* was investigated using nine allozyme loci. Genetic diversity was moderate (mean $H_E = 0.154$). In this study a small but significant excess of homozygotes was found, as F_{IS} was estimated as 0.069. Very low among-population differentiation was recorded as mean F_{ST} was estimated at 0.010 (Chung *et al.*, 2002).

Genetic variation of *Quercus suber* in Spain was investigated using 13 isozyme loci. Genetic diversity was high for enzyme studies, as an average H_E value of 0.304 was recorded, but an excess of homozygosity was recorded at most loci, with an average F_{IS} value of 0.173. Differentiation among populations was high, with average F_{ST} estimated at 0.169 (Elena-Rossello and Cabrera, 1996).

Fragmented *Quercus robur* populations close to the species limit in Finland were studied using 13 allozyme loci (Vakkari *et al.*, 2006). Overall F_{ST} was 0.066. There was

some variation with the size of populations; larger populations had greater allelic richness and also higher F_{IS} values. The higher F_{IS} values were explained by the presence of a Wahlund effect, or cryptic population subdivision, in the larger populations.

Eucalyptus marginata in south-western Australia was studied using RFLP loci. A moderate level of diversity was found with total heterozygosity (H_T) equal to 0.345 and weak differentiation among populations (F_{ST} = 0.034). However, the differentiation among populations was significantly correlated with distance (r = 0.568) (Wheeler *et al.*, 2003).

In severely fragmented Scottish populations of *Sorbus aucuparia*, genetic diversity was found to be only slightly lower than that found in European populations of the same species (H_E = 0.195) (Bacles *et al.*, 2004). Population differentiation was significant but not surprisingly high (θ = 0.043 for six isozyme loci, θ = 0.131 for cpPCR-RFLP). In this insect-pollinated species it is likely that much of the population differentiation results from restricted pollen flow, as pollinators may not travel for long distances over the barren landscape between fragments, while seed flow may be significant as birds may have to travel between small fragments as seed supplies become rapidly exhausted.

There is variation among the patterns found in different broadleaved species; although F_{ST} values are generally found to be low, as expected (Hamrick and Godt, 1996), this is not always the case. It is clear that different modes of pollen and seed dispersal may affect the degree of differentiation among populations, and that severe disturbance (e.g. Bacles *et al.*, 2004) may not always have the predicted effects.

2.2.4.3 Small scale spatial genetic structure

Small scale studies of aim to determine the degree of isolation by distance or small scale genetic structure. This is the extent to which individuals which are geographically close to each other are more closely related genetically than individuals sampled at random from a population. As detailed in section 2.1.4, Mantel tests and spatial autocorrelation are frequently used in studies at this scale.

Fagus crenata was studied in Japan using three microsatellite loci (Asuka *et al.*, 2004). Weak genetic structure over distances up to 30 m was shown by correlograms of Moran's *I*. The structure observed was probably due to limited seed dispersal, but over

long distances pollen and secondary seed dispersal meant that there was no structure. Observed genetic structure was stronger in individuals of small diameter class (≤ 12 cm DBH (diameter at breast height)).

Spatial genetic structure in *Fagus crenata* and *Fagus japonica* was compared using four microsatellite loci (Takahashi *et al.*, 2003). Only weak spatial genetic structure was observed at small distances; the number of alleles in common was significantly greater than the grand mean of alleles in common up to 10 m in *Fagus crenata* and up to 30 m in *Fagus japonica.* The weakness of the genetic structure was caused by extensive pollen dispersal. The differences between the two species may be due to the different extent of overlapping generations. *Fagus crenata* generally regenerates by seed in clearings and canopy gaps, while *Fagus japonica* can coppice vigorously from stools, so genotypes may persist for up to 1000 years.

Spatial genetic structure in *Fagus crenata* was also studied using 13 allozyme loci on five populations located at different elevations on a mountain ridge and isolated summits (Kitamura *et al.*, 2005). The autocorrelation coefficient first intercepted the *x*-axis at distances between 27 m and 97 m in different populations, indicating that small-scale spatial genetic structure extended up to these distances.

The broadleaved Japanese species *Cercidiphyllum japonicum* was studied using five microsatellite loci and no small-scale spatial genetic structure was found (Sato *et al.*, 2006). Simultaneous parentage analysis of seedlings showed extensive dispersal of seeds and pollen, with at least 28.8% of seedlings being the result of pollen flow from beyond the 20 hectare study site.

In Indiana, three closely related oak species, *Quercus rubra, Q. shumardii*, and *Q. palustris,* were found to have significant spatial genetic structure up to 90 m, although it was quite weak (r at 20 m = 0.08) (Aldrich *et al.*, 2005).

In Korea, *Quercus acutissima* was investigated using nine allozyme loci (Chung *et al.*, 2002). In undisturbed natural populations of mature trees (DBH ≥15 cm) no small scale spatial genetic structure was observed. Contrastingly, in a disturbed, isolated population, significant (but weak) positive, fine-scale spatial genetic structure was observed up to almost 50 m. Similarly weak but significant fine-scale spatial genetic structure was found in two further disturbed populations (Chung *et al.*, 2004). This structuring of

disturbed populations may be due to the effects of habitat disturbance on seed dispersal and recruitment.

Also in Korea, spatial genetic structure was studied in three populations of *Quercus crenata*, using multilocus allozyme genotypes (Chung *et al.*, 2005). Significant fine-scale structure was found in the population which was putatively established from old on-site individuals, in contrast to the other populations which were putatively established from off-site individuals.

A mixed population of *Quercus petraea* and *Quercus robur* was studied in north-west France (Streiff *et al.*, 1998). Data from six microsatellite loci were compared with data from four isozyme loci used in a previous study of the same stand. This study showed similar results from isozyme and microsatellite loci, although greater interlocus variance in isozymes was observed. Weak fine-scale genetic structure was observed in both species, although it was stronger in *Quercus petraea*. It was suggested that the acorns of *Quercus robur* are more efficiently dispersed, perhaps by jays, leading to an erosion of spatial structure. Streiff *et al.* (1998) also compared the use of R_{ST} and F_{ST} in order to determine if in this case the step-wise mutational model was important. Less structure was shown by the use of R_{ST}, suggesting that either mutations were occurring independently in different areas, or gene flow was of sufficient magnitude to erode any structuring in allele sizes.

Populations of *Quercus lobata,* the California Valley oak, are severely fragmented due to human activities. There is concern that this threatened species may be increasingly threatened by limited gene flow. Using six microsatellite loci, significant fine-scale genetic structure was detected. These data were also used to examine the extent of anisotropic gene flow but no anisotropy was detected (Dutech *et al.*, 2005).

The wild service tree, *Sorbus torminalis,* was investigated in northern Switzerland using six allozyme loci (Hoebee *et al.*, 2006). In small, isolated populations, some degree of spatial genetic structure was found up to distances of 15 and 30 m. However, when duplicated multilocus genotypes (clones) were excluded, there was no spatial genetic structure, presumably due to efficient pollen and seed dispersal.

Fifteen isozyme loci were used to elucidate the clonal structure of *Quercus havardii,* which forms dense patches of vegetatively-spreading scrub (Mayes *et al.*, 1998). It was

found that the population was largely formed of sexually reproducing individuals, which were spreading clonally to increase their size. However, the genetic makeup of the population was consistent with an outbreeding system.

Genetic structure including clonality of *Populus tremuloides* was examined in two Canadian stands, using four microsatellite loci (Namroud *et al.*, 2005). The G/N ratio (number of genets divided by number of ramets) varied from 0.67 to 0.92 among different stands and age cohorts, showing a relatively low number of ramets per genet. Spatial genetic structure was apparent up to around 30 m, but was weaker in older cohorts (> 100 yrs) than in younger cohorts (≤ 40 years).

In *Populus nigra*, Barsoum *et al.* (2004) studied populations along 30 km of the river Garonne in France. Twenty-two percent of all trees sampled had replicate genotypes, showing clonal reproduction to be important. In this study the authors also identified the importance of the translocation of fragments in long-distance clonal reproduction. Even with the limited sampling strategy, 11 replicate genotypes were discovered at distances apart of up to 19 km. The microsatellites used gave very high probabilities that these identical multi-locus genotypes had not occurred independently.

Although small-scale spatial genetic structure has been observed in many of these studies, in most cases it is weak, and in some negligible. The explanations for this are likely to be found in the overlapping generations as well as extensive gene flow common to many tree species. Where clonal reproduction is significant, genetic markers can help to elucidate the extent of clonal genets, as this can vary between different environments.

2.2.5 Genetic effects of regeneration and other management practices

It is well recognised that forest management and silvicultural practices may affect genetic diversity within tree populations. A recent review focussing on Central Europe (Hosius *et al.*, 2006) discusses historical factors, a range of silvicultural practices and the possible effects of the breeding and domestication of trees. Lefevre (2004) also reviews human impacts on forest genetic resources, and discusses breeding, seed transfer, management and environmental alterations. It is the silvicultural practices (reviewed by Finkeldey and Ziehe, 2004) and particularly the method of regeneration that are of interest in this study.

In forestry practice, regeneration may be by natural or artificial means, and the details of the regeneration stage vary between silvicultural systems (e.g. Matthews, 1991). A number of authors have studied the effects on genetic diversity of different regeneration practices.

Genetic diversity of *Eucalyptus consideniana* in Australia was measured under alternative regeneration strategies: clear-fell followed by aerial sowing, and seed tree treatments, where four trees were left in each coupe and natural regeneration was allowed to take place (Glaubitz *et al.*, 2003a). A significant loss of rare alleles was recorded in the seed tree plots compared to the clear-fell plots, perhaps because the seed used for sowing had been collected from a greater number of trees. The authors suggest that this locally-uncommon species may be more vulnerable than the locally-common species *Eucalyptus sieberi*; in a similar study of the latter species there were no differences in genetic diversity between the different treatments (Glaubitz *et al.*, 2003b).

No differences in genetic diversity were found between uncut stands, shelterwood leave trees, seed crops in shelterwoods and natural regeneration in shelterwoods (using ten allozyme loci) of *Pseutdotsuga menziesii* in southwest Oregon (Neale, 1985). Contrastingly, although populations were little altered by natural or artificial regeneration, cutting the smallest trees to leave shelterwoods in *Pseudotsuga menziesii* (also in Oregon) was found to slightly reduce the number of effective alleles per locus (Adams *et al.*, 1998).

In a large-scale investigation of *Picea glauca* in Saskatchewan, old-growth stands, natural regeneration, plantations and phenotypically-selected trees were studied using around 50 RAPD loci (Rajora, 1999). A significant reduction of genetic diversity was found in the plantations and the phenotypically-selected trees. Similarly, in a study of *Picea abies* using eight isozyme loci, no difference was found between old-growth forests and naturally-regenerated managed stands, but artificially-regenerated stands had decreased genetic diversity and a change in allele frequencies (Gomory, 1992). In a study of *Pinus contorta* using RAPD and microsatellite loci naturally-regenerated stands were found to have slightly lower diversity than unharvested stands, but the differences were found not to be significant (Thomas *et al.*, 1999). In addition, Thomas *et al.* (1999) discuss the relative magnitude of differences in genetic diversity found in different

studies, and consider the difficulties in comparing studies, especially where significance is not tested (e.g. Gomory, 1992).

In some cases, regenerative material is grown in nurseries, before planting out for afforestation schemes or on the restock sites created by clearfelling. In a study of *Fagus sylvatica* using 14 allozyme loci, differences in genetic diversity among seedlings grown in greenhouses and outdoors, in open beds and in plugs, and among seedlings sorted for sizes, were investigated (Konnert and Ruetz, 2003). Almost no differentiation was found among groups of seedlings grown in different conditions, and amongst seedlings sorted by size only very small differences were found. Similarly *Pseudotsuga menziesii* seedlings were not found to be selectively influenced under standard nursery procedures (Konnert and Ruetz, 2006). In contrast to these studies, a previous study by the same workers found that size-sorted seedlings of *Picea abies* and *Abies alba* differed in genetic structure (Konnert and Schmidt, 1996, cited in Konnert and Ruetz, 2003).

In another nursery study, the changing contribution of different half-sib families to a seedling crop of *Pseudotsuga menziesii* was studied. The seedlings were sown in cavity blocks with three seedlings per cavity due to the poor germination rate. Largely due to variation in germination rates and time, when families were mixed the proportion of families changed after thinning to one seedling per cavity, as the largest (earliest germinated) seedlings were preferentially selected (El-Kassaby and Thomson, 1996). To avoid this, the authors suggest sowing seed-lots separately by family to maximise the genetic variation provided by using multiple seed parents.

Due to the different methods used by different workers it is inevitably difficult to compare results. However, while there are reports of both slight but significant effects and of no significant effects of artificial regeneration on genetic diversity, no studies report highly reduced diversity. Many authors discuss the significance of the number of seed trees used in the case of artificial regeneration, and it may be that when high (> 20) numbers of seed trees are used, as commonly recommended, there is rarely a substantial loss of genetic diversity.

Other silvicultural practices such as thinning, used increasingly under conversion to continuous cover forestry systems, may also affect genetic diversity. In comparisons of

different thinning methods, stands of *Fagus sylvatica* were found to have suffered a loss of genetic variation compared to unthinned reference plots (Dounavi *et al.*, 2002). Target-diameter harvesting in *Fagus sylvatica* was also demonstrated to lead to loss of genetic variation and reduce the potential of subsequent generations to reach larger diameters (Ziehe and Hattemer, 2002). It is possible that thinning and harvesting systems may be more important than regeneration in terms of genetic diversity. However, under natural regeneration systems, the genetic diversity of the shelterwood or seed trees will be passed on to the next generation, and so it is important to consider the genetic consequences of thinning and harvesting operations.

2.3 *Fraxinus excelsior* L. (Oleaceae): common ash

2.3.1 Description and ecological classification

Fraxinus excelsior is a deciduous tree, usually 12-18 m in height but occasionally reaching 43 m (Wardle, 1961). The crown is open and narrow. The bark is smooth and grey, becoming furrowed in mature trees; the twigs are thick, with black buds. The leaves are pinnately compound and opposite, opening in late spring. The purplish flowers open before the leaves, and the fruit are winged samarae commonly known as "keys".

Fraxinus excelsior is the third most common broadleaved tree in British woodlands (12% of the total area of broadleaved trees, after *Quercus* spp. and *Fagus sylvatica*), and is second only (16%) to *Quercus* spp. in Wales (Locke, 1987). It frequently forms a part of mixed deciduous woodland, although pure stands can be found. *Fraxinus excelsior* does not easily colonise undisturbed ground, but rapid growth allows seedlings which may be suppressed in the ground layer of woodland to take advantage of gaps which form in the canopy (Grime *et al.*, 1988). *Fraxinus excelsior* prefers base-rich soils (Wardle, 1961) and is usually absent where the pH of the surface soil is less than 4.2. In Wales it is found on lower slopes and valley floors (Clark, 2000). In Britain, *Fraxinus excelsior* is a dominant species of the habitat defined as '*Tilio-Acerion* forests of slopes, screes and ravines', which is defined as a priority habitat under Annex I of the European Habitats Directive (JNCC, 2004). Several areas of this habitat have therefore been designated as European Special Areas of Conservation (SACs).

2.3.2 Reproductive biology

The reproductive system of *Fraxinus excelsior* is curious, being described as 'total sexual confusion' (Mitchell, 1974). The species is polygamous, with trees being male, female, hermaphrodite, or a mixture, with flowers of different types being found on different branches, or in different years, on the same tree (Wardle, 1961). *Fraxinus excelsior* is wind-pollinated or anemophilous, and the fruits are also dispersed by wind.

2.3.3 Distribution and species history

Fraxinus excelsior is widely distributed throughout Britain, where it is described as native except in the far north of Scotland (Preston *et al.*, 2002). *Fraxinus excelsior* is also native to most of Europe (Wardle, 1961) except in the steppe regions and parts of the Mediterranean. The widespread distribution of *Fraxinus excelsior* in Europe is thought to be the result of post-glacial re-colonisation. The recolonisation of Britain by *Fraxinus excelsior* is described by Birks (1989) using isochrone maps based on pollen cores. This palynological approach shows that while *Fraxinus excelsior* was present in southern and central England between 7000 and 6000 BP, its gradual north-western spread into the remainder of Britain continued until around 3000 BP. The distribution of *Fraxinus excelsior* in Britain is now largely the result of anthropogenic factors. *Fraxinus excelsior* has been planted widely in many areas, and extensively cleared from many more. In studies such as this one, there is a risk that the patterns recorded may be the result of artificial planting rather than natural patterns. However, by selecting study populations which are believed to be relatively natural, such as those on steep slopes and putatively ancient woodland sites, it is hoped to minimise this risk.

2.3.4 Uses of *Fraxinus excelsior*

Fraxinus excelsior can produce a high-quality hardwood timber, and so the species may be planted for commercial as well as conservation purposes, and is in fact the second most widely-planted broadleaved tree in Britain (Harmer and Forrester, 1994). Seasoned *Fraxinus excelsior* timber is tough and flexible, being the wood of choice for the handles of tools and some sports goods (Savill, 1991). The timber may also be used for building and furniture making. *Fraxinus excelsior* coppices easily and produces firewood of a high quality. The seeds, when green, may be pickled (Phillips, 1983). Various parts of *Fraxinus excelsior* have also been used in traditional herbal medicine (Culpeper, 1652).

2.3.5 Genetic diversity studies of *Fraxinus excelsior* – the current state of knowledge

Within the last decade, with the general trend of increasing interest in molecular ecology, a number of studies have used molecular markers to study aspects of the genetics of *Fraxinus excelsior*. Nuclear microsatellite markers have been developed (Brachet *et al.*, 1999; Lefort *et al.*, 1999) that show good levels of polymorphism. Chloroplast markers have also been used for studies of larger scale patterns of genetic diversity.

In a Europe-wide study, samples were collected from around 200 widely-distributed populations. Chloroplast microsatellites were used and identified twelve distinct haplotypes (Heuertz *et al.*, 2004a). As shown in Figure 2.1 the different haplotypes were found in different areas, with five haplotypes accounting for most samples (haplotypes 6-12 were found in six or fewer individuals). As with other species discussed above, different western and eastern lineages are found. More chloroplast diversity is found in south-eastern Europe. In Britain, haplotypes were almost exclusively of the Iberian type.

In a second Europe-wide study, Heuertz *et al.* (2004b) used five nuclear microsatellite loci and found that populations in western Europe had little differentiation over large scales, and could be characterised as a single deme with high allelic richness and genetic diversity. In contrast strong genetic differentiation occurred over short distances in Sweden and south-eastern Europe, where allelic richness and genetic diversity were lower. It seems possible that significant admixture occurred in western areas during migration from several different refugia, while in south-eastern areas post-glacial mixing was limited by topography.

Heuertz *et al.* (2001) used six of the primer pairs identified by Brachet *et al.* (1999) and Lefort *et al.* (1999) to analyse variation in microsatellites within and among *Fraxinus excelsior* populations in Bulgaria, from a region within a putative refugium of the species during the last glaciation. Their results showed high levels of polymorphism in the markers studied, indicating high genetic diversity (overall mean H_E within populations = 0.731), similar to that found in other tree species. However, a low but significant level of inbreeding was also found (F_{IS} = 0.014, F_{IT} = 0.104). This could be due to the presence of null alleles, or to biparental inbreeding. A low level of differentiation among populations was recorded (F_{ST} = 0.087), 80% of which resulted

from variation among populations within regions (F_{SR} = 0.070) with only 20% resulting from variation among regions (F_{RT} = 0.018). Genetic structure was consistent with isolation by distance, with Wright's neighbourhood size calculated at 38-66 individuals from the intrapopulation analyses and 126 from the interpopulation analysis. These estimates are an order of magnitude smaller than those calculated by other workers for *Quercus petraea* and *Quercus robur* (Le Corre *et al.*, 1998; Streiff *et al.*, 1999).

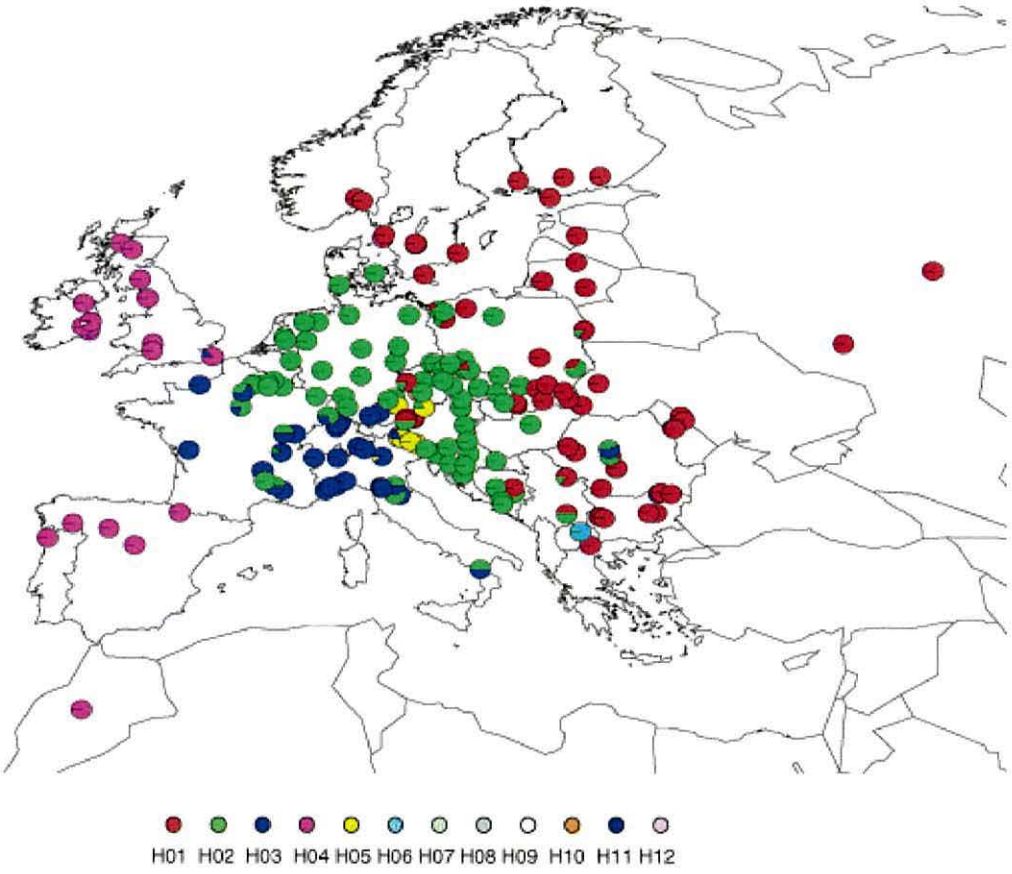

Figure 2.1 Geographical distribution and frequency of chloroplast microsatellite haplotypes in *Fraxinus excelsior*. From Heuertz et al. (2004a).

Nuclear microsatellites were also used to analyse genetic structure among twelve French populations of *Fraxinus excelsior* (Morand *et al.*, 2002). As in the study by Heuertz *et al.* (2001), a high level of intrapopulation diversity was found (mean H_E = 0.899), but a significant deficiency of heterozygotes was also observed. F_{IS} was calculated at 0.163 in seeds and 0.292 in adult trees, indicating once again either the presence of null alleles, a degree of mating among relatives or partial selfing, or the existence of a Wahlund effect (this would imply that temporal or spatial breeding subunits exist within the populations studied).

Another study of spatial genetic structure was carried out in a Romanian population of *Fraxinus excelsior* (Heuertz *et al.*, 2003). In this case the patterns of isolation by distance were analysed in order to obtain information about the relative dispersal of seeds and pollen. The slope of the plot of kinship against logarithmic distance is known to give information about the level of gene flow, while the shape may give information about the relative importance of seed vs. pollen dispersal. The data obtained were compared with simulations, suggesting that the initial steepness of the slope indicates limited seed dispersal. Suggested mean dispersal distances are up to 14 m for seeds and between 70 m and 140 m for pollen.

Four nuclear and five chloroplast microsatellite loci were used to compare genetic variation among populations of *Fraxinus excelsior* from different provenance regions of southern Germany (Hebel *et al.*, 2006). The nuclear microsatellites showed high levels of polymorphism and heterozygosity. The overall F_{IS} value was 0.080, while the F_{ST} value was estimated at 0.012. Six chloroplast haplotypes were found and while a mixture of two common haplotypes and one or two rare ones was found in two regions, only one haplotype was found in the third region.

In highly fragmented remnant populations of *Fraxinus excelsior* in Scotland, (Bacles *et al.*, 2005) found high levels of genetic diversity. Expected heterozygosity, H_E, was 0.849 overall, very similar to that found in France, and slightly higher than that found in Bulgaria. Differentiation among remnant populations was low (average $\theta = 0.080$, 95% confidence interval 0.047-0.121) and effective pollen dispersal distances were high, averaging 328 m. A proposed explanation is that the open landscape facilitates airborne pollen movement.

The effects of discontinuous marginal habitats were also addressed at the northern limit of *Fraxinus excelsior* on islands in southern Finland (Höltken *et al.*, 2003). Lower numbers of alleles were found at four microsatellite loci in more isolated populations, and the more isolated populations were also found to be more genetically differentiated from the overall gene pool than the less isolated populations. The overall estimated F_{ST} value was 0.123.

Morand-Prieur *et al.* (2002) used microsatellites from the chloroplast genome to study inheritance in controlled hybrids between *Fraxinus excelsior* and narrow-leaved ash,

Fraxinus angustifolia Vahl. Only one marker was found to be polymorphic between *Fraxinus excelsior* and *Fraxinus angustifolia* but in controlled crosses this marker was found to be the same in the hybrids as in the female parent, regardless of which species was used as the female. This provides evidence of maternal inheritance of the chloroplast genome in these *Fraxinus* species.

Another study investigating hybrids between *Fraxinus excelsior* and *Fraxinus angustifolia* used a combination of microsatellite markers and RAPDs, which showed that in controlled crosses a large number of hybrids was easily produced (Raquin *et al.*, 2002).

In another study of *Fraxinus excelsior* using nuclear microsatellites, paternity analysis of controlled crosses was used to show that pollen from male flowers was more successful in producing seed than pollen from hermaphrodite flowers (Morand-Prieur *et al.*, 2003). While pollination by pollen from hermaphrodite trees produced some seed when used in isolation, pollen from male trees produced ten times more fruits, and when a mixture of pollen from both types of trees was used, 106 seedlings out of 107 were sired by male trees and only one by a hermaphrodite tree. Three seedlings were also found to be the result of selfing by hermaphrodite flowers, providing evidence of some self-compatibility, although selfing has usually been found to lead to general failure of fruit production (Morand *et al.*, 2002).

2.4 Summary and conclusions

The study of patterns of genetic structure and diversity can provide much information about the history and biological processes that characterise a species. Developing technology in recent decades has allowed scientists to collect and analyse increasingly large data sets, which have been used to draw an ever more detailed picture of gene flow, mating systems and migration patterns in a wide range of species including trees.

Genetic structure and diversity are of interest in trees for scientific reasons, but also for a range of social and economic reasons. Genetic patterns found in trees are frequently significantly different from those found in smaller, shorter-lived, plants. The typical patterns of high within-species diversity but low among-population diversity may result from the tall stature and low population density of many trees. This pattern is found in many trees although mating systems and clonal reproduction vary widely among

species. It is possible for forest management and silvicultural practices to affect levels of genetic variation in trees, but while some studies have shown significant effects, many have found only slight effects or no effects.

Genetic diversity in *Fraxinus excelsior* has been studied in a number of countries and at different scales. In general, patterns of diversity have been similar to those found in many other tree species, with low differentiation among populations, high diversity within populations, and relatively small neighbourhood sizes. In Europe-wide studies, different regional patterns have been found, with the south-east displaying a greater number of chloroplast haplotypes and greater genetic differentiation among populations at nuclear microsatellite loci. Western Europe, in contrast, has only a few chloroplast haplotypes and very low genetic differentiation among populations based on nuclear microsatellites.

Although a number of studies exist of genetic diversity in *Fraxinus excelsior* in continental Europe, the only study in Britain is one of severely fragmented Scottish populations (Bacles *et al.*, 2005). It was therefore decided to investigate diversity within less isolated populations in Wales. Studies of small scale genetic structure have so far been limited to those in eastern Europe (Heuertz *et al.*, 2001; Heuertz *et al.*, 2003), and it was felt that given the genetic differences outlined above, it would be interesting to carry out a small-scale study of genetic structure in western Europe.

Although the results of some controlled crossing experiments (Morand-Prieur *et al.*, 2003) were available, little information was available on the natural breeding system of *Fraxinus excelsior* although a report including this has recently been published (FRAXIGEN, 2005). A number of workers, as described above, have carried out research into the effects of silvicultural practices on genetic diversity, but these studies have mainly focussed on coniferous species. A small number of studies have investigated changes in genetic diversity in *Fagus sylvatica* (Konnert and Ruetz, 2003; Ziehe and Hattemer, 2002), but it was felt that temperate broadleaved species are underrepresented in these studies and it was decided to investigate the effects of regeneration practices in *Fraxinus excelsior*.

3. Materials and methods

3.1 Field sites

3.1.1 Selection of field sites and description

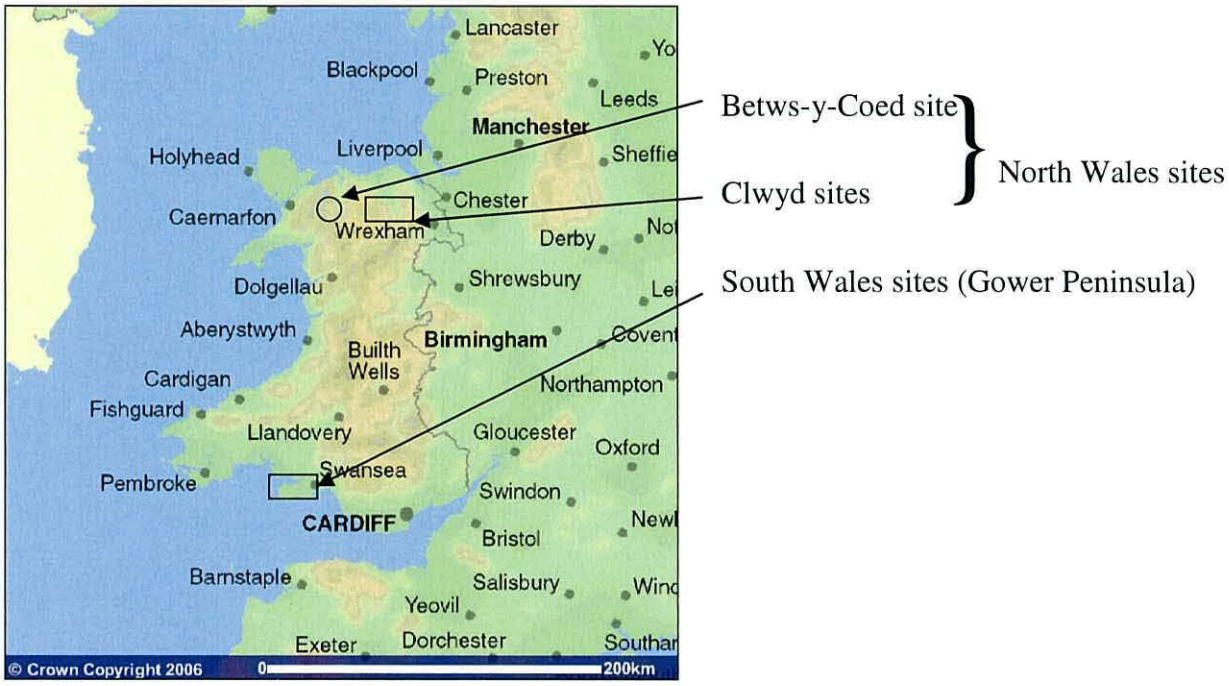

Figure 3.1 Map of Wales showing the location of the field sites.

Six field sites were selected: three in South Wales and three in North Wales (two in Clwyd and one at Betws-y-Coed). The locations of the sites are shown in Figure 3.1. Apart from the Betws-y-Coed site, all the sites are parts of Special Areas of Conservation (SACs), designated primarily because of the Annexe I habitat type 9180: 'Tilio-Acerion forests of slopes, screes and ravines' (JNCC, 2004). In Britain this habitat type is characterised by woodland dominated by ash. All the sites are also believed to carry ancient semi-natural woodland (Walker and Richardson, 1989). The trees sampled are, therefore, putatively of autochthonous origin.

The South Wales sites (map in Chapter 5) form part of the Gower Ash Woods SAC (JNCC, 2004). This comprises a number of steep-sided valleys and coastal slopes cut into Carboniferous limestone. The first site, Bishopston Valley (NGR SS574890), is a steep sided valley about three km long. The other two sites, Nicholaston Woods (NGR

SS520881) and Oxwich Woods (NGR SS504860), are on coastal slopes, approximately two km apart on either side of Oxwich Bay, while Bishopston Valley runs north to south around five km to the east. Approximately 200 km to the north-east, the Clwyd sites (map in Chapter 5) are part of the Alyn Valley Woods (NGR SJ197627) and Elwy Valley Woods (NGR SJ019685) SACs. Both valleys run from south to north, approximately 10 km apart on either side of the Clwydian Range. At these sites also the geology is primarily limestone. Mixed deciduous woodland covers all the sites and is either dominated by or contains a high proportion of ash. Maps of each area are shown in Chapter 5 and large-scale maps of each site are shown in Chapter 4 (Bishopston Valley) and in Appendix II (remaining sites).

The original intention was to collect seed from at least one of the South Wales or Clwyd sites. However, in 2005, the second field season, there was very little ash seed in Wales (or elsewhere in Britain). A sixth field site was therefore selected, because seed had already been collected from this site in 2004, and a sub-sample of seed was available for the current study. This site is an area of predominantly ash woodland between the junction of the A470 and the A5 (NGR SH799555), near Betws-y-Coed in North Wales, approximately 30 km west of the Clwyd sites. This woodland is also believed to be ancient semi-natural woodland.

Sites were selected that consisted of woodlands (with owners sympathetic to scientific research) with a high proportion of *Fraxinus excelsior*. The initial intention was to study anisotropic gene flow in a number of parallel steep-sided valleys on the Gower peninsula. However, as preliminary data from the three Gower sites was analysed it became clear that genetic structure and differentiation were limited at these sites. It was therefore decided to include sites from a greater distance, and the Clwyd sites were chosen, providing another pair of sites with slightly greater separation and a significant range of hills between them, but with a great degree of separation from the Gower sites. These five sites all contained similar habitat with a high proportion of *Fraxinus* excelsior on steep limestone slopes. The Betws-y-Coed site does not fit this description, but was chosen due to the availability of seed and ease of sampling, and provides a somewhat intermediate distance between sites. It might have been ideal to sample another site in mid-Wales, but there are no limestone valleys conveniently positioned, and ultimately constraints of time and money limited the collection of further samples.

3.1.2 Sample collection

Samples were collected during May and June from the South Wales sites in 2004 and the Clwyd sites in 2005. Leaves were collected from mature trees approximately every 20 m ($\bar{x}$ = 26.6 m, σ = 15.8 m) along transects through the woodlands. At each site the transect began at one end of the woodland and continued lengthwise, following the direction of the river (in the valley sites) or parallel to the coast, in as constant a direction as possible, given the irregular positions of the trees. The locations of trees within the site were recorded by starting at a known location and measuring the distance, bearing and slope between each pair of trees using a 50 m tape, compass and clinometer. The young leaves were put into plastic bags containing silica gel and transported to the laboratory. Samples were collected from approximately 30 mature trees at all sites except Bishopston Valley where samples were collected from 90 mature trees and also from 83 naturally regenerating seedlings or saplings (<2 cm diameter) nearby. For all sampled mature trees at the South Wales and Clwyd sites, diameter at breast height (DBH) was also recorded with a diameter tape. The tape measured diameters up to 60 cm, so trees with a greater diameter were included in the 60 cm diameter class.

Seeds had been collected by staff of Forest Research (the research agency of the Forestry Commission of Great Britain) in 2004 from 20 trees at the site in Betws-y-Coed. A subsample of this seed was obtained from Forest Research, and 20 seeds were sampled from ten of the parent trees, while two seeds were sampled from the remaining ten. In July 2005 samples were collected from 32 mature trees and 32 naturally regenerating seedlings or saplings (<2cm diameter) in the population at Betws-y-Coed. Some of the collected seed had been sown in plugs in spring 2005 at the Carmarthen Tree Nursery (near Carmarthen, South Wales, NGR SN470165). Thirty-five of these nursery-grown seedlings were also sampled.

3.2 Laboratory work

3.2.1 Extraction of DNA and visualisation

DNA was extracted from approximately 20 mg of dried young leaves using the DNeasy Plant Mini kit (QIAGEN). Samples were ground in 1.5 ml tubes using plastic micropestles, some with liquid nitrogen. However, it was found that when leaves were

fully dried grinding could be successfully achieved without the addition of liquid nitrogen. Some samples were also ground using the Qiagen TissueLyser mill.

Dried seeds were separated from the samarae and soaked overnight in distilled water. The embryos were then dissected from the rehydrated seeds and immediately ground with liquid nitrogen prior to extraction using the DNeasy Plant Mini kit (QIAGEN). Because of the small quantity of starting material, the volume of the final elution buffer (Buffer AE) was reduced to 2 × 50 µl instead of 2 × 100 µl.

In order to verify the success of the extraction, 5 µl of each extract was run out on a 1% agarose gel (either a mini gel, run at 70 V for 30-45 minutes, or a larger gel run at up to 100 V for 45-60 minutes) containing 0.005% ethidium bromide. Gels were run in 1 × TBE buffer. A marker such as the SuperLadder-Low 100BP Ladder (ABgene) was run in an additional lane. An ultra-violet light box was then used to visualise the DNA fragments and the gels were photographed.

3.2.2 PCR and genotyping

Six pairs of microsatellite primers which had previously shown good levels of polymorphism (Brachet *et al.*, 1999; Lefort *et al.*, 1999) were used in a polymerase chain reaction (PCR). Details of the markers are shown in Table 3.1. The PCR reactions were performed in a mix containing 0.4 µM of each primer and 1 µl of template DNA (extract diluted 20 times) with 1.1 × Thermo-Start® PCR Master Mix (ABgene). The reactions contained either 1.5 mM (FEMSATL2, FEMSATL4, FEMSATL16, FEMSATL19, M2.30) or 4.5 mM (FEMSATL11) of $MgCl_2$. After an initial activation step at 95°C for 15 minutes, amplification for four pairs of primers comprised 35 cycles of 30 s denaturing at 94°C, 45 s annealing at either 56°C (FEMSATL11, FEMSATL19), 52°C (FEMSATL4) or 60°C (M2.30) and 30 s extension at 72°C. For the remaining two primer pairs the annealing temperature was reduced during the first 10 cycles from 57°C to 52°C (FEMSATL16) or 50°C to 46°C (FEMSATL2) followed by a further 30 cycles (FEMSATL16) or 25 cycles (FEMSATL2) at the lower temperature. The PCR reactions were performed on a Perkin Elmer GeneAmp® PCR System 9700.

To confirm that the PCR had been successful, 5 µl of each reaction was run out on an agarose gel as described above.

Table 3.1 The six microsatellite loci used. T_a, annealing temperature. Published in [1]Lefort et al. (1999), [2]Brachet et al. (1999). [3]The forward primer for M2.30 was redesigned from the published sequence. [4]Size range in original publication. [5]Size range from Bulgarian populations (Heuertz *et al.*, 2001)

Locus	Genbank Accession no.	Primer sequence (5'-3')	T_a (this study)	Repeat motif	Size range (bp)[4]
FEMSATL2[1]	AF023473	F: TCTTTATCATCAAAAAATAA	46	$(CT)_{28}$	174-212
		R: TACAAGGTGATATCACTTCT			
FEMSATL4[1]	AF006069	F: TTCATGCTTCTCCGTGCTTC	52	$(CA)_2(AG)_{24}$	164-228[4] 158-251[5]
		R: GCTGTTTCAGGCGTAATGTG			
FEMSATL11[1]	AF020394	F: GATAGCACTATGAACACAGC	56	$(GT)_{23}$	138-188[4] 176-266[5]
		R: TAGTTCTACTACTTCAAGAA			
FEMSATL16[1]	AF029882	F: TTTAACAGTTAACTCCCTTC	52	$(CA)_3(CG)(CA)_{10}(TA)_2(CA)_3$	180-226[4] 176-204[5]
		R: CAACATACAGCTACTAATCA			
FEMSATL19[1]	AF020400	F: CTGTTCAATCAAAGATCTCA	56	$(CA)_6CGGC(CA)_{13}$	180-200[4] 142-229[5]
		R: TGCTCGCATATGTGCAGATA			
M2.30a[2]	AF021337	F: CGCACGTTCTTTCTATTTG	60	$(TG)_{15}(AG)_{23}$	182-248[4] 182-294[5]
		F2[3]:CACGTTCTTTCTATTTGCAGTCG			
		R: GACCGGCTGACTATTTTCTC			

The forward primers were WellRED oligos (Proligo®) labelled with fluorescent dyes at the 5' end: D2-PA for FEMSATL19 and M2.30, D3-PA for FEMSATL4 and FEMSATL16 and D4-PA for FEMSATL2 and FEMSATL11. This allowed the pooling of three PCR products together with an internal size standard. As the size ranges of all loci are overlapping it was not possible to multiplex more than three PCR products. Genotyping of the PCR products was carried out using the CEQ* Genetic Analysis System (Beckman Coulter, Inc.).

To confirm that no contamination had occurred a negative control was run in each batch of PCRs, including 1 µl ultrapure H_2O instead of template DNA. In order to test the reproducibility of the genotyping, four samples were selected as positive controls and these samples were repeatedly amplified and genotyped with different batches of PCRs. Genotyping was found to be highly reproducible although the size of amplified fragments could vary up to one base pair in length.

3.3 Analysis

3.3.1 Scoring of alleles and preliminary analysis

Fragments were sized automatically with the use of an internal size standard. In all cases sizes were rounded to the nearest whole number of base pairs. The microsatellites are all formed by dinucleotide repeats, and so FEMSATL2, FEMSATL4, and FEMSATL11 were binned into even numbers of base pairs and FEMSATL16 was binned into odd numbers (because most alleles were closer to odd numbers in this case). In the cases of FEMSATL19 and M2.30, it was not possible to determine whether odd or even numbered alleles were more appropriate, and so allele lengths were kept at odd and even numbers of base pairs. From Table 3.1 it may be observed that these two loci do not consist of perfect dinucleotide repeats but are made up of more than one motif. This may help explain the departure from two-base-pair spaced alleles.

The programme Microchecker (van Oosterhout *et al.*, 2004) was used to check for alleles beyond the expected size range, for alleles not consistent with dinucleotide microsatellites (FEMSATL19 and M2.30 were classed as mononucleotide microsatellites for this purpose) and for the likelihood of null alleles.

Further analysis was carried out using the programmes FSTAT 2.9.3 (Goudet, 2001) and GenAlEx 6 (Peakall and Smouse, 2006) and details are given in the following chapters.

4. Bishopston valley: genetic diversity within a site

4.1 Introduction

Samples were collected in Bishopston Valley from mature trees along a transect which followed the line of the valley, and from natural regeneration close to each mature tree. A map of the site is shown in Figure 4.1.

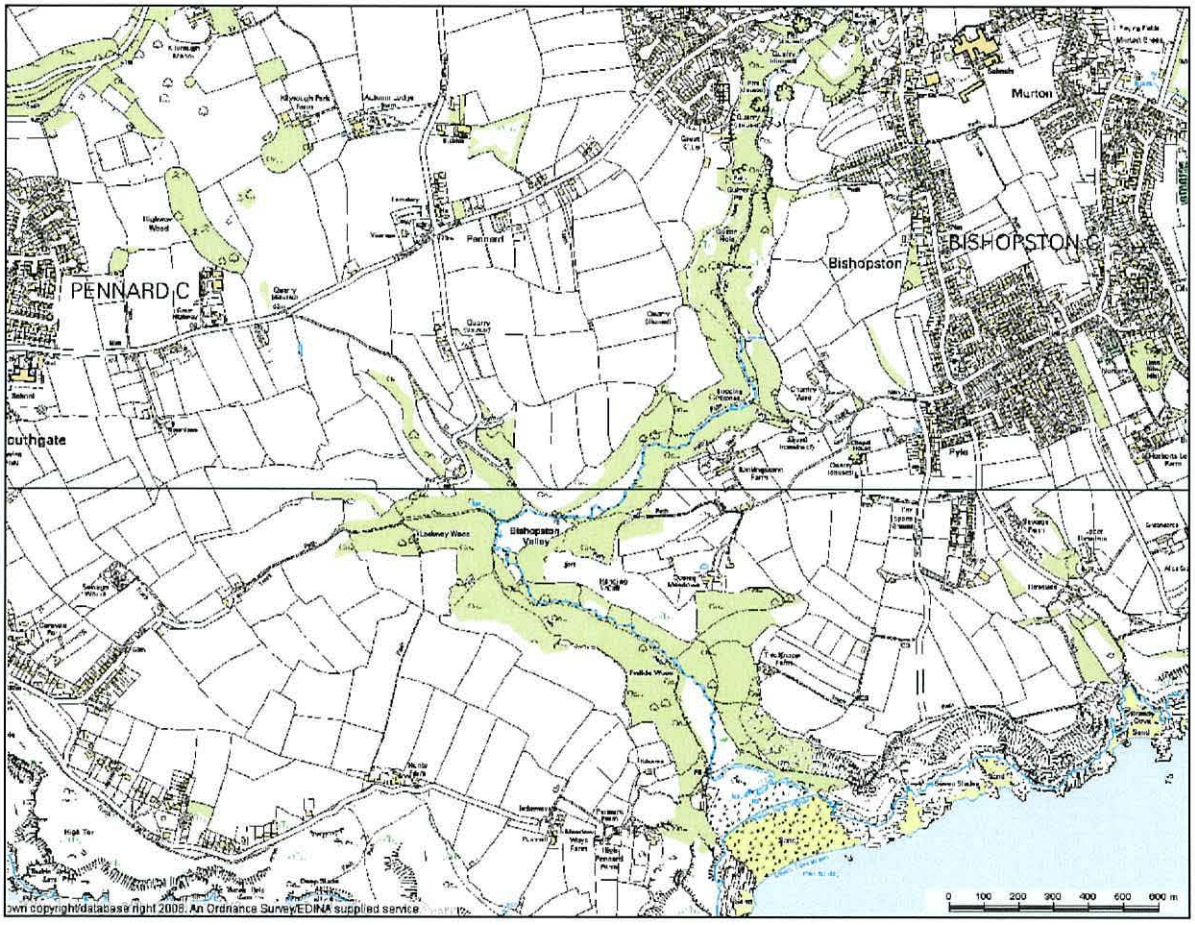

Figure 4.1 Map showing Bishopston Valley, South Wales (NGR SS574890).

The aims of this part of the study were to determine (1) the genetic diversity of the mature *Fraxinus excelsior* trees in the valley, and to compare this with the genetic diversity of the natural regeneration, (2) the degree of spatial genetic structure within the population and (3) the magnitude of geneflow within the population.

4.2 Analysis

The programme Micro-checker 2.2.3 (van Oosterhout *et al.*, 2004) was used to determine the likelihood of null alleles being present at any loci, based on the frequency of heterozygotes and homozygotes for alleles of different sizes.

Allele frequencies and summary statistics (number of alleles, allelic richness, *F*-statistics, observed and expected heterozygosity, departure from Hardy-Weinberg Equilibrium and probability of linkage disequilibrium) were calculated using GenAlEx 6 (Peakall and Smouse, 2006) and FSTAT 2.9.3 (Goudet, 2001). These were calculated for the mature trees, for the natural regeneration, and for all samples together. They were calculated over all loci and at each locus, and then, due to the excess homozygosity observed at locus FEMSATL16, over five loci excluding FEMSATL16.

A matrix of pairwise genetic distances between individuals was calculated using the method in GenAlEx (Peakall and Smouse, 2006). This uses a multilocus measure of genetic distance described in detail by Smouse and Peakall (1999). A matrix of pairwise geographic distances between individuals was also produced, and a Mantel test was carried out between the two matrices. In order to investigate in more detail the nature of small-scale genetic structure, a spatial autocorrelation analysis (Smouse and Peakall, 1999) was carried out. In this method, the correlation coefficient (r) between pairs of individuals separated by distances in a predetermined distance class is calculated. In order to calculate statistical confidence, the matrix of genetic distances is permutated 999 times amongst the distance classes in order to determine the values of r under the null hypothesis of random distribution of genotypes. In addition, confidence intervals around the calculated values of r are calculated by bootstrapping within each distance class.

Initially spatial autocorrelation was carried out using nine sets of even-sized distance classes from 5 m to 1600 m. These distance classes were chosen as they included the minimum and close to the maximum distances between sampled trees within the valley. The nine values of r for the first distance class in each of the nine analyses were used to produce a correlogram. A spatial autocorrelogram was also constructed using distance classes of increasing size, in order to minimise the problems caused by smaller sample sizes within classes.

Spatial autocorrelation was also carried out using an alternative measure of distance. It was suggested that gene flow might be anisotropic; that is that wind-mediated gene flow might be more significant along the valley than in other directions. To test this, autocorrelation was carried out using a one-dimensional measure of distance along the transect rather than distances between tree positions (given by two-dimensional x and y coordinates).

4.3 Results

The diameter distribution for the mature trees sampled in Bishopston Valley is shown in Figure 4.2. Diameter at breast height ranged from 8 cm to over 60 cm ($\bar{x}$ = 26.0, σ = 14.2).

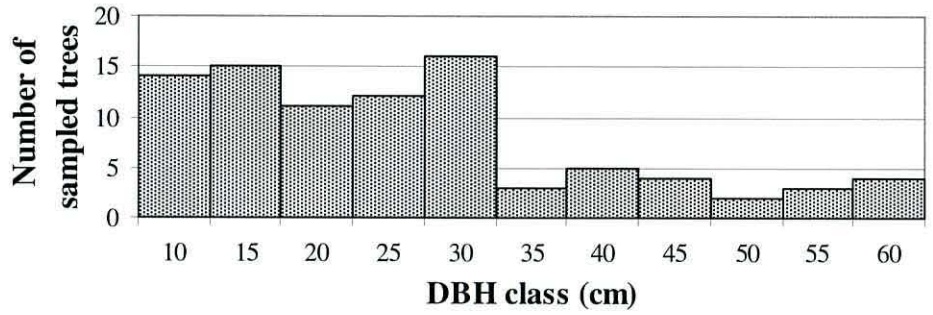

Figure 4.2 Distribution of diameter at breast height (DBH) of sampled mature trees of *Fraxinus excelsior* in Bishopston Valley, South Wales.

The locations for all the samples collected in Bishopston Valley are shown in Figure 4.3. A fairly uniform spacing was achieved along the transect which was nearly 3 km long, although due to the bends in the valley the maximum straight line distance between samples is 1730 m.

Results from Micro-checker (van Oosterhout *et al.*, 2004) showed that there was a possibility of null alleles at all loci. Estimated frequencies are shown in Table 4.1; when the mature trees and the regeneration are treated as separate populations null alleles are possible at all loci except FEMSATL4 and M2.30 (in mature trees). When mature trees and regeneration are treated as one population null alleles are possible at all loci, varying from 3% at FEMSATL4 to 24% at FEMSATL16. As FEMSATL16 had a greater estimated frequency of null alleles than the other loci, further analyses were carried out both including and excluding the locus.

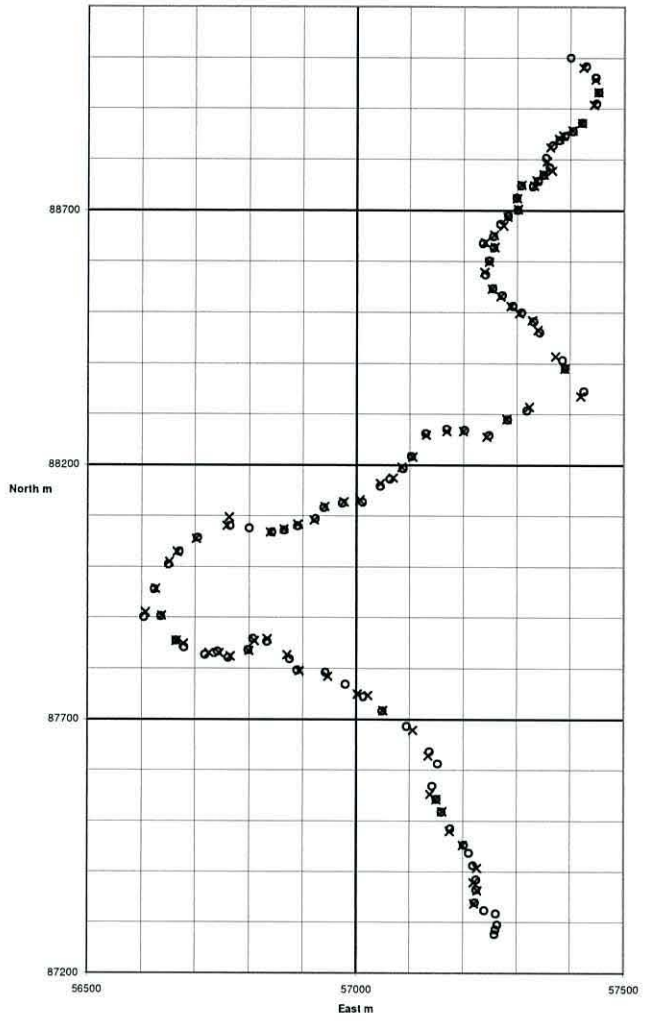

Figure 4.3 Distribution of the 173 samples in Bishopston Valley. Grid numbers shown are from the Ordnance Survey National Grid. Small squares are 100 m × 100 m. O, mature sample; X, regenerating seedling.

Table 4.1 Estimated percentage of null alleles at six microsatellite loci in samples of *Fraxinus excelsior* from Bishopston Valley, South Wales. Percentages are given for mature trees and regeneration separately and for the overall population.

	FEMSAT2	FEMSAT4	FEMSAT11	FEMSAT16	FEMSAL19	M2.30
Mature	10.9%	no	13.5%	22.5%	5.4%	no
Regen.	15.9%	no	12.5%	26.3%	5.3%	10.0%
Overall	13.0%	3.0%	5.9%	24.4%	5.5%	13.6%

Table 4.2 Microsatellite diversity in a population of *Fraxinus excelsior* in Bishopston Valley, South Wales. For each locus in each age cohort and over all trees the number of samples successfully genotyped (N), the number of observed alleles (A_O allelic richness, the expected (H_E) and observed (H_O) heterozygosities, and F values are reported. Results are shown for each locus, over all six loci, and over five loci, excluding FEMSATL16. Significance of F statistics is indicated by asterisks. ns, nonsignificant; *p ≤0.05; **p ≤0.01; ***p ≤0.001.

	Age cohort																	
Locus	**Mature**						**Regeneration**						**All**					
	N	***A_O***	***Allelic richness***	***H_E***	***H_O***	***F_{IS}***	***N***	***A_O***	***Allelic richness***	***H_E***	***H_O***	***F_{IS}***	***N***	***A_O***	***Allelic richness***	***H_E***	***H_O***	***F_{IT}***	***F_{ST}***
FEMS2	90	26	43.8	0.914	0.856	0.218 ***	83	22	45.4	0.870	0.831	0.342 ***	173	29	43.3	0.897	0.844	0.277**	0.003
FEMS4	89	17	23.9	0.870	0.629	0.069 *	83	18	21.9	0.899	0.687	0.051 ns	172	19	25.3	0.887	0.657	0.062***	0.005
FEMS11	89	34	17.2	0.919	0.820	0.282 ***	82	24	17.9	0.912	0.817	0.242 ***	171	36	16.3	0.918	0.819	0.262***	0.003
FEMS16	90	13	11.4	0.758	0.389	0.491 ***	83	11	10.9	0.789	0.386	0.516 ***	173	13	12.5	0.775	0.387	0.503***	0.003
FEMS19	90	45	28.5	0.960	0.756	0.113 ***	82	46	23.8	0.956	0.634	0.111 **	172	52	32.6	0.961	0.698	0.111***	0.002
M2-30	90	36	36.2	0.950	0.900	0.058 *	79	36	36.0	0.953	0.772	0.196 ***	169	41	35.2	0.954	0.840	0.122***	0.003
All loci				0.895	0.725	0.195 ***				0.897	0.688	0.239 ***				0.899	0.707	0.216***	0.003
Excl. 16				0.923	0.792	0.143				0.918	0.748	0.183				0.923	0.772	0.167***	0.003

Summary statistics are shown in Table 4.2. The number of alleles and allelic richness varied among loci as did heterozygosity. However, there were only very slight differences in the number of alleles and heterozygosity between mature trees and natural regeneration. All loci showed significant departures from Hardy-Weinberg equilibrium (HWE), although FEMSATL4 did not show a significant departure from HWE in the natural regeneration. The greatest excess of homozygotes was recorded in FEMSATL16; when this locus was excluded higher overall estimates of heterozygosity and lower overall estimates of F_{IS} and F_{IT} were obtained. No significant linkage disequilibrium was found between any loci (data not shown).

The Mantel test showed a low but significant correlation between genetic and geographic distances between samples (R = 0.104, p $\leq$ 0.001). When the locus FEMSATL16 was excluded, no correlation was found (R = -0.012, p = 0.295).

Spatial autocorrelation showed significant spatial genetic structure at a small scale. Figure 4.4 shows the average genetic autocorrelation for pairs of trees separated by distances up to an increasing maximum. Two sets of confidence intervals are shown: (a) 95% confidence intervals around the null hypothesis (r = 0), determined by permutations, and (b) 95% confidence error bars around the value of r, determined by bootstrapping. Values are non-significant if (a) the value of r is lower than the upper confidence limit of permuted r, or if (b) the lower confidence limit of bootstrapped r is lower than zero.

When all loci were included, the correlation coefficient, r, had a maximum value of 0.127 (±0.052, p (r > permuted r) $\leq$ 0.001) for pairs of trees up to 5 m apart. It had a similar value for pairs of trees up to 10 m apart (r = 0.117 ± 0.041, p $\leq$ 0.001), but then declined steadily with larger distance classes. For pairs of trees up to 800 m apart it was still significantly greater the permuted value of r (r = 0.007 ±0.003, p $\leq$ 0.001), but very close to zero. For trees up to 1600 m it was not significantly different from zero.

When the locus FEMSATL16 was excluded from the spatial autocorrelation analysis, genetic autocorrelation was lower for pairs of trees at all distance classes (maximum r (up to 5 m) = 0.059 ± 0.039, p $\leq$ 0.001). However, values of r remain significantly greater than permuted r up to 400 m (r = 0.004 ± 0.003, p = 0.006).

Figure 4.5 shows spatial autocorrelograms for pairs of trees within distance classes. When even-sized distance classes were used, there was an overall decline in autocorrelation with distance, with considerable fluctuation around zero in the middle range distances (around 500 m), as shown in Figure 4.5A for distance classes of 50 m. The *r* value first intercepts the y axis at approximately 320 m. With larger distance classes (200 m), less fluctuation is seen and the intercept is around 660 m (Figure 4.5B). With variable and increasing sized distance classes (Figure 4.5C), a rapid decline over the initial 20 m is seen, followed by a more gradual decline. In this case, the intercept is around 780 m.

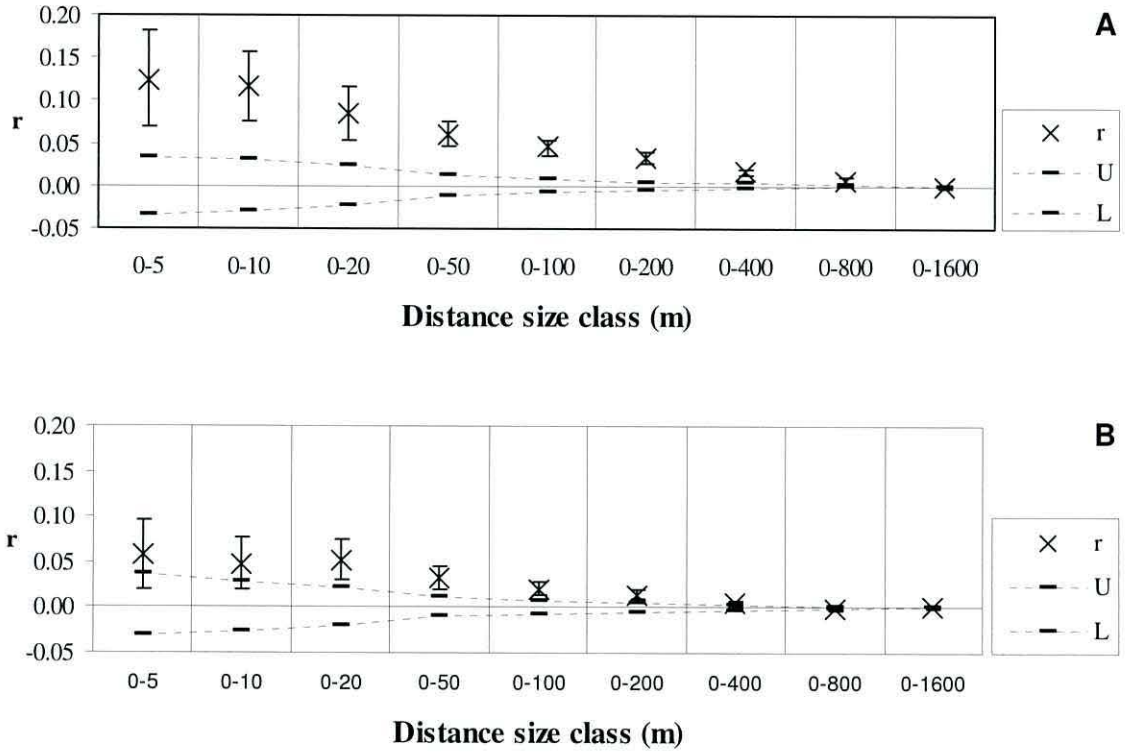

Figure 4.4 Spatial genetic structure in a population of *Fraxinus excelsior* in Bishopston Valley, South Wales. Genetic autocorrelation *r* for the first distance class with different distance class sizes, upper (U) and lower (L) limits for 95% confidence interval about the null hypothesis of a random distribution of genotypes as determined by permutations, 95% confidence error bars about *r* as determined by bootstrapping. (A) Analysis including all six loci. (B) Analysis excluding locus FEMSATL16.

When data from the locus FEMSATL16 was excluded from the spatial autocorrelation analysis, as shown in Figure 4.5D, the values of *r* were lower than with the six loci. Although at most distances genetic autocorrelation remained significant, in the 5-10 m distance class *r* was not significantly different from zero or from permuted *r*.

When the distance used was linear (one-dimensional) distance along the transect (Figure 4.5E) the *r* values were in general similar. It is not possible to compare *r* values for a given distance class because the classes in Figures 4.5C and 4.5E are not equivalent. The *r* value for the 0 m class, representing the mature trees with the closest regenerating seedlings, was 0.110 (± 0.04, $p \leq 0.001$), slightly lower than the value for the 0-5 m distance class of Euclidean distance (r = 0.127 ± 0.052, $p \leq 0.001$). The intercept occurred around 980 m in linear distance.

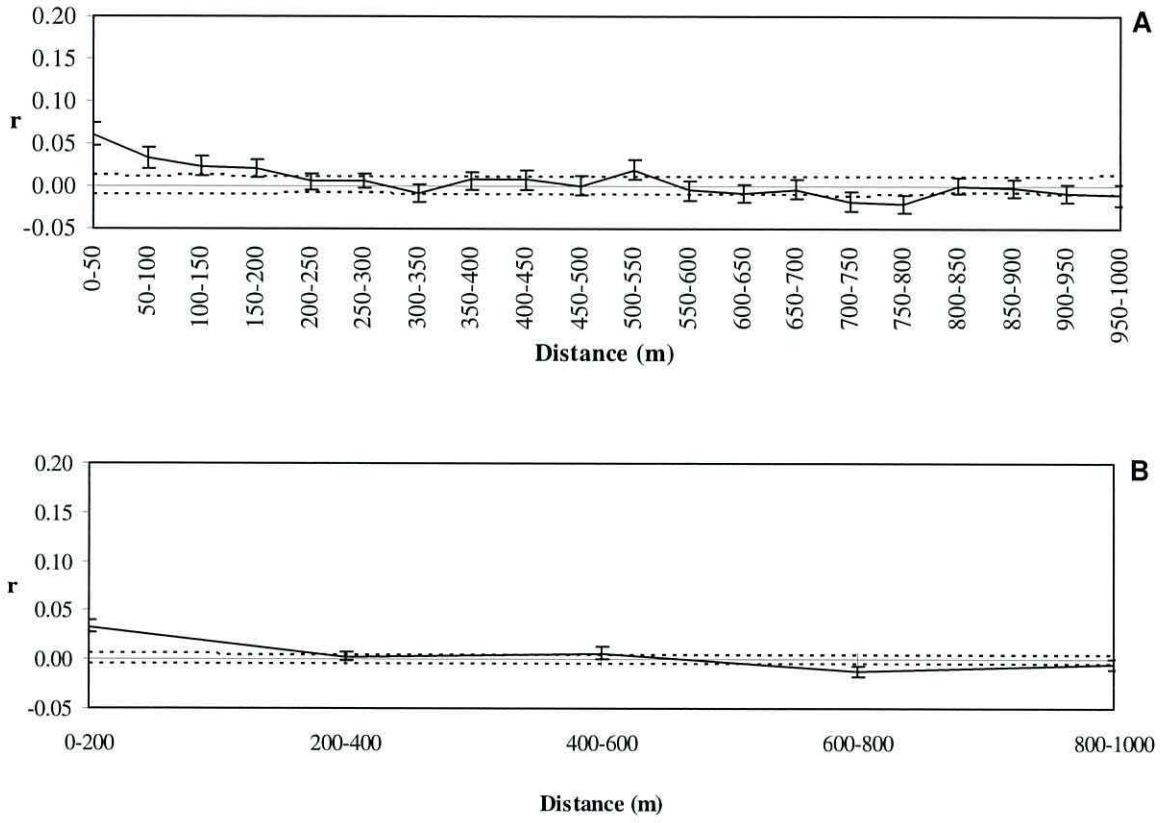

Figure 4.5 Spatial genetic structure in a population of *Fraxinus excelsior* in Bishopston Valley, South Wales. Correlograms showing the combined genetic correlation *r* as a function of distance, 95% confidence interval about the null hypothesis of a random distribution of genotypes, and 95% confidence error bars about *r* as determined by bootstrapping. (A) Autocorrelation for distance class sizes of 50 m, based on data from six microsatellite loci. (B) Autocorrelation for distance size classes of 200 m, based on data from six microsatellite loci.

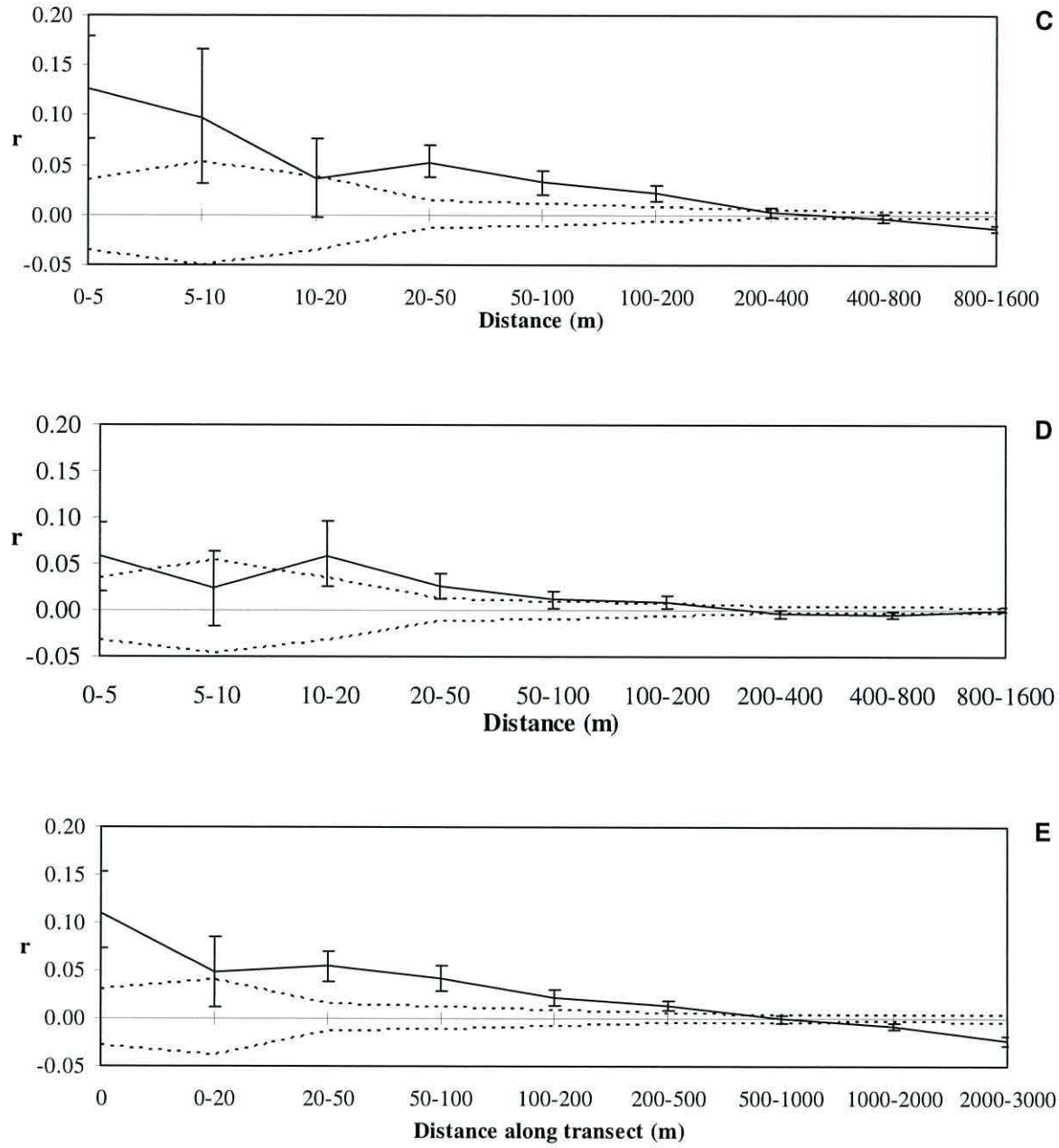

Figure 4.5 cont. (C) Autocorrelation for distance classes of increasing size, based on data from six microsatellite loci. (D) As (C), but based on data from five microsatellite loci, excluding FEMSATL16. (E) Autocorrelation for variable size classes of distance measured in one dimension along the transect, based on data from six microsatellite loci. Naturally regenerated seedlings were classed as 0 m distant from the closest mature tree.

4.4 Discussion

As in many studies of trees, an excess of homozygotes was shown at the six nuclear microsatellite loci used in this study. This is similar to the results of Morand *et al.* (2002) for French populations of ash. These workers discussed in detail the possible

reasons for the observed excess, including nonMendelian inheritance of alleles, the presence of null alleles, a Wahlund effect, and assortative mating. The results given by the programme Micro-checker (van Oosterhout *et al.*, 2004) indicate the possibility of null alleles at all loci, but if the population is not in Hardy-Weinberg equilibrium then the assumptions of Micro-Checker are violated.

There were only slight differences in heterozygosity, number of alleles or inbreeding (F_{IS}) between the mature trees and the naturally regenerated seedlings. This suggests that the natural regeneration is genetically representative of the mature trees and not dominated by offspring from a small number of parent trees. Under continued natural regeneration genetic diversity seems likely to be maintained.

Some small scale spatial genetic structure was observed. A Mantel test gave a low but significant overall correlation between genetic and geographic distances between pairs of individuals. An autocorrelation coefficient, r, of 0.127 ($p < 0.001$) was calculated for all pairs of individuals between 0 m and 5 m apart. Due to the nature of the sampling, and the distances between mature trees, these pairs of individuals all consist of a mature tree and the nearest naturally regenerated seedling. In effect, a seedling growing near to a mature tree stands a greater than average chance of being related to that tree. This is probably due to the relatively limited dispersal of seeds, rather than to limited pollen flow.

Although the autocorrelation coefficient, r, is a proper correlation coefficient, with possible values between 1 and -1, autocorrelation coefficients are not expected to give high values approaching 1 (unless clonal groups were sampled). If all pairs of individuals within a distance class were outcrossed parent-offspring pairs, they would only be expected to share half their alleles. A value of 0.127 with $p < 0.001$ (as calculated by permutations and bootstrapping) may therefore be considered a strong autocorrelation.

The use of the intercept of the r values with zero as an indication of the extent of spatial genetic structure is subject to some difficulties, as it varies greatly according to the choice of distance classes. With smaller distance classes, there are fewer pairs of individuals within each class; the stochastic variation in r masks the overall trend, and causes the intercept to occur at around 320 m for distance classes of 50 m. However, the

comparison in Figure 4.4 of the r value for increasing class sizes shows the value approaching 0 around 800 m, and this is similar to the intercept (660 m to 780 m) with distance classes that are larger than 200 m or variable in size. The maximum extent of spatial genetic structure ($r > 0$, $p \leq 0.05$) is therefore believed to be around 700-800 m.

Unexpectedly, when data from the locus FEMSATL16, which had the highest estimated frequency of null alleles, was excluded from the spatial analysis, a Mantel test showed that there was no overall correlation between genetic and geographic distances. There was still some significant autocorrelation at smaller distance classes, but the values of r were lower than when data from FEMSATL 16 were included in the analysis. This is illustrated in Figure 4.4 and in Figure 4.5C and D. The values of r remain significantly different from permuted r and significantly different from zero at most of the smaller distance classes, although in the 5-10 m distance class r is non-significant.

This is unexpected, because if there is a high frequency of null alleles at locus FEMSATL16, data from this locus are expected to be less informative. It would therefore be expected that when data from FEMSATL16 were excluded from the analysis, similar or slightly higher levels of spatial genetic structure would be observed. The higher levels observed when FEMSATL16 is included in the analysis suggest that a significant part of the observed multilocus spatial genetic structure is due to spatial genetic structure at this locus. This suggests that some unanticipated factor is influencing the locus. One possibility is that FEMSATL16 does not act as a neutral locus, but is linked to a gene which is under selective pressure at the small scale observed here. This could be due to micro-environmental effects such as soil moisture, soil nutrient status, or incidence of frost, wind or sunlight. Such a linkage has never previously suggested for this locus, but it would explain the departure from Hardy-Weinberg equilibrium.

To conclude, this study found high genetic diversity in the *Fraxinus* excelsior population in Bishopston Valley. Natural regeneration was found to be genetically similar to mature trees, suggesting that a representative proportion of mature trees are contributing to pollen and seed production. An excess of homozygosity was recorded, similar to that found in other studies. This may be partially due to the presence of null alleles, but similar excesses have been found in other studies and so null alleles are not expected to be the only explanation. Weak spatial genetic structure was found up to

around 700-800 m and this is thought to be caused by limited seed dispersal. When data from the locus with the greatest estimated frequency of null alleles was excluded from the analysis, lower levels of spatial genetic structure were observed. This suggests that the locus is, possibly, linked to a gene under selection.

5. Genetic diversity among sites

5.1 Introduction

At present, guidelines governing the collection, marketing and use of Forest Reproductive Material (FRM) are based on designated Seed Zones (Herbert *et al.*, 1999). These seed zones are based on major geoclimatic and landform boundaries, including major watersheds and, in one case where no strong features are present, a motorway. They are applied to all native species, although it is recognised that scientific understanding of the genetic diversity of native species is still limited. Wales is divided into east (304) and west (303) zones, using the main watershed of the Cambrian Mountains, as shown in Figure 5.1.

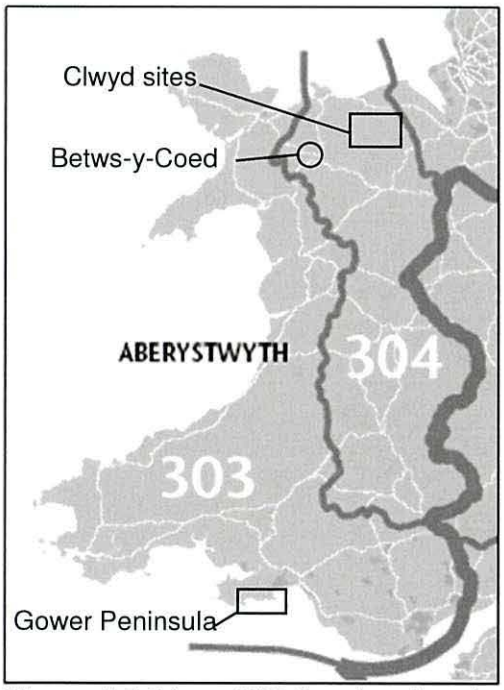

Figure 5.1 Map of Wales showing the seed zones from Herbert *et al.* (1999). The areas of the field sites are marked.

If tree populations are in equilibrium, and gene flow is limited, then it is expected that there would be greater differentiation among populations that are further apart. In effect, there would be some degree of isolation by distance, so that there would be a positive correlation between genetic distance and geographic distance between pairs of populations. If this is not the case, then either the populations are not in equilibrium, or there are no restrictions to gene flow.

The aim of this part of the study was to quantify both the genetic diversity of *Fraxinus excelsior* populations at different sites within Wales, and the differentiation among these populations. With reference to the planting of semi-natural woodlands, the aim was to find out if the designated seed zones could be supported by genetic studies in ash.

As detailed in Chapter 3, samples were collected from three sites in South Wales (the Gower Peninsula) and from three sites in North Wales: two in Clwyd and one near Betws-y-Coed. The locations of the field sites are shown in Figure 5.1. The Gower Peninsula sites in the south fall into the west Wales seed zone 303, while the northern sites fall into zone 304. Maps showing the location of the Gower, Clwyd and Betws-y-Coed sites are shown in Figure 5.2 - 5.4; a large-scale map of the Bishopston Valley site is shown in Figure 4.1 (Chapter 4); and large-scale maps of the remaining sites may be found in Appendix II.

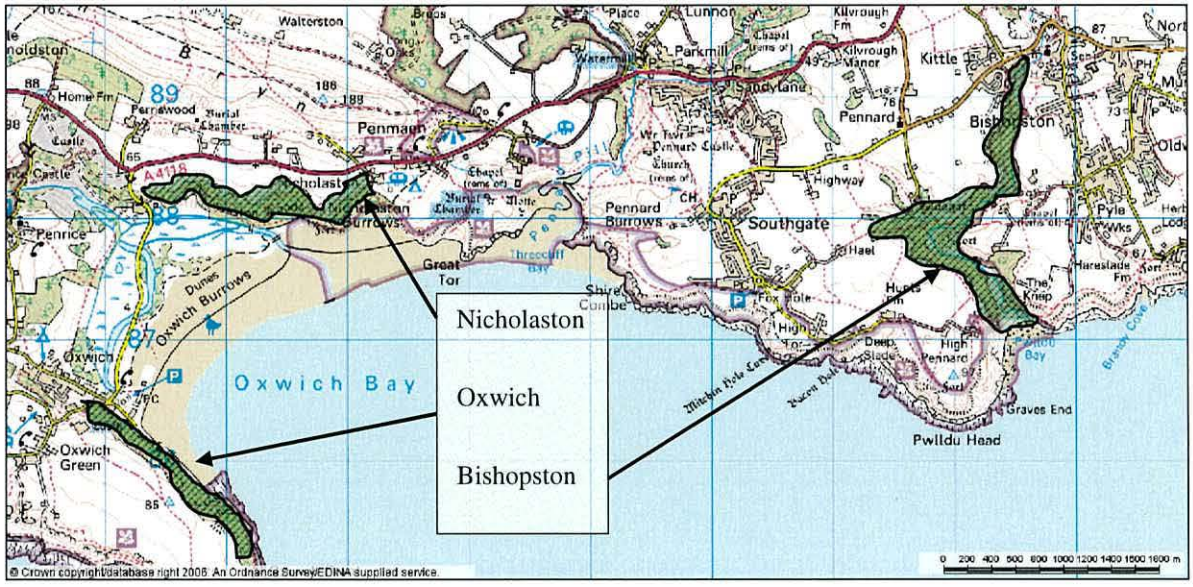

Figure 5.2 Map showing the location of the three sites on the Gower Peninsula, South Wales (NGR for Bishopston Valley SS574890).

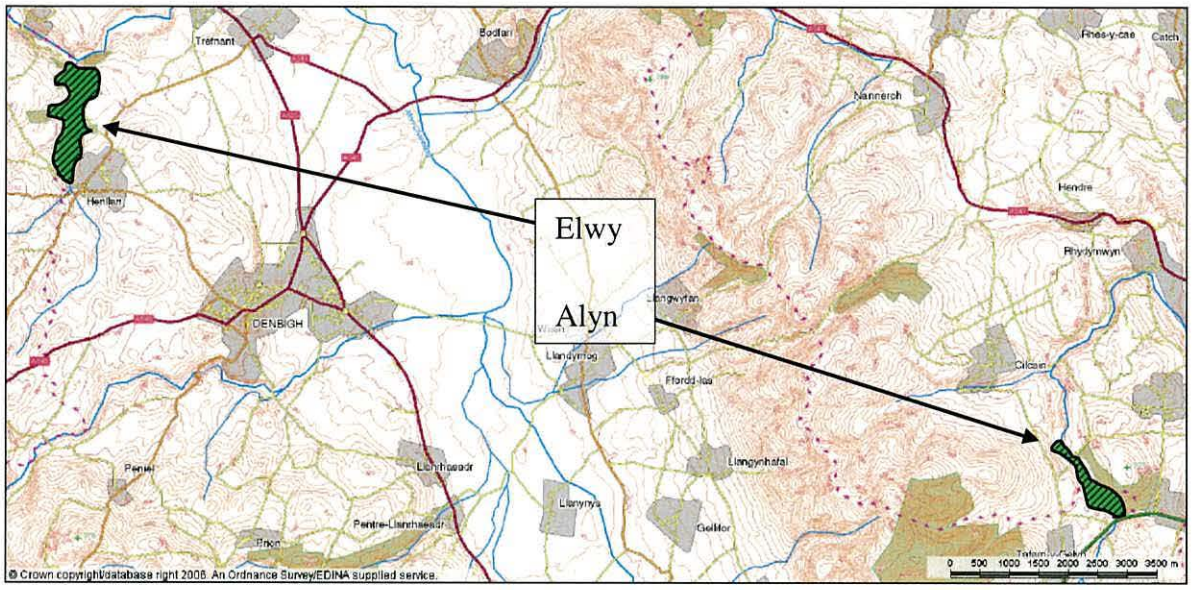

Figure 5.3 Map showing the location of the two sites in Clwyd, North Wales (NGR for Elwy SJ019685).

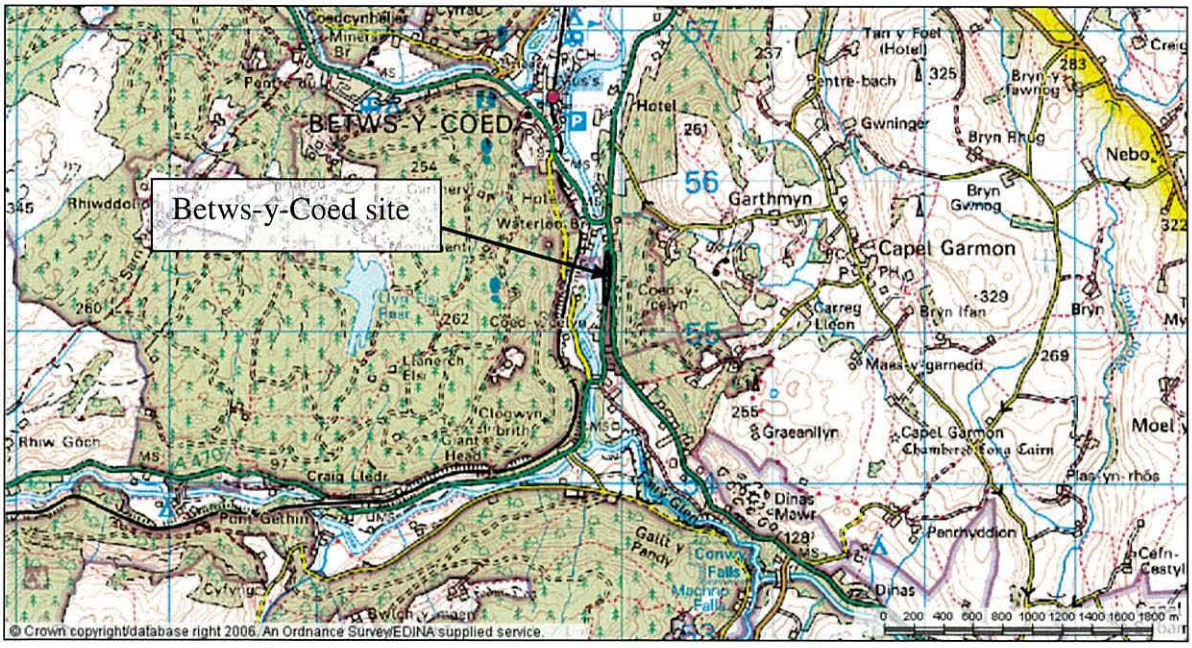

Figure 5.4 Map showing the location of the site at Betws-y-Coed, North Wales (NGR SH799555).

5.2 Analysis

The programme Micro-Checker 2.2.3 (van Oosterhout *et al.*, 2004) was used to estimate the likelihood of null alleles at each microsatellite locus.

The programmes FSTAT 2.9.3 (Goudet, 2001) and GenAlEx 6 (Peakall and Smouse, 2006) were used to calculate allelic frequencies and summary statistics (allelic richness, expected and observed heterozygosity, and *F* statistics). These were calculated for each

locus individually and averaged over all loci, and for each population and averaged over all populations. Pairwise F_{ST} values were calculated for all pairs of populations. The significance of F statistics was tested by randomisation, and adjusted by Bonferroni correction in FSTAT. FSTAT was also used to test for the existence of linkage disequilibrium. Due to the high number of null alleles estimated to be present at locus FEMSATL16, multilocus statistics were calculated twice, once including all six microsatellite loci, and a second time over five loci, excluding FEMSATL16.

A standardised measure of genetic differentiation, $F_{ST} / (1- F_{ST})$, was plotted against the log of geographic distance for all pairs of populations, and the correlation coefficient between them was calculated.

The method of Dyer and Nason (2004) was used to construct a "Population Graph". This method can allow the interpretation of patterns of genetic structure and differentiation among populations. Compared to F statistics, which provide averages, and tree-based methods, which are limited by their more rigid structure, the "Population Graph" can graphically show hierarchical relationships among populations. Populations are represented by nodes with sizes proportional to their genetic diversity, and linked by varying numbers of edges with lengths representing the similarities between populations.

5.3 Results

Null alleles were estimated by Microchecker (van Oosterhout *et al.*, 2004) to be present at all loci except FEMSATL4 (Table 5.1). Average frequency of null alleles at a given locus in a population varied from five percent (M2.30) to 18 percent (FEMSATL16).

Table 5.1 Estimated percentages of null alleles at six microsatellite loci in six populations of *Fraxinus excelsior*. Percentages are given for each population and over all populations.

	FEMS2	FEMS4	FEMS11	FEMS16	FEMS19	M2.30
Bish	10.9%	no	13.5%	22.5%	5.4%	no
Ox	12.7%	no	no	12.9%	no	no
Nich	13.6%	no	18.1%	no	15.7%	14.1%
Alyn	15.7%	no	14.9%	16.3%	no	7.2%
Elwy	14.2%	no	no	23.9%	6.8%	8.1%
Betws	8.7%	no	no	21.8%	8.9%	no
Overall	13.0%	no	12.8%	18.3%	7.6%	5.4%

Table 5.2 Microsatellite diversity in six populations of *Fraxinus excelsior*. For each of six microsatellite loci in each population, the number of samples successfully genotyped (*N*), the number of observed alleles (A_O), the allelic richness, the expected (H_E) and observed (H_O) heterozygosities, and F_{IS} values are reported. Significance of F_{IS} is indicated by asterisks. ns, nonsignificant; *p ≤0.05; **p ≤0.01; ***p ≤0.001.

Population	Locus	N	A_O	Allelic richness	H_E	H_O	F_{IS}
Bishopston	**FEMSATL2**	88	44	29.0	0.960	0.750	0.224***
	FEMSATL4	88	26	18.9	0.913	0.864	0.06ns
	FEMSATL11	87	19	12.8	0.871	0.632	0.28***
	FEMSATL16	88	13	9.3	0.769	0.420	0.458***
	FEMSATL19	87	33	20.5	0.908	0.816	0.107**
	M2-30	88	36	26.1	0.952	0.920	0.038ns
Oxwich	**FEMSATL2**	30	30	29.5	0.953	0.700	0.281***
	FEMSATL4	30	18	17.8	0.874	0.867	0.026ns
	FEMSATL11	30	11	11.0	0.883	0.767	0.149*
	FEMSATL16	30	7	6.9	0.686	0.500	0.287**
	FEMSATL19	30	13	12.9	0.863	0.867	0.012ns
	M2-30	30	23	22.7	0.922	0.833	0.113*
Nicholaston	**FEMSATL2**	30	25	24.7	0.935	0.667	0.302***
	FEMSATL4	30	17	16.8	0.844	0.767	0.108ns
	FEMSATL11	30	10	9.9	0.829	0.533	0.372***
	FEMSATL16	30	8	7.9	0.775	0.700	0.114ns
	FEMSATL19	30	13	12.8	0.723	0.533	0.278**
	M2-30	29	22	22.0	0.930	0.655	0.312***
Alyn	**FEMSATL2**	31	32	31.0	0.955	0.645	0.339***
	FEMSATL4	32	18	17.1	0.854	0.844	0.028ns
	FEMSATL11	32	10	9.9	0.863	0.625	0.291***
	FEMSATL16	32	7	6.9	0.737	0.500	0.336***
	FEMSATL19	31	20	19.3	0.907	0.839	0.092ns
	M2-30	31	28	27.4	0.948	0.806	0.166***
Elwy	**FEMSATL2**	29	26	31.7	0.926	0.655	0.309***
	FEMSATL4	31	18	15.7	0.881	0.871	0.028ns
	FEMSATL11	30	11	10.8	0.751	0.633	0.173*
	FEMSATL16	31	6	6.9	0.700	0.355	0.506***
	FEMSATL19	29	20	18.7	0.924	0.793	0.159**
	M2-30	29	29	18.0	0.954	0.828	0.15**
Betws-y-	**FEMSATL2**	31	33	31.4	0.953	0.806	0.169***
Coed	**FEMSATL4**	31	16	19.2	0.808	0.774	0.058ns
	FEMSATL11	31	11	12.0	0.828	0.710	0.159*
	FEMSATL16	31	7	8.0	0.648	0.387	0.417***
	FEMSATL19	30	19	20.5	0.899	0.733	0.201**
	M2-30	29	18	26.6	0.916	0.897	0.039ns

The summary statistics are shown for each locus in population in Table 5.2 and as multilocus means for each population in Table 5.3. Statistics for all samples at each locus are shown in Table 5.4. Observed number of alleles varied among loci but was similar among populations. A greater number of alleles was found in the Bishopston population, which had 88 samples compared to around 30 at the other sites. Allelic richness, based on the minimum population size of 29 individuals, was slightly higher in the Bishopston population (average over six loci 19.4) and slightly lower in the

Nicholaston population (15.7, Table 5.3). As illustrated in Figure 5.5A, the greatest number of alleles was found at the FEMSATL2 locus, and fewest at FEMSATL16.

A one-way analysis of variance was carried out to test the significance of variation in genetic diversity and inbreeding among populations and among loci. No significant difference in allelic richness [F(5,30)=0.23, p=0.95], expected heterozygosity [F(5,30)=0.33, p=0.89] or F_{IS} [F(5,30)=0.41, p=0.84] was found among populations. However, significant differences among loci were found in allelic richness [F(5,30)=63.07, p<0.001], expected heterozygosity [F(5,30)=20.08, p<0.001] or F_{IS} [F(5,30)=8.20, p<0.001]. Post-hoc comparisons were carried out using the Tukey test to investigate which loci differed significantly; these are indicated in Table 5.4.

Table 5.3 Microsatellite diversity by population in *Fraxinus excelsior*. For each of six populations the number of samples successfully genotyped at all six loci (*N*), the number of observed alleles (A_O), the expected (H_E) and observed (H_O) heterozygosities, and F_{IS} values are reported over six microsatellite loci, and over five loci, excluding FEMSATL16. Averages over five loci are shown in brackets.

Population	*N*	A_O	Allelic richness	H_E		H_O		F_{IS}	
Bishopston	87	28.5	19.4	0.895	(0.920)	0.734	(0.796)	0.186***	(0.141***)
Oxwich	30	17	16.8	0.864	(0.899)	0.756	(0.806)	0.142***	(0.120***)
Nicholaston	29	15.8	15.7	0.839	(0.852)	0.643	(0.631)	0.251***	(0.275***)
Alyn	31	19.2	18.6	0.878	(0.905)	0.710	(0.751)	0.207***	(0.186***)
Elwy	29	18.3	18.2	0.856	(0.887)	0.689	(0.756)	0.211***	(0.165***)
Betws-y-Coed	29	17.3	17	0.842	(0.880)	0.718	(0.784)	0.164***	(0.126***)
Overall								0.202***	(0.172***)

Table 5.4 Microsatellite diversity by locus in *Fraxinus excelsior*. For each of six microsatellite loci the total number of samples successfully genotyped (*N*), the number of observed alleles (A_O), allelic richness, the expected (H_E) and observed (H_O) heterozygosities, overall F_{IS} values and F_{IT} values are recorded. All values of F_{IT} are significant ($p < 0.001$) based upon randomization of alleles in FSTAT. Values marked by the letters *a, b* and *c* are not significantly different to values sharing the same letter.

Locus	*N*	A_O	*Allelic richness*	H_E	H_O	F_{IS}	F_{IT}
FEMSATL2	239	61	31.4	0.967 *a*	0.715	0.271 *ab*	0.263
FEMSATL4	242	36	19.2 *a*	0.890 *bc*	0.839	0.051 *c*	0.061
FEMSATL11	240	19	12.0 *b*	0.874 *b*	0.646	0.237 *ab*	0.267
FEMSATL16	242	14	8.0 *b*	0.751	0.463	0.353 *a*	0.387
FEMSATL19	237	41	20.5 *a*	0.921 *abc*	0.776	0.142 *bc*	0.166
M2.30	236	44	26.6	0.956 *ac*	0.847	0.136 *bc*	0.117

A general deficiency of heterozygotes was observed, with most populations deviating from HWE at most loci, as shown by the significance of most F_{IS} values in Table 5.2.

Again there was variation among loci, as illustrated in Figure 5.5B, where the F_{IS} values are shown. The locus FEMSATL4 did not deviate from HWE in any population (Table 5.2), and had an F_{IT} value of 0.061 (Table 5.4), while FEMSATL16 deviated from HWE in all populations except Nicholaston ($p < 0.01$, Table 5.2) and had an F_{IT} value of 0.387 (Table 5.4). When data from FEMSATL16 were excluded, multilocus estimates of heterozygosity were higher and F_{IS} estimates were lower in most populations. Compared to variation among loci, there was relatively little variation in heterozygosity among populations. However, Nicholaston had lower expected and observed heterozygosity than the other populations, and the highest estimate of F_{IS} (Table 5.3). In addition, while Nicholaston had the highest single-locus estimates of F_{IS} at four loci, it was the population with lowest single-locus F_{IS} at locus FEMSATL16 (Table 5.2).

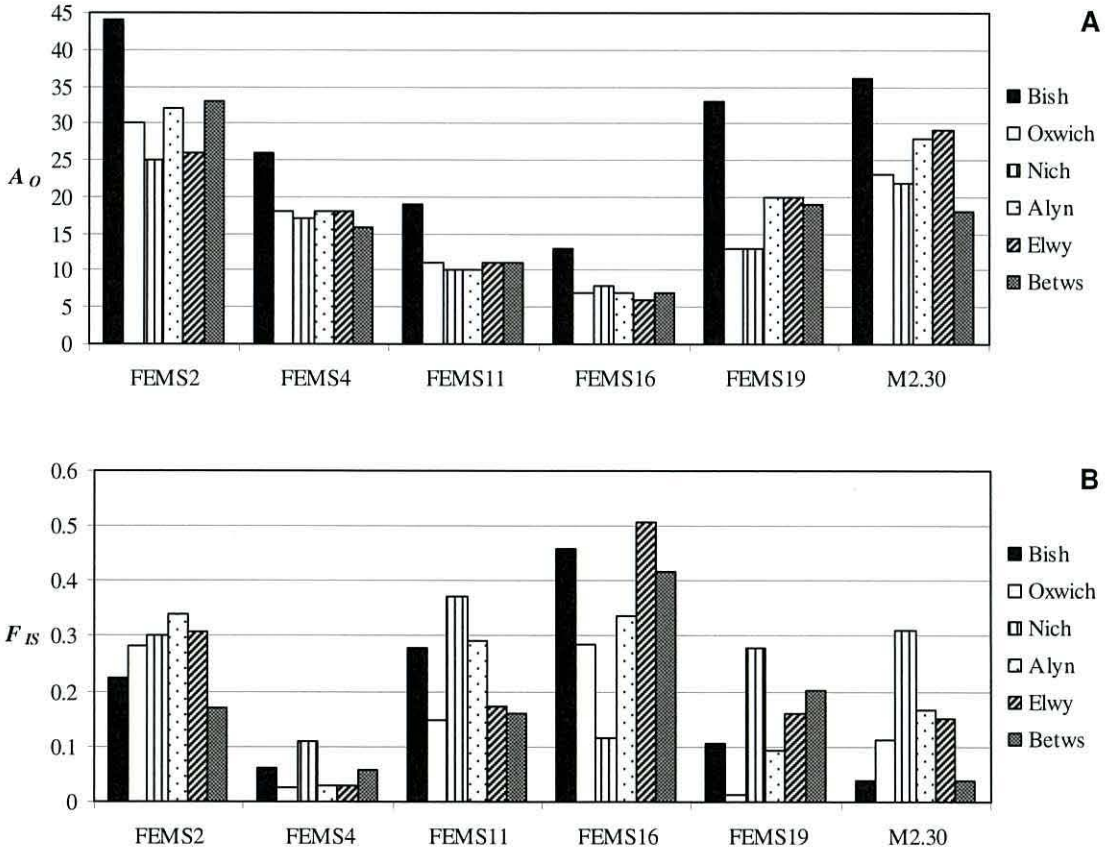

Figure 5.5 For each of six microsatellite loci in six populations of *Fraxinus excelsior* at six microsatellite loci (A) the observed number of alleles and (B) F_{IS}. Populations are shown by shading. Black, Bishopston; white, Oxwich; vertical hatching, Nicholaston; light dots, Alyn; diagonal hatching, Elwy, grey, Betws-y-Coed.

Pairwise multilocus F_{ST} values are shown in Table 5.5. These varied from 0.0414 between the Nicholaston and Betws-y-Coed populations, to 0.0041 between the Alyn and the Oxwich and Betws-y-Coed populations. However, despite these low values of F_{ST}, all except three values were significant ($p < 0.05$, Table 5.5). F_{ST} was also calculated excluding data from FEMSATL16, but estimates were only very slightly different and in no consistent direction, and are not shown here. Corrected pairwise F_{ST} values are plotted against the log of geographic distance between populations in Figure 5.6. There was no significant correlation ($r = 0.29$) between population differentiation and geographic distance.

Table 5.5 Pairwise multilocus F_{ST} values among six populations of *Fraxinus excelsior*. Significance of F_{ST} is indicated by asterisks. ns, nonsignificant; *p ≤0.05; **p ≤0.01; ***p ≤0.001.

	Oxwich	Nicholaston	Alyn	Elwy	Betws-y-Coed
Bishopston	0.0078 ***	0.0309 ***	0.0068 **	0.0108 ***	0.0142 **
Oxwich		0.0330 ***	0.0041 ns	0.0164 ***	0.0149 **
Nicholaston			0.0193 ns	0.0309 ***	0.0414 ***
Alyn				0.0109 *	0.0041 ns
Elwy					0.0293 ***

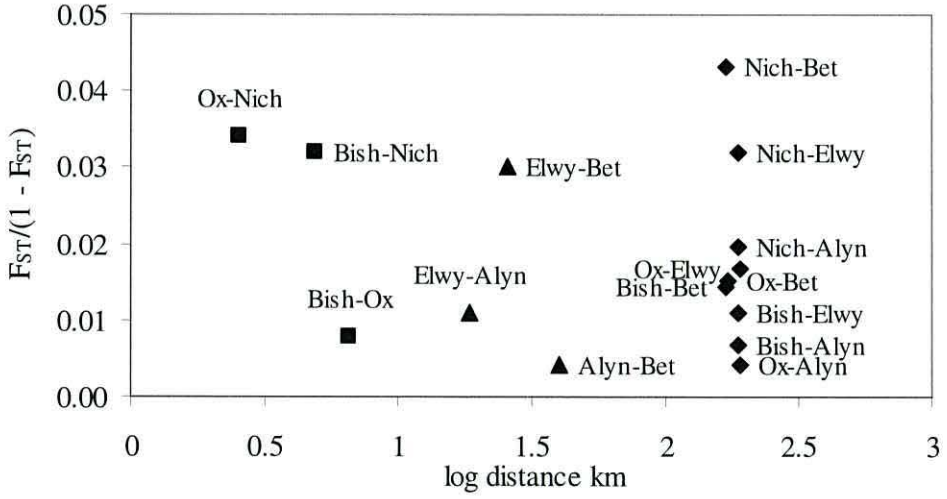

Figure 5.6 Pairwise genetic differentiation (F_{ST} / (1-F_{ST})) plotted against the log of the geographic distance between pairs of populations of *Fraxinus excelsior*. Squares indicate two South Wales populations; triangles indicate two North Wales populations; diamonds indicate a North-South pair of populations.

The highest values of pairwise F_{ST} were found when one of the populations was Nicholaston. Overall F_{ST} was estimated at 0.017 in all populations, but at 0.011 when

data from Nicholaston were excluded. Single-locus estimates of F_{ST} showed considerable variation, as shown in Figure 5.7. The highest values of F_{ST} were found at locus FEMSATL19, where estimates between pairs of populations including Nicholaston were between 0.089 and 0.130, considerably higher than any other values of F_{ST}. At other loci most estimates of pairwise F_{ST} were nonsignificant, with only one or two values being significant after Bonferroni corrections.

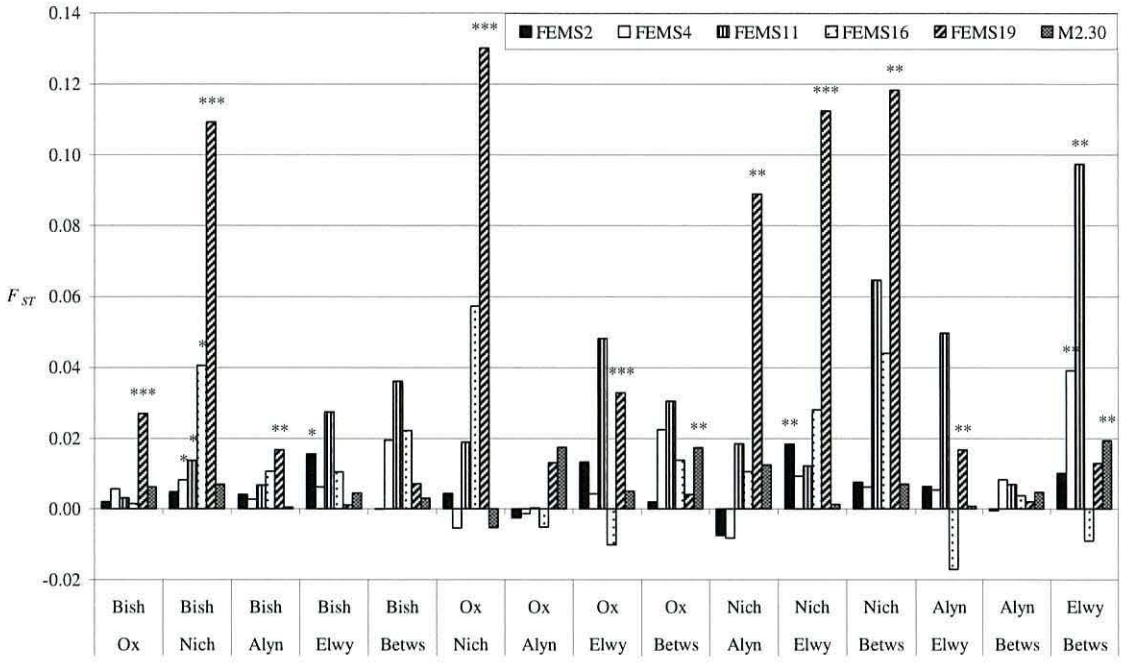

Figure 5.7 Pairwise single-locus estimates of F_{ST} for six microsatellites and six populations of *Fraxinus excelsior*. Significance is indicated by asterisks. No asterisk, nonsignignificant; *p ≤0.05; **p ≤0.01; ***p ≤0.001. Loci are shown by shading. Black, FEMSATL2; white, FEMSATL4; vertical hatching, FEMSATL11; light dots, FEMSATL16; diagonal hatching, FEMSATL19, grey, M2.30.

A "Population Graph" showing the genetic relationships between the populations is shown in Figure 5.8. No major pattern is evident, as all populations (nodes) are joined to three, four or five other populations and there is little variation in edge length. The number and length of edges connecting nodes indicates the genetic similarity among populations, so in a more structured set of populations different nodes might be separated by only one edge.

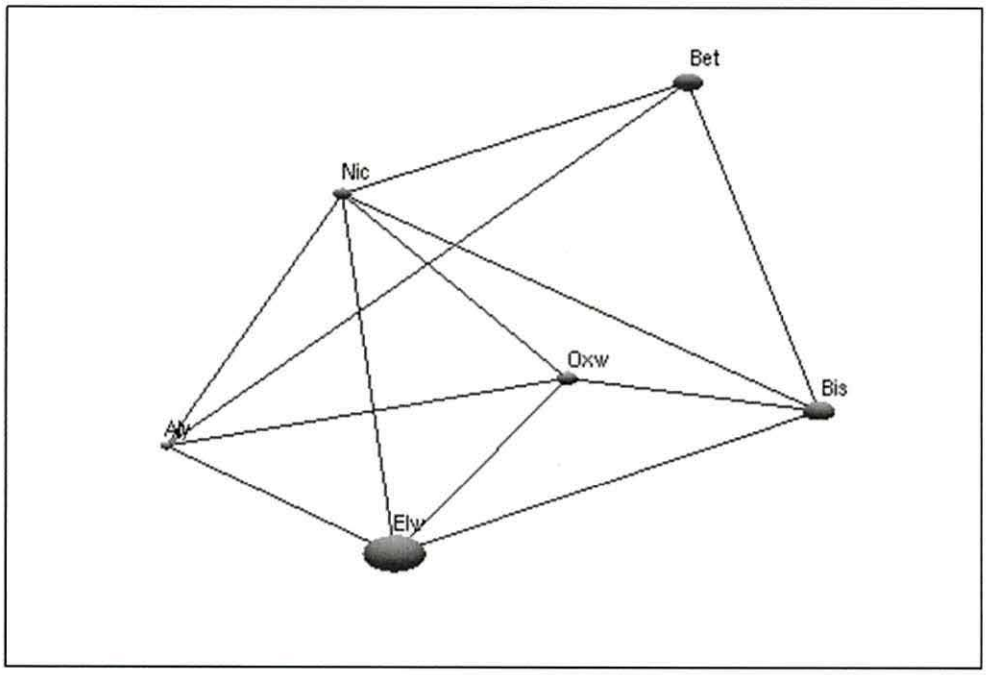

Figure 5.8 "Population Graph" (Dyer and Nason, 2004) representing the genetic relationships among the six populations of *Fraxinus excelsior*. Node diameter reflects within population microsatellite variation while edge length reflects a multivariate measure of genetic covariance between the connected nodes. Bis, Bishopston; Oxw, Oxwich, Nic, Nicholaston; Aly, Alyn; Elw, Elwy; Bet, Betws-y-Coed.

5.4 Discussion

As with the Bishopston population, Micro-checker estimated high frequencies of null alleles. The locus FEMSATL16 had the highest estimated frequencies of null alleles, and so further analyses were performed both including and excluding data from this locus. However, if the populations are not in Hardy-Weinberg equilibrium then an excess of homozygotes is expected, and it is this excess of homozygosity which leads to the estimates of null alleles. This is discussed in greater detail below.

The six microsatellite loci showed high levels of polymorphism in the populations studied here. High numbers of alleles were recorded, in common with other studies of *Fraxinus excelsior* using the same loci. Table 5.6 shows numbers of alleles recorded by different researchers, along with the total number of samples included in each study. In Figure 5.9 these data are plotted and as expected, the number of alleles can be seen to increase with the number of samples scored. Due to the small number of data points, the statistical significance of the relationships and R^2 values should be treated with caution, but it may be concluded that much of the variation in number of observed alleles is explained by the number of samples included in the study. Holtken *et al.* (2003)

observed that Heuertz *et al.* (2001) found two to three times as many alleles in Bulgaria than in the discontinuous marginal habitats of their Finnish study. However, this may be largely due to the greater number of samples included rather than to the nature of the populations.

Table 5.6 Number of alleles found for each of six microsatellite loci in studies by different workers. - not scored for a study; [1]Lefort et al. (1999); [2]Brachet et al., (1999); [3]Heuertz et al. (2001); [4]Holtken et al. (2003); [5]Bacles et al. (2005); [6]Hebel et al. (2006).

	This study	Original primer notes[1,2]	Bulgarian ash[3]	Finnish ash[4]	Scottish ash[5]	German ash[6]
N (approx.)	230	16, [2]50	320	160	80	700
Locus						
FEMSATL2	61	5	-	-	40	-
FEMSATL4	36	9	50	22	-	41
FEMSATL11	19	11	32	-	-	-
FEMSATL16	14	4	10	11	6	27
FEMSATL19	41	12	33	-	19	-
M2.30a	44	[2]18	59	-	30	-

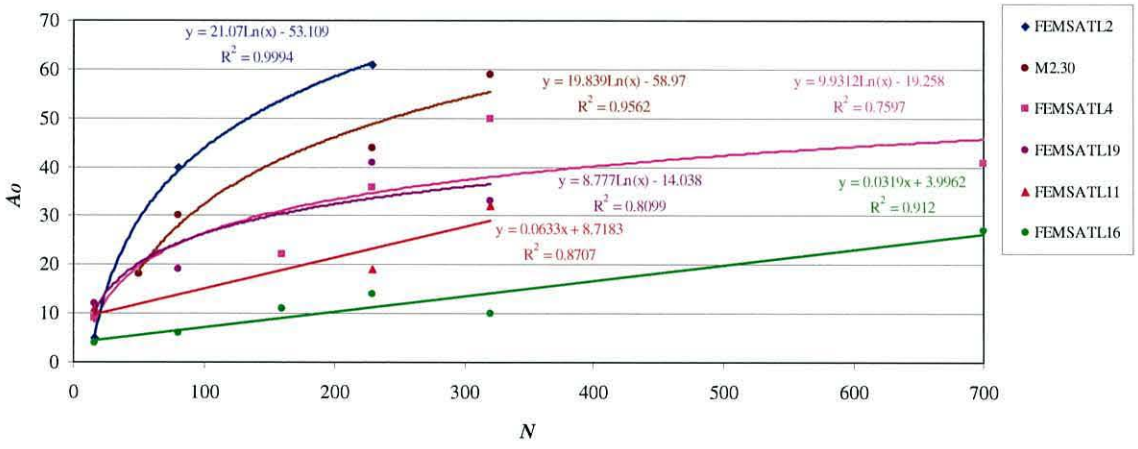

Figure 5.9 Data from Table 5.6 plotted to show the relationship between number of observed alleles and number of samples recorded for each locus. Linear or logarithmic trend lines are shown with their equations and R^2 values.

There was little pattern in the variation in number of alleles observed in the populations in this study. In line with expectations, more alleles were found at Bishopston, where 88 samples were taken, than in the other populations, with around 30 samples each. When allelic richness was calculated based on the minimum population size the variation among populations was much lower. Between loci, however, there were distinct differences, with FEMSATL16 showing fewer alleles, and FEMSATL2 and M2.30 showing more.

Heterozygosity also varied among loci but not among populations. The lowest heterozygosity was recorded at the FEMSATL16 locus, as expected due to the low number of alleles found at this locus. This is consistent with findings from other workers. FEMSATL16 also had the highest values of F_{IS} and greatest deviation from HWE. One possible explanation for these findings is the presence of null alleles at a relatively high frequency at this locus.

Statistics for Nicholaston were slightly different from those for other populations, as this population had the lowest allelic richness and heterozygosity, but highest F_{IS} of all the populations. Single-locus patterns were also interesting, as in this population the locus FEMSATL16 did not have a significant excess of homozygotes. Thus the Nicholaston population shows reduced heterozygosity overall, but at FEMSATL16, the locus with the lowest heterozygosity overall, the Nicholaston population shows higher heterozygosity. The F_{ST} values in Table 5.5 show that Nicholaston was rather different from the other populations. When the pairwise F_{ST} values were ranked, the five values including Nicholaston were in the top six positions, with only the pairwise value between Nicholaston and Alyn being non-significant. However, when single-locus F_{ST} values are examined, it may be seen that most of the differentiation between Nicholaston and other populations is accounted for by locus FEMSATL19.

The level of differentiation observed is remarkable considering that Nicholaston is less than 10 km from either Bishopston or Oxwich, but nearly 200 km from Alyn. It does suggest that there is something different about the population in Nicholaston Woods. One explanation is that there has been some planting at this site in the past. This seems unlikely given that the site is an ancient woodland site and on steep seaside slopes. Another explanation is that one or more environmental factors are different at this site, and so selection has contributed to the genetic differentiation. A possible selective factor would be exposure to salt, as Nicholaston Woods (like Oxwich Wood) are on seaside slopes. It is possible that the south-facing aspect of Nicholaston allows more salt deposition than the sheltered north-west aspect of Oxwich. The possibility of locus FEMSATL16 (as suggested in Chapter 4) and FEMSATL19, from these data, being linked to a gene or genes under selection would help to explain the different patterns found at these loci.

An overall deficiency of heterozygotes was found in this study. This could be partially accounted for by the presence of null alleles. However, the high F_{IS} values are not uncharacteristic of those recorded for populations of *Fraxinus excelsior*. The average value of F_{IS} over populations and loci was 0.184. This is lower than the F_{IS} value of 0.292 found for mature trees in a study of French *Fraxinus excelsior* populations (Morand *et al.*, 2002), but similar to the average value of 0.177 in remnant populations in Scotland (Bacles *et al.*, 2004). However, it is considerably higher than overall F_{IS} values of 0.080 recorded in southern Germany (Hebel *et al.*, 2006), 0.014 in Bulgaria (Heuertz *et al.*, 2001), and 0.030 in Romania (Heuertz *et al.*, 2003). It seems possible that this reflects patterns recorded across Europe (Heuertz *et al.*, 2004b), with western areas having greater within-population diversity than eastern areas, but lower population differentiation.

The F_{ST} values shown in Table 5.5 are mostly significant, as tested by FSTAT (Goudet, 1995) by randomisation of genotypes among samples. However, the differentiation represented is very low, accounting for between 0.4% and 4% of the total genetic variation. The average value of F_{ST} was 0.012. These values are very similar to F_{ST} values of 0.012 found in Germany (Hebel *et al.*, 2006) and slightly lower than the value of 0.043 found in France (Morand *et al.*, 2002). A higher value of 0.087 was found in Bulgaria (Heuertz *et al.*, 2001) but the significance of this is uncertain. No correlation can be seen between genetic differentiation and geographic distances between populations, plotted in Figure 5.6. The "Population Graph" shown in Figure 5.8 also shows that there is no pattern in the relationships among populations, as no populations group together, but almost all nodes are interconnected.

The low differentiation found in this study among populations of *Fraxinus excelsior* is similar to that found in many other studies of trees. It may be explained by extensive gene flow, as pollen and seeds, both wind-dispersed, travel far enough to erode any genetic differentiation during the long generation time of the species. Another explanation is that current patterns of genetic diversity are the result of historical factors. During the rapid post-glacial advance of the forests trees of the same genetic origin spread over the British Isles and there have not been sufficient generations for differentiation to occur by mutations and genetic drift or restricted gene flow.

The results of this study suggest that genetic diversity as reflected by microsatellites is not structured across Wales. Data from the FRAXIGEN (2005) project and from Heuertz *et al.* (2004b) also suggest little differentiation across Britain and western Europe. This suggests that the seed zones of Herbert *et al.* (1999) may be more rigorous than required in order to maintain genetic diversity in *Fraxinus excelsior.* The relationship between microsatellite diversity and any adaptive genetic variation is not well known. Microsatellites are regarded as neutral markers, and used as such in the current study, but in some cases some markers may be linked to adaptive traits. Reciprocal transplant experiments on eight populations of *Fraxinus excelsior* in England and Wales (Boshier and Stewart, 2005; FRAXIGEN, 2005) show little local adaptation on a scale up to 250 km. The authors suggest that locally-adapted seed may be collected within areas up to this scale, and in order to maintain diversity it may be more important to maintain good seed collection practices, such as using a large (> 20) number of seed trees.

6. Genetic diversity and regeneration

6.1 Introduction

During the creation or replacement of woodlands, it is important to know the likely effects of the choice of regeneration methods, including the effects on genetic diversity. The effects of various management operations have been studied by a number of workers, as reviewed in section 2.2.5. However, relatively few studies have investigated the effects of natural and artificial regeneration of broadleaved species.

As described in section 3.1, seeds and leaf samples were collected from a putatively autochthonous stand of *Fraxinus excelsior* near Betws-y-Coed (NGR SH799555, details and location map in Chapter 5, large-scale map in Appendix II). The samples comprised leaf samples from 32 mature trees and 32 natural regenerated seedlings/ saplings, 35 seedlings grown in a nursery, and seeds from 20 parents: 20 from ten parents and two from the remaining ten.

The aim of this part of the study is to determine the direction and magnitude of any changes in genetic diversity from mature trees to seeds, and from seeds to naturally- and artificially-regenerated seedlings.

6.2 Analysis

The frequency of null alleles was estimated using Micro-checker 2.2.3 (van Oosterhout *et al.*, 2004). In order to determine genetic diversity for the different cohorts, summary statistics (allelic richness, observed and expected heterozygosity and *F* statistics) were calculated using the programmes FSTAT 2.9.3 (Goudet, 2001) and GenAlEx 6 (Peakall and Smouse, 2006). *F* statistics were tested for significance, and tests were also carried out for linkage disequilibrium in FSTAT. This analysis was the same as that described in Chapter 5 and will not be described in more detail here.

6.3 Results

Estimated frequencies of null alleles are given in Table 6.1. Null alleles are possible at all loci except FEMSATL4. Frequencies for each locus over all cohorts vary from 3.8% at M2.30, to 20.3% at FEMSATL16.

Table 6.1 Estimated percentages of null alleles at six microsatellite loci in samples of *Fraxinus excelsior* from Betws-y-Coed, North Wales. Frequencies are given for different cohorts separately and over all cohorts

	FEMSAT2	FEMSAT4	FEMSAT11	FEMSAT16	FEMSAT19	M2.30
Mature	8.7%	no	no	21.8%	8.9%	no
Seeds	11.0%	no	10.4%	20.1%	8.2%	4.8%
Nursery	10.6%	no	10.0%	24.1%	no	no
Nat regen	7.3%	no	no	16.2%	6.4%	no
Overall	10.7%	no	10.5%	20.3%	8.1%	3.8%

Summary statistics for each cohort are given in Table 6.2. Most samples were successfully genotyped. A greater number of seeds was genotyped than individuals of the other cohorts, and a greater number of alleles was observed. However, almost no differences in allelic richness (based on the minimum sample size of 29) were recorded. Observed and expected heterozygosity were also similar across cohorts, with expected heterozygosity between 0.842 and 0.862, and observed heterozygosity lower, between 0.717 and 0.757. F_{IS} values for cohorts were all highly significant, between 0.119 and 0.170.

Table 6.2 Microsatellite diversity in different cohorts of *Fraxinus excelsior* from a population at Betws-y-Coed, North Wales. For each cohort the number of samples successfully genotyped at all six loci (N), and over six microsatellite loci the average number of observed alleles (A_O), the average allelic richness, expected (H_E) and observed (H_O) heterozygosity and F_{IS} values are given. H_E, H_O and F_{IS} were also calculated over five loci, excluding FEMSATL16; these values are shown in brackets. F_{IS}, tested by randomisation of alleles within samples in FSTAT, is significant ($P \leq 0.001$) in all cohorts, over five and over six loci.

Cohort	N	A_O	Allelic richness	H_E	H_O	F_{IS}
Mature trees	29	17.3	17.0	0.842 (0.880)	0.718 (0.784)	0.164 (0.126)
Seeds	209	32.8	17.4	0.862 (0.906)	0.717 (0.788)	0.170 (0.133)
Natural regeneration	30	16.8	16.6	0.843 (0.892)	0.757 (0.830)	0.119 (0.086)
Nursery seedlings	34	18.0	16.8	0.862 (0.896)	0.728 (0.811)	0.169 (0.110)

Table 6.3 gives summary statistics by locus; variation among loci is apparent. Figure 6.1A shows that FEMSATL2 had the greatest allelic richness and FEMSATL16 had the least. Figure 6.1B shows that at FEMSATL4 and M2.30 F_{IS} was close to zero, while at the other loci it was significantly greater than zero ($p \leq 0.05$, Table 6.3). The highest values of F_{IS} were recorded at FEMSATL16.

Differentiation among cohorts is shown as a matrix of pairwise F_{ST} in Table 6.4, and displayed in a bar chart in Figure 6.2. Values of F_{ST} were low between all cohorts, with the highest values being 0.0198 between mature trees and seeds, 0.0178 between mature trees and nursery seedlings and 0.0140 between natural regeneration and seeds. However, all the values were significant ($p \leq 0.01$) except the low value (0.0016) between mature trees and natural regeneration. There was little variation in F_{ST} among loci.

Table 6.3 Microsatellite diversity by locus in different cohorts of *Fraxinus excelsior* from a stand at Betws-y-Coed, North Wales. For each locus in each cohort the number of samples successfully genotyped (N), the number of observed alleles (A_O), the allelic richness, the expected (H_E) and observed (H_O) heterozygosities, and F_{IS} values are given. Significance of F_{IS}, tested by randomisation of alleles within samples in FSTAT, is indicated by asterisks. ns, nonsignificant; *p ≤0.05; **p ≤0.01; ***p ≤0.001.

Cohort	Locus	N	A_O	Allelic richness	H_E	H_O	F_{IS}
Mature trees	FEMSATL2	31	33	31.75	0.953	0.806	0.169 ***
	FEMSATL4	31	16	15.73	0.808	0.774	0.058 ns
	FEMSATL11	31	11	10.81	0.828	0.710	0.159 *
	FEMSATL16	31	7	6.93	0.648	0.387	0.417 ***
	FEMSATL19	30	19	18.73	0.899	0.733	0.201 **
	M2-30	29	18	18.00	0.916	0.897	0.039 ns
Natural regeneration	FEMSATL2	31	27	26.45	0.953	0.806	0.169 ***
	FEMSATL4	31	15	14.60	0.825	0.903	0.079 ns
	FEMSATL11	31	8	7.94	0.837	0.710	0.168 *
	FEMSATL16	31	7	6.93	0.593	0.387	0.362 ***
	FEMSATL19	30	23	22.70	0.921	0.800	0.148 **
	M2-30	30	21	20.76	0.929	0.933	0.012 ns
Nursery seedlings	FEMSATL2	35	30	27.28	0.941	0.743	0.225 ***
	FEMSATL4	35	15	14.20	0.856	0.886	0.021 ns
	FEMSATL11	35	11	10.46	0.857	0.686	0.213 **
	FEMSATL16	35	6	5.97	0.687	0.314	0.553 ***
	FEMSATL19	35	18	16.85	0.889	0.800	0.114 *
	M2-30	34	28	25.95	0.940	0.941	0.014 ns
Seeds	FEMSATL2	218	56	26.60	0.947	0.739	0.222 ***
	FEMSATL4	219	31	15.77	0.882	0.918	0.038 ns
	FEMSATL11	220	19	12.17	0.872	0.686	0.215 ***
	FEMSATL16	216	10	6.50	0.636	0.361	0.434 ***
	FEMSATL19	218	38	19.42	0.895	0.748	0.167 ***
	M2-30	217	43	23.97	0.937	0.853	0.093 ***

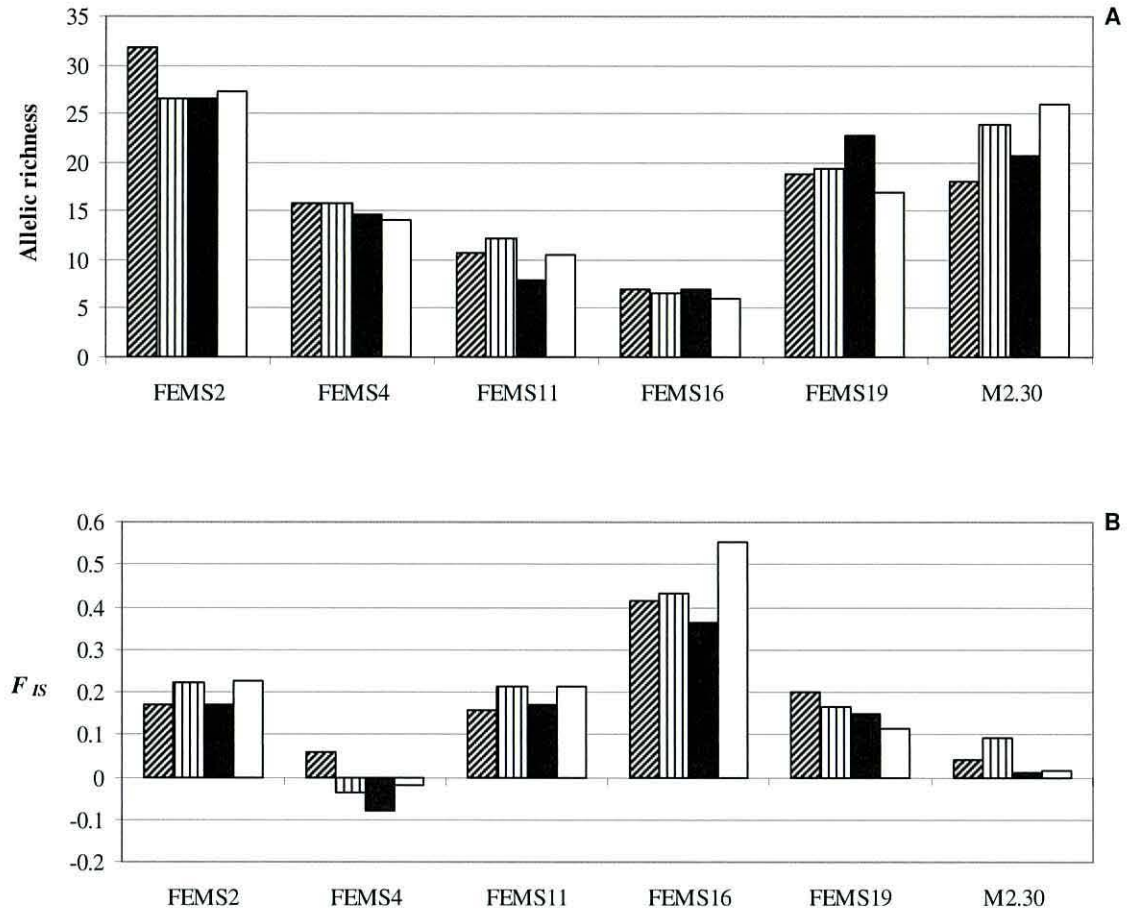

Figure 6.1 Microsatellite diversity in different cohorts of *Fraxinus excelsior* from a single population. (A) allelic richness and (B) F_{IS}, for each cohort at each locus. Diagonal hatching, mature trees; vertical hatching, seeds; black, natural regeneration; white, nursery seedlings;.

Table 6.4 Genetic differentiation (F_{ST}) between different cohorts of *Fraxinus excelsior* from a single population. F_{ST} between cohorts is shown. Significance of F_{ST}, tested by randomisation of alleles within samples in FSTAT (Goudet, 2001), is indicated by asterisks. ns, nonsignificant; **p ≤0.01.

	Seeds	**Natural regeneration**	**Nursery seedlings**
Mature trees	0.0198 **	0.0016 ns	0.0178 **
Seeds		0.0140 **	0.0033 **
Natural regeneration			0.0082 **

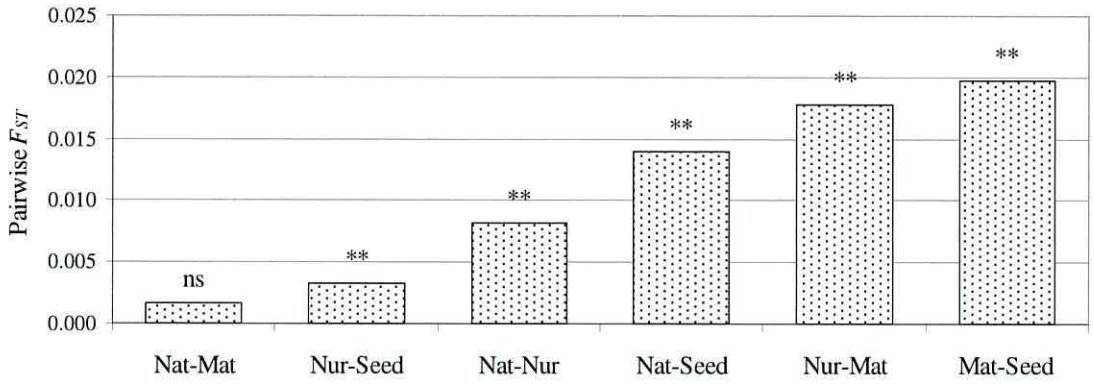

Figure 6.2 Pairwise F_{ST} between cohorts of *Fraxinus excelsior*, displayed in order of ascending F_{ST}. Nat, natural regeneration; Mat, mature trees; Seed, seeds; Nur, nursery seedlings. ns, not significant; **, $p \leq 0.01$.

6.4 Discussion

Analysis by Micro-checker (van Oosterhout *et al.*, 2004) showed that there was a possibility of null alleles at all loci except FEMSATL4. The highest estimated frequency of null alleles was found at FEMSATL16, and further analyses were carried out both including and excluding data for FEMSATL16. The exclusion of this locus resulted in higher estimates of heterozygosity and lower (but still significant) estimates of F_{IS}. The possible causes of the excess of homozygotes, especially at this locus, have been discussed in earlier chapters and will not be explored further at this point.

Almost no differences were recorded in genetic diversity among cohorts. Allelic richness (corrected for the minimum sample size), heterozygosity and F_{IS} were all remarkably constant across the different life stages sampled. This shows that the regeneration, whether natural or nursery-grown from collected seed, is representative of the mature trees sampled.

Very low levels of genetic differentiation were calculated as pairwise F_{ST} values. Values of less than 0.05 can be considered as showing little differentiation (Wright, 1978) although randomisation tests even with Bonferroni corrections suggested that most of these values were significant. The one value which was not significant was between the mature trees and the natural regeneration. The comparison between the seeds and the nursery seedlings gave the lowest of the significant values, as shown in Figure 6.2.

In explaining these differences a consideration of the sampling methodology is important. As outlined above, the seed samples were collected by staff of Forest Research in 2004, and the nursery seedlings were grown from a sample of the seed. It would therefore be expected that there would be a great similarity between the seeds and the seedlings. The mature trees and natural regeneration were sampled for this study in 2005, from the same area of woodland, but probably from different mature trees as the woodland was located using the grid reference provided but no micro-geographic information. As the woodland extends in a strip around 50 m wide for approximately 200 m, it is quite possible that the trees sampled in 2004 and 2005 were in different parts of the woodland. However, the mature trees and natural regeneration were sampled from the same area of woodland and so it is likely that much of the regeneration had grown from seed donated by the mature trees sampled, which would account for the lack of genetic differentiation.

It had been hypothesized that when comparing natural regeneration with seedlings grown in a nursery some differences might be seen. It was thought that by collecting seed systematically from 20 trees and then sowing a stratified sample in plugs all seeds might have had a more equal chance of germination, while under natural regeneration selection and chance (drift) before germination might reduce genetic diversity. Alternatively, it was possible that if seed collection from 20 trees was not a large enough sample of the population, or if the 20 trees were not widely enough spaced, natural regeneration might originate from a greater number of parent trees than collected seed and therefore show greater genetic diversity. However, it seems that in this case there was no effect of regeneration methods on genetic diversity.

At the present time few, if any, studies have compared genetic diversity among cohorts under differing regeneration practices in European broadleaved tree species. Some studies have, however, considered the differences between different age cohorts of trees in natural populations. In the study of French populations of *Fraxinus excelsior*, for example, most data were collected from seedlings germinated *in vitro*, but for comparison adult trees were sampled in three populations and almost identical values were recorded for allelic richness, heterozygosity and F_{IS} (Morand *et al.*, 2002).

The influence of nursery practices on genetic diversity was studied in *Fagus sylvatica* (Konnert and Ruetz, 2003). Seedlings grown under different conditions (greenhouse,

open seedbeds and plugs) showed no significant differences in genetic diversity, although sorting the seedlings by size, keeping the larger plants, did result in slightly reduced genetic diversity. In the present study *Fraxinus excelsior* seedlings were grown in plugs and there had been no sorting, and genetic diversity was not found to be different from that of mature trees. In the *Fagus sylvatica* experiment there was no comparison with mature trees or natural regeneration.

In conifers and eucalypts, frequently grown commercially, the effects of thinning and regeneration on genetic diversity have been investigated in a number of species. Genetic diversity in two species of *Eucalyptus* was studied in Australia under alternative regeneration strategies. In one species, *Eucalyptus consideniana,* lower genetic diversity was found in a seed tree treatment than in clear-fell followed by aerial sowing. In the other species, *Eucalyptus sieberi*, no significant differences in genetic diversity were found between treatments (Glaubitz *et al.*, 2003a; Glaubitz *et al.*, 2003b).

In several studies of regeneration methods and thinning types in *Pseudotsuga menziesii* and *Pinus contorta*, no significant differences were found between treatments (Neale, 1985; Thomas *et al.*, 1999). Contrastingly, in another study of *Pseudotsuga menziesii* and in one of *Picea glauca*, slight reductions in genetic diversity were found after phenotypic selection (Adams *et al.*, 1998; Rajora, 1999). Artificially-regenerated stands of *Picea abies* were also found to have decreased genetic diversity and altered allele frequencies compared with old-growth and naturally-regenerated stands (Gomory, 1992).

It may be seen that in some conifer species under certain conditions, management techniques may have negative effects on genetic diversity. Similar effects are possible in broadleaved trees if phenotypic selection is carried out. However, the present study suggests that negative effects of this type are unlikely to result from allowing natural regeneration where there is a reasonable population of mature trees, or from planting nursery seedlings that are unsorted for size.

When making recommendations for managing regeneration to conserve genetic diversity, it can therefore be concluded that natural regeneration is broadly equivalent to collecting seeds and growing seedlings in a nursery. On sites where natural regeneration is present, it is likely to be best to take advantage of this. In situations where natural

regeneration is not present and it is necessary to plant with forest reproductive material from external sources, similar levels of genetic diversity are likely to be maintainied, providing seeds are collected from a suitably large number of trees. The results obtained here suggest that a seed collection from 20 trees is sufficient to capture most of the diversity in the mature trees of a population.

7. Analysis of parentage for seed arrays

7.1 Introduction

Fraxinus excelsior, as described above, is wind-pollinated and has seeds which are dispersed by wind. In addition, trees may be male, female or hermaphrodite. This combination of characters means that the mating system and the nature of gene flow in *Fraxinus excelsior* may be assumed to be relatively complex. Gene flow is mediated by the dispersal of pollen and seed. In a model to fully describe gene flow, each functionally male tree would be described by a dispersal function (not necessarily symmetrical) giving proportions of pollen dispersed at varying distances. Each functionally female tree would then be allocated pollen proportionally according to the overlapping pollen dispersal distributions, and seeds would then be dispersed in another distribution.

The original intention of this study was to return to Bishopston Valley, which was intensively sampled for mature trees and natural regeneration in 2004, and to sample seeds from selected mature trees within the site. This would have allowed a detailed analysis of gene flow between generations on this site. However, as explained above, when it was intended to carry out seed collection in 2005, almost no *Fraxinus excelsior* seed was produced across Wales and indeed the rest of Britain.

It was therefore decided to carry out a study of genetic diversity in seeds which had already been collected (in 2004) by staff of Forest Research. These seeds had been collected from the site at Betws-y-Coed (NGR SH799555). The initial intention was to determine the maternal genotype of the seeds by extracting DNA from the samarae. Several trial DNA extractions were carried out, and visualisation after electrophoresis on 1% agarose mini-gels confirmed that these extractions were successful. However, PCRs failed to amplify microsatellite loci from the DNA extracts. It was considered likely that the mature tissue of the samara contained PCR inhibitors and due to time constraints it was decided not to persevere with these PCRs after initial attempts proved futile.

Another possible way of obtaining the maternal genotypes that was considered was to obtain data from staff at East Malling Research, who had also carried out genetic

analysis on seeds from this collection. However, it was eventually decided that time constraints precluded this approach also, as it would have been necessary to carry out a number of control PCRs in order to cross-validate the results and thus justify the use of data from a different laboratory.

Despite intentions, therefore, to sample seeds from trees with known genotypes or to obtain the maternal genotypes for the seed sample obtained from the stand at Betws-y-Coed, this was not possible, and the seeds sampled did not have a known maternal genotype. As described in chapters 3 and 6, the sampled seeds consisted of collections from 20 trees. From these samples, 20 embryos were extracted and genotyped for ten trees, and two for the remaining ten.

The aim of this part of the study is to estimate the number of parents contributing pollen to the seeds collected.

7.2 Analysis

7.2.1 Inferring maternal genotypes

For the seeds where 20 seeds per parent were analysed, a maternal genotype was inferred manually by exclusion. For each locus maternal alleles were chosen that resulted in the fewest inconsistent alleles between offspring and maternal genotypes.

7.2.2 CERVUS 2.0

The programme CERVUS 2.0 (Marshall *et al.*, 1998) was used to determine the number of seed samples for which the sampled trees had acted as pollen donors. CERVUS uses simulation to determine the likelihood of any matches. A simulation was carried out using the allele frequencies from the entire sample set. With 10,000 simulation samples (offspring) and 100 candidate parents, the simulation assumed that 90% of loci were typed and 1% of typed loci were mistyped. The simulation also assumed that 40% of the candidate male parents had been sampled and genotyped.

CERVUS allocates a most likely candidate parent (pollen donor) to each offspring (seed) and assesses the likelihood of this parent being the true parent using the LOD score and the delta (Δ) statistic (Marshall *et al.*, 1998). The LOD score (the log of the product of the likelihood ratios at each locus) gives the likelihood of the candidate

parent being the true parent compared to a randomly chosen individual. In an exhaustive study of parentage in red deer, *Cervus elaphus,* on the island of Rum, Scotland, the reliability of the method was assessed and a threshold value of LOD ≥ 3 was suggested (Slate *et al.*, 2000). The Δ statistic assesses the reliability of assigning parentage to the most likely parent, and is defined as the difference in LOD scores between the most likely candidate parent and the second most likely candidate parent. During the simulation, confidence levels are calculated so that values of Δ may be assigned to strict and relaxed levels. The confidence levels in CERVUS were set so that the strict level was 95% and the relaxed level was 80%.

The paternity analysis was carried out using the inferred maternal genotypes as known parents. The candidate male parents were the 32 sampled trees and the ten inferred genotypes (since any one of these trees could have acted as pollen donors for any of the remainder). Initially these were all used; then to assess the significance of selfing the known maternal parent for each seed genotype was excluded from the candidate male parent list. Analysis in CERVUS was carried out over six microsatellite loci and over five loci, excluding FEMSATL16, because of the possible high frequencies of null alleles at this locus.

7.2.3 GERUD 2.0 and single-locus minimum method

In order to determine the minimum number of pollen donors for each half-sib family, the programme GERUD 2.0 (Jones, 2005) was used to attempt the reconstruction of parental genotypes. However, when more than six pollen donors have contributed to a half-sib family, the algorithms used by GERUD are too computer-intensive, and GERUD no longer functions.

The single-locus minimum method was also used to determine the number of sires. The inferred maternal genotype was used as above, and maternal alleles subtracted from the genotype for each seed. The remaining alleles were considered paternal alleles; for offspring with the same single-locus genotype as the mother, one allele was retained at random. At each locus, the number of paternal alleles was divided by two, and then rounded up. This procedure was carried out for each locus in each half-sib family, and the locus with the highest number of paternal alleles in each family was used to calculate the single-locus minimum number of pollen donors. Thus a minimum was

obtained for ten families and summary statistics (minimum, maximum, average, standard deviation) were calculated.

7.2.4 MLTR 3.0

The programme MLTR 3.0 (Ritland, 2002) was used to estimate single- and multi-locus outcrossing rates and correlated paternity from progeny arrays. In order to allow the analysis to proceed, individual seed genotypes that were inconsistent with the inferred maternal genotype were excluded from the analysis. The half-sib families were resampled using 1000 bootstraps in order to calculate the standard error of the estimates. Once again, analysis was carried out both including and excluding data from the locus FEMSATL16.

7.3 Results

7.3.1 Inferred maternal genotypes

In most families, it was not possible to infer a single maternal genotype with no mismatches. In some cases a number of half-sibs had alleles one base pair apart; in this case it was likely that rounding had occurred in the wrong direction, and so the alleles were re-binned together (e.g. alleles of 188 or 189 bp in FEMSATL19). It was also observed that with larger-sized alleles scoring was less accurate due to multiple peaks, so in some cases alleles of size 256, 258, 260, and 262 bp in FEMSATL2 were re-binned together. In some cases there were a large number of mis-matches and a high proportion of homozygotes with different alleles. This was interpreted as the result of the presence of a null allele, which was present in all apparent homozygotes not consistent with the inferred maternal allele(s). Other mismatches could have been due to incorrect sample identification or errors other than scoring during genotyping.

7.3.2 CERVUS 2.0

The simulated parentage analysis gave low success rates. Using data from six loci, parents were assigned with 95% confidence to 7% of offspring and with 80% confidence to 21% of offspring. Using data from five loci, 5% and 19% of offspring were assigned parents at the same confidence levels.

Results from the parentage analysis of the 220 genotyped seeds are shown in Table 7.1. Using data from six loci, CERVUS allocated candidate male parents with LOD scores

≥3 to 37 (16.8%) of the 220 seeds analysed. When selfing was not permitted this was reduced to 24 seeds (10.9%). The Δ scores gave confidence levels for the candidate male parents allocated to two (0.9%) of the 37 seeds at 95% and 15 (6.8%) at 80%. The remaining seeds were simply the most likely candidate parent according to their LOD scores. For the 24 candidate male parents allocated to seeds when selfing had been excluded, none had 95% confidence but 10 (4.5%) had 80% confidence.

When all assignments (including those with LOD scores <3) were considered, a greater number of candidate parents was assigned at the relaxed confidence level.

The background paternity (pollen donors not accounted for by the sampled trees or inferred maternal genotypes) can be assigned depending upon whether selfing is permitted or not, and depending upon the threshold confidence level. With selfing, the background paternity was 99.1 % (95% confidence) or 93.1% (80% confidence). Without selfing the same confidence levels gave 100% or 95.4% background paternity.

Table 7.1 Results of parentage analysis in a population of *Fraxinus excelsior*. Number of offspring (out of a total of 220) assigned a parent by CERVUS (Marshall *et al.*, 1998) under different criteria.

	Six loci				Five loci			
	Including selfing		Without selfing		Including selfing		Without selfing	
All LOD scores								
Δ confidence ≥ 95% (strict)	2	(0.9%)	0	(0%)	4	(1.8%)	2	(0.9%)
Δ confidence ≥ 80% (relaxed)	23	(10.4%)	15	(6.8%)	25	(11.3%)	20	(9%)
Total selfed	12	(5.4%)	0		10	(4.5%)	0	
Background paternity								
Relaxed	197	(89.5%)	205	(93.1%)	195	(88.6%)	200	(90.9%)
Strict	218	(99%)	220	(100%)	216	(98.1%)	218	(99%)
LOD scores ≥ 3	37	(16.8%)	24	(10.9%)	22	(10%)	11	(5%)
Δ confidence ≥ 95% (strict)	2	(0.9%)	0	(0%)	4	(1.8%)	2	(0.9%)
Δ confidence ≥ 80% (relaxed)	15	(10%)	10	(10.9%)	14	(3.6%)	7	(1.8%)
Total selfed	14	(6.3%)	0		11	(5%)	0	
Background paternity								
Relaxed	205	(93.1%)	210	(95.4%)	206	(93.6%)	213	(96.8%)
Strict	218	(99%)	220	(100%)	216	(98.1%)	218	(99%)

When data from locus FEMSATL16 were excluded from the analysis slightly different results were obtained. When only assignments with LOD scores of three or above were considered, candidate parents were assigned to fewer offspring, except at the strict confidence level. When LOD scores were not restricted, candidate parents were assigned to a slightly greater number of offspring.

With respect to the number of seeds from each half-sib family of 20 that were allocated parents, when selfing was permitted 2-10 seeds ($\bar{x}$ = 3.7, σ = 2.7) were allocated parents. Without selfing, 0-7 seeds ($\bar{x}$ = 2.4, σ = 2.1) from each family were allocated parents.

7.3.3 GERUD 2.0

In order to reconstruct genotypes using GERUD (Jones, 2005) the half-sib families had to be compatible with a single maternal genotype, with no mismatches, so adjustments were made as detailed above and some individuals were deleted. However, GERUD was unable to reconstruct the possible paternal genotypes as in every half-sib family more than six fathers were inferred.

7.3.4 Single-locus minimum method

A spreadsheet was used to calculate the single-locus minimum number of fathers and after the subtraction of the inferred maternal alleles the number of paternal alleles was counted and divided by two. The locus with the greatest number of paternal alleles in seven out of ten families was M2.30, in five out of ten families it was FEMSATL2, and for one family it was FEMSATL4 (N.B. in several families M2.30 and FEMSATL2 had equally high numbers of alleles and in one family all three loci had the same number of alleles). The single-locus minimum number of fathers varied from six to nine ($\bar{x}$ = 7.3, σ = 0.82) per half-sib family.

7.3.5 MLTR 3.0

The multilocus estimate of outcrossing (t_m) was 1.2 (σ = 0). The single-locus estimate (t_s) was 0.907 (σ = 0.014). The multilocus correlation of paternity (r_p) was estimated as -0.134 (σ = 0.026). Estimates were very similar when calculated over five loci, excluding locus FEMSATL16.

7.4 Discussion

All the methods used here point to a similar conclusion: there is a high level of outcrossing; a high level of pollen flow from beyond the trees sampled; and a large number of trees are contributing pollen to the progeny arrays sampled.

Due to the various uncertainties involved in these calculations, it is impossible to be precise about the conclusions drawn, but given that all the methods lead to similar conclusions it is possible to have a certain degree of confidence in the general pattern.

When using CERVUS, a number of assumptions must be made: for example, the number of unsampled candidate parents. When the number of unsampled candidate parents is unknown it is difficult to estimate the effect of cryptic gene flow – that is when pollen from beyond the sampled area has haplotypes identical to those that could be produced within the local population (Oddou-Muratorio *et al.*, 2003). To make an estimate of the number of unsampled candidate parents is very difficult without prior information about the dispersal of *Fraxinus excelsior* pollen or the population of *Fraxinus excelsior* in the surrounding area. It is certain that this is not an entirely isolated population, but the density of trees in the surrounding area, and the size of the area that could contribute pollen, are unknown. It is probably safest to use the higher confidence levels, and in that case we have to conclude that if selfing is excluded, none of the embryos genotyped was fathered by the sampled trees. Cryptic gene flow is therefore not a problem in this case.

The error rate in genotyping is also important, because even small rates of genotyping errors can result in the false exclusion of true parents (Slavov *et al.*, 2005). This type of false exclusion becomes increasingly likely with an increasing number of loci. CERVUS allows for this type of error by calculating likelihood values rather than using simple exclusion, but when estimating the genotyping error rate an additional area of uncertainty is introduced.

The possibility of the presence of null alleles could have a significant effect on the results of parentage analysis. In some cases it might result in false exclusions, although in other cases it could cause individuals to appear more related than they actually are. For example, two individuals scored as homozygotes, AA and BB, could in fact share a null allele, but would not be considered to share any alleles. In the case of a seed and candidate parent with these genotypes, the parent could be falsely excluded. Conversely, in the case of two individuals both scored as AA, if one had a null allele they could in fact only share one allele when they are recorded as sharing two. If the maternal genotype was AB and the offspring apparently AA (but with a null allele), a paternal genotype of AB or true homozygote AA could be falsely included.

The changes in the results from CERVUS when FEMSATL16 is excluded may be partly due to the effects of possible null alleles. However, the reduction in the number of loci also results in reduced statistical power, and thus although fewer parents may be excluded, the confidence in parentage assignments must be reduced.

In this study no attempt was made to infer directly the average distances of pollen and seed dispersal. However, a study of *Fraxinus excelsior* in Bulgaria suggested restricted seed flow (mean dispersal distance up to 14 m) and moderate pollen flow (mean dispersal distance between 70 -140 m) (Heuertz *et al.*, 2003). Another study, in Britain and Sweden, found that 18-34% of seeds were sired by fathers outside the 10 and 12 ha plots, and estimated mean pollen dispersal distances of 41-72 m (FRAXIGEN, 2005). Considerable variation was observed in gene flow between a mast year and a non-mast year.

This may be compared with another species of ash from Japan, *Fraxinus mandshurica* var. *japonica*, a dioecious species that is also wind-pollinated and has wind-dispersed seeds. A study of gene flow in this species within an exhaustively studied 10.5 ha plot suggested that the pollen dispersal curve was "fat-tailed" (Goto *et al.*, 2006). The results fitted a two-component pollen flow model, with 26.1% of the pollen being dispersed relatively short distances (mean dispersal distance 7.2 m) and the remainder much further (mean dispersal distance 209.9 m). Seed dispersal was not successfully fitted to a similar model, but 31.5% of established seedlings could not be assigned maternal parents within the plot, indicating some longer-distance seed dispersal (30% > 100 m).

These studies, both in *Fraxinus excelsior* and in another *Fraxinus* species, found high levels of pollen and seed dispersal, although this varied considerably. These variations may be partially due to the methods used in each study. However, they support the findings of this study that many seeds were sired by pollen beyond the trees sampled here.

In GERUD, the single-locus minimum method, and MLTR the methods depend upon inferring the maternal genotype from the progeny array. In several cases no one maternal genotype matched all the seeds from one array. One possible explanation for this is the presence of a null allele, which was assumed when a high proportion of the seeds from one array consisted of homozygotes for different alleles. This occurred in

four out of ten arrays in FEMSATL16, in three out of ten in FEMSATL2, and in one in FEMSATL11. The second possible explanation is that an error occurred during the genotyping and scoring of the microsatellite loci. This was particularly likely when one maternal genotype matched most of the progeny array but in one or two seeds there was a mismatch by one or two base pairs. However, it is also possible that an error occurred during collection, DNA extraction, or amplification, and that the identity of a sample was incorrect.

Despite the potential errors and uncertainties in the methods used, the single-locus minimum method implies a minimum of between six and nine pollen donors for each progeny array. It is important to recognise that this method gives a maximum number of pollen donors of N/2, which would be ten in this case. Hence the numbers recorded are close to the maximum possible with this method and sample size.

The multilocus outcrossing rate given by MLTR was 1.2, which effectively means that 100% of the seeds were the result of outcrossed as opposed to selfed matings. The single-locus outcrossing rate was lower than the multilocus rate, which suggests that biparental inbreeding (matings between relatives) is significant. This is likely to occur as a result of small scale genetic structure within populations, as suggested in Chapter 4. The correlated paternity rate was small and negative, which means that progeny within an array do not share fathers.

Other studies of *Fraxinus excelsior* have found similarly high levels of outcrossing. In controlled crosses studied with microsatellites, selfed progeny were found to be very uncommon (Morand-Prieur *et al.*, 2003). Controlled crossing in another experiment also found that although selfed seed could be produced, it was very rare (selfing 0.3-1.7%) (FRAXIGEN, 2005; Stewart *et al.*, 2006).

Many studies have found similarly high outcrossing rates in other temperate tree species, although some species also have considerable selfing. Mating system is known to vary considerably among species. In *Prunus mahaleb*, a gynodioecious species, high outcrossing (t_m = 1.01) was recorded in females, but in the self-compatible hermaphrodite individuals, much lower outcrossing was recorded (t_m = 0.41-0.49) (Garcia *et al.*, 2005). In *Pinus sylvestris*, normally highly outcrossing, selfing rates up to 25% were recorded in a small, isolated population, compared to selfing rates of 1-6% in

larger populations (Robledo-Arnuncio *et al.*, 2004b). However, in another study where contrasting silvicultural practices (shelterwood and group selection) were compared, outcrossing was high (t_m = 0.93-0.99), and no difference was found between the stands (Robledo-Arnuncio *et al.*, 2004a). Similarly, in a comparison of old-growth and shelterwood stands of *Pseudotsuga menziesii*, no significant difference was found between stands (t_m = 0.94-1) (Neale and Adams, 1985). Although these species are highly diverse, it is clear that a high outcrossing in *Fraxinus excelsior*, as found in this study, is far from atypical among temperate tree species.

To conclude, there are some limitations to the methods used in this part of the study. However, even with these limitations, due to the correspondence between methods it is possible to have some confidence that the *Fraxinus excelsior* seeds sampled were produced by outcrossing. Close to 100% of the pollen is believed to have originated from outside the patch of woodland sampled, and a high number of parents (a minimum of six to nine for an array of 20 half-sibs, but probably closer to 20) are believed to have contributed pollen to produce these seeds.

8. General discussion and conclusions

8.1 Suitability and limitations of methods used

8.1.1 Microsatellite loci

It was decided to use microsatellites in this study as a number had already been developed for *Fraxinus excelsior* and used in other studies. As codominant markers, microsatellites are preferable to dominant markers such as AFLPs. Other advantages include the relative ease of scoring (with only two peaks to be scored for each locus) and the fine-scale resolution, which allows closely-related individuals to be distinguished. The use of chloroplast markers, either RFLPs or microsatellites, was discussed, but based on other studies in the UK it seemed unlikely that polymorphisms would be found within Wales.

The six microsatellite loci used in this study were a selection of those developed by workers in France (Brachet *et al.*, 1999; Lefort *et al.*, 1999). These microsatellite loci have been used successfully in studies on a number of scales across Europe. They were selected because they had shown high levels of polymorphism in all the populations studied, and had been found to amplify reliably without high frequencies of null alleles. It was initially hoped to use eight microsatellite loci, and so two additional sets of primers, for loci FEMSATL8 (Lefort *et al.*, 1999) and 1.19 (Brachet *et al.*, 1999) were tested. These loci, despite being used successfully in some laboratories, failed to amplify reliably here. However, the six loci used all showed high levels of polymorphism in this study and so even with fewer loci than originally hoped a large amount of information was available.

In this study, the use of four samples as positive controls for PCR and genotyping showed that allele sizes were highly reproducible but could vary in length within one base pair. The scoring of dye peaks was therefore critical. At four loci (FEMSATL2, FEMSATL4, FEMSATL11 and FEMSATL16), peaks seemed naturally to group into alleles two base pairs apart, and so at these loci all alleles were recorded as either even or odd- numbers of base pairs. At the remaining two loci (FEMSATL19 and M2.30), peaks did not seem to segregate in this manner, and so peaks were rounded up or down

to the nearest whole number of base pairs. It is apparent that these choices affected the number of alleles observed at each locus.

The analysis of half-sib arrays during the parentage analysis reported in Chapter 7 revealed a few inconsistencies, with genotypes recorded in 20 individuals that could not be accounted for by a single maternal genotype. Some of these were attributed to the presence of a null allele (this is discussed in more detail below). Others were rounded up or down (in the opposite direction to previously) and this solved the problem. A few more cases did not have an obvious solution, and these cases were deleted for the parentage analysis. It is probable that these cases (approximately five out of 220 seeds genotyped) represent a mixture of errors in genotyping and errors of sample identification, possible at any stage from sample collection to PCR.

It is clear that the process of sampling, extraction, amplification and genotyping of microsatellites was not entirely error-free. However, as alleles at each locus were scored consistently, and errors observed in the half-sib arrays were at a relatively low frequency, the results obtained are in general robust.

One possible problem with the use of microsatellites is the possibility of the existence of null alleles. The loci used were chosen because high frequencies of null alleles had not been reported. However, analysis using the programme Micro-Checker (van Oosterhout *et al.*, 2004) estimated that there was a possibility of null alleles in most populations at all loci except FEMSATL4. The difficulty with this estimation is that it is calculated from the excess of apparent homozygotes at all size ranges (with excess of homozygotes at only smaller sizes, a different phenomenon, large allele dropout, is indicated, but this was not found in this study). An excess of apparent homozygotes could also be due to an excess of actual homozygotes, due to a Wahlund effect, selfing or biparental inbreeding. The excess homozygosity found in this study is similar to that found in French populations of *Fraxinus excelsior* (Morand *et al.*, 2002), as well as that found in fragmented Scottish populations (Bacles *et al.*, 2004).

One problem with accepting the presence of null alleles as an explanation for the excess homozygosity recorded is that at loci with higher frequencies of null alleles a higher number of samples would be expected to entirely fail to amplify. If the frequency of null alleles was p, heterozygotes with null alleles would be recorded as homozygotes at a

frequency of $2pq$, where q is the frequency of all recorded alleles. However, homozygotes for null alleles would be observed as samples which failed to amplify at a frequency of p^2. The estimated null allele frequency at FEMSATL16 was around 0.2, which would lead to an expected frequency of null allele homozygotes of around 0.04. With over 617 samples in total, approximately 25 samples should have failed to amplify at FEMSATL16. However, of the 617 samples collected, only eight were not genotyped at FEMSATL16. Of these, four were not genotyped at any loci, probably due to a failed extraction or the presence of PCR inhibitors. Therefore, only four out of 613 samples failed to amplify at FEMSATL16 but were successfully amplified at the other loci, indicating the possibility of a null allele homozygote. This is considerably less than the expected estimate so casts some doubt on the null allele explanation.

The genotyping of sibling arrays in this study allowed additional information on the likelihood of null alleles to be collected. The presence of a null allele was inferred in four out of ten arrays at FEMSATL16, in three out of ten at FEMSATL2, and in one at FEMSATL11. In a diploid species, this would imply roughly estimated null allele frequencies of 20% at FEMSATL16, 15% at FEMSATL2 and 5% at FEMSATL11. These estimates are approximately consistent with estimated null allele frequencies in chapters 4-6.

The highest estimated frequencies of null alleles were found at FEMSATL16, with estimates up to 26% (in natural regeneration in Bishopston Valley) and F_{IS} values up to 0.511 (in the same population). In order to test the effect that this might have on the overall results, analyses were repeated with the exclusion of locus FEMSATL16. Overall heterozygosity increased while estimates of F_{IS} and F_{IT} decreased. Among-population differentiation, estimated by F_{ST}, was not found to change significantly.

The most noticeable change was the loss of small-scale spatial genetic structure in Bishopston Valley. With all six loci, spatial autocorrelation showed that pairs of individuals from smaller distance classes were more likely to be closely related than pairs of individuals drawn randomly from the population. A Mantel test also showed a slight, but significant overall correlation between genetic and geographic distances between pairs of individuals. However, when FEMSATL16 was excluded the Mantel test showed no correlation. A spatial correlogram produced from data for five loci showed that individuals in smaller distance classes were still slightly more likely to be

related. However, at several distance classes, this effect was not significant and both the r value in the smallest distance class and the distance at which the r value intercepted the x axis were much lower.

This effect was entirely unexpected and is somewhat difficult to explain. If FEMSATL16 was less informative than the other loci, it would be expected to decrease any genetic structure, so that when it was excluded a higher degree of structure would be observed. Due to the testing by permutations carried out during spatial autocorrelation and a Mantel test, it is clear that there is significant structure at FEMSATL16. It is extremely unlikely that this pattern arose by chance ($p \leq 0.001$). It seems that FEMSATL16 must be informative, and some explanation must be sought for the excess homozygosity at this locus. One possibility, not investigated in this study, is that FEMSATL16 is closely linked to a gene under selection, which would not be expected to be in Hardy-Weinberg equilibrium.

In order to determine whether selection was involved in the anomalous patterns associated with FEMSATL16, a number of complex experiments would be necessary. It can be speculated, however, that if selection occurs within the relatively small distances in Bishopston Valley, it must be based on small-scale environmental variation, for example in soil moisture, frost incidence, sunlight received, or soil chemistry.

When single-locus F_{ST} values were calculated it became clear that there was considerable variation among loci. In particular, high F_{ST} values were observed at FEMSATL19. When single-locus pairwise estimates of F_{ST} were compared, it was seen that these high values came from pairs of populations including Nicholaston. Variation between loci in differentiation could also imply that these loci were linked to genes under selection in some populations. To explain this, one or more environmental factors that vary between sites would be required. One suggestion is that Nicholaston is the most exposed coastal site and might be exposed to salt spray.

In the French study of *Fraxinus excelsior*, the authors discuss extensively the possible causes for their observations of excess homozygosity (Morand *et al.*, 2002). They consider null alleles unlikely for the following reasons: the microsatellite loci were developed species-specifically for *Fraxinus excelsior*; the high estimated frequencies of null alleles compared to the frequencies of other alleles; and the Mendelian inheritance

of microsatellite loci demonstrated in their (limited number of) controlled crossing experiments. A Wahlund effect, or the existence of spatial or temporal breeding subunits, is another explanation, but there was no evidence of lower homozygosity when the Bishopston Valley population was subdivided. The limited spatial genetic structure in Chapter 4 does not seem enough to account for the excess homozygosity. Temporal genetic structure, if trees breed in small groups separated by time, is possible, but given the low values of F_{ST} estimated among populations, it would seem odd to find much higher differentiation among temporal breeding subunits. Biparental inbreeding, assortative mating, or selfing are all possible, but results from Chapter 7 indicate a high level of outcrossing. This is supported by controlled crosses in another study, where only three out of 110 seedlings were the result of selfing (Morand-Prieur *et al.*, 2003).

The excess homozygosity found in this study does complicate the interpretation of results, as it is currently impossible to determine the cause. It seems likely from the sibling arrays that the presence of null alleles at least contributes to the excess of homozygotes, but the spatial structure recorded at FEMSATL16 in Bishopston Valley suggests that a different explanation may be needed. The suggestion that one or more microsatellite loci may deviate from the assumption of neutrality raises questions about how the interpretation of the data. If neutral markers are used then all patterns of diversity detected may be explained by mutation, migration and genetic drift. When selection is suspected, this may also influence the patterns found. However, the use of microsatellite loci remains a highly informative exercise.

8.1.2 Sampling

In practical experiments, in contrast to theoretical experiments, the extent of sampling is necessarily limited by constraints of time, finance, and logistics. In theory it would have been desirable to genotype every individual of *Fraxinus excelsior* in each study site, if not every individual in Wales. However, under practical constraints, decisions were made about the number and distribution of trees sampled in each site.

Bishopston Valley was the most intensively studied site, with samples from over 80 mature trees and a similar number of naturally regenerated seedlings or saplings. These were collected along a transect following the line of the valley (see Chapter 4), at a relatively wide spacing in order to detect spatial structure at a medium scale. Given the small-scale nature of the genetic structure detected, it might have been interesting to

sample every mature tree, and a greater number of naturally regenerating seedlings, over a relatively small scale. Also, due to the possibility of anisotropic gene flow linked to the prevailing wind, a second transect perpendicular to the first might have allowed interesting contrasts to be made.

From the remaining sites around 30 mature trees were sampled. This was regarded as a suitable sample size to estimate genetic diversity within the populations. Although a greater number of alleles was observed in Bishopston Valley, due to the greater number of samples, other statistics such as allelic richness, heterozygosity and *F* statistics were fairly similar among populations, suggesting that the sample size was sufficient to avoid the random effects of small sample sizes.

The sampling of different cohorts from Betws-y-Coed was not ideal as the original intention, as explained in Chapter 7, was to return to Bishopston Valley and collect seeds from a number of the trees already genotyped there. In this case a larger number of candidate parents would have been genotyped for the parentage analysis.

While the comparison of different cohorts, regenerated by different means, in Chapter 6, was relatively robust, with sufficient sample size, there were weaknesses in the parentage analysis described in Chapter 7. More robust results could have been obtained if the maternal genotypes had been known with more certainty, and if a greater number of mature trees had been sampled. Ideally, to fulfil the requirements of CERVUS (Marshall *et al.*, 1998), the local population would have been sampled exhaustively and the number of candidate parents in the surrounding area would also have been determined.

8.1.3 Overall success of methods

In order to make a judgement about the overall success of the methods used in an experiment, it is necessary to determine to what extent the questions addressed have been answered. Conversely, if the questions have not been answered as fully as hoped, the areas in which the methods were lacking and the modifications that would be made retrospectively can be considered.

As discussed above, microsatellites were chosen as molecular markers in as they have several advantages over other types of markers. Thus despite the likelihood of a low rate

of genotyping errors and the likely presence of null alleles, it is considered that they remain the best option. With more resources it might have been desirable to develop primers for a greater number of microsatellite loci, but the development of new microsatellite loci is a complex process that can be unpredictable, especially in plants (Squirrell *et al.*, 2003).

In the sampling stage, with hindsight it might have been advantageous to adopt a more complex sampling strategy within Bishopston Valley, in order to assess small-scale genetic structure in more detail. In addition, more complete sampling of parents in the parentage study would have been desirable. However, given the likely distances of pollen flow in *Fraxinus excelsior*, it is likely that even with much more extensive sampling a large number of pollen donor trees would have remained unsampled.

In conclusion, the methods used in this study were subject to limitations due to the resources available. However, these types of difficulties are inevitable in ecological studies such as this one where by collecting a reasonable number of samples an attempt is made to simplify and understand inevitably complex real world processes. In this study a large number of samples were collected and microsatellites were genotyped in a repeatable manner.

8.2 Contribution of this study to the understanding of the ecology of *Fraxinus excelsior*

8.2.1 Within-population genetic structure

The genetic spatial autocorrelation analysis carried out on samples from Bishopston Valley showed that naturally regenerated seedlings were likely to be related to the mature trees closest to them. Furthermore, weak spatial structure existed up to around 700 m, meaning that individuals within that distance were more likely to be related than individuals picked at random from the population.

Previous studies of small-scale genetic structure in *Fraxinus excelsior* have been carried out in Bulgaria (Heuertz *et al.*, 2001) and in Romania (Heuertz *et al.*, 2003). In the Romanian populati

on neighbourhood sizes, calculated as the variance of gene dispersal distances, ranged from 38 to 66 individuals. A pattern, similar to that found in this study, of isolation by

distance was found as neighbour individuals were found to be more highly related than pairs of individuals in larger distance classes. A similar result was found in the Bulgarian population, and in addition simulations were used to estimate seed flow at 14 m while pollen flow was estimated at between 70 m and 140 m. The results reported in this study suggest that patterns of small-scale spatial genetic structure found in Wales and probably western Europe are similar to those found in eastern European populations.

A review showed that 14 out of 19 outcrossing woody perennials showed some small scale genetic structure, although in five of these cases this structure was linked to founding effects. Pure isolation by distance was only suggested in nine cases out of 19 (Ennos, 2001). Weak genetic structure, probably caused by restricted pollen and seed flow, was found in studied populations of *Fagus crenata* (some in cut forest) (Asuka *et al.*, 2004; Kitamura *et al.*, 2005; Takahashi *et al.*, 2000; Takahashi *et al.*, 2003), *Fagus japonica* (Takahashi *et al.*, 2003), *Quercus crenata* (Chung *et al.*, 2005), *Quercus petraea* and *Q. robur* (Streiff *et al.*, 1998) and *Quercus lobata* (Dutech et al., 2005). In contrast, no spatial genetic structure was found in a population of *Fagus crenata* in primary forest (Takahashi *et al.*, 2000), in *Quercus acutissima* (Chung *et al.*, 2004) or in *Cercidiphyllum japonicum* (Sato *et al.*, 2006).

Similar results have been found in studies of coniferous forest trees; no significant genetic structuring has been found in *Pinus contorta* (Epperson and Allard, 1989), in a field population of *Larix laricina* (Knowles *et al.*, 1992), or in chloroplast DNA markers in *Pinus ponderosa* (Latta et al., 1998). However, significant spatial genetic structure was found in some populations of *Pinus banksiana* (Xie and Knowles, 1991), and in mitochondrial DNA in *Pinus ponderosa* (Latta *et al.*, 1998). In a number of other studies genetic structure has also been found which can be explained by founding events and so does not imply restricted geneflow (e.g. Furnier *et al.*, 1987; Knowles *et al.*, 1992; Perry and Knowles, 1991).

It may be seen that the weak genetic structure recorded in this study in *Fraxinus excelsior*, as well as in Bulgarian and Romanian populations, is common in outcrossing forest trees, although no structure is apparent in some species or populations. Stronger structure has been observed in clonal species, which may differ in many aspects from the outcrossing species discussed here.

8.2.2 Within population genetic diversity

This study, in common with other studies of *Fraxinus excelsior*, found high levels of allelic richness and heterozygosity in all sampled populations. The number of alleles observed was generally similar to that in other studies, taking sample size into account. Heterozygosity was also similar to levels found in other investigations, including that of fragmented populations in Scotland (Bacles *et al.*, 2005), where the overall value of expected heterozygosity, H_E, was 0.849, very similar to an average for the mature trees in this study of 0.891.

A general excess of homozygotes was found in this study, in common with findings from Scotland (Bacles *et al.*, 2005) and France (Morand *et al.*, 2002). However, a much lower excess of homozygotes was found in more eastern populations, including southern Germany, Bulgaria and Romania (Hebel *et al.*, 2006; Heuertz *et al.*, 2001; Heuertz *et al.*, 2003). As discussed above, this effect may be due to the presence of null alleles, to some form of selfing or biparental inbreeding, or (at least in the case of FEMSATL16) because the locus in question is not selectively neutral. The difference between western and eastern Europe may therefore be due to the distribution of null alleles with more being present in western countries. However, given that all the microsatellite loci used were developed in France, this seems unlikely. It is therefore possible that differences in species history and ecology have determined these differences.

8.2.3 Among-population genetic differentiation

Low levels of differentiation were estimated among populations in this study. Pairwise F_{ST} values were found to vary between 0.004 and 0.041, with an average of 0.017. These values are similar to those found in Germany (average 0.012, Hebel *et al.*, 2006) and slightly lower than those found in France and Scotland (average 0.043, Morand *et al.*, 2002); Bulgarian populations had higher values (average 0.087, Heuertz *et al.*, 2001).

Compared to studies of other broadleaved trees, F_{ST} values calculated for *Fraxinus excelsior* are low. For example, in populations of *Sorbus torminalis* overall F_{ST} was recorded at 0.167 (Bednorz *et al.*, 2006) although in fragmented populations of the congener *Sorbus aucuparia* overall θ (equivalent to F_{ST}) was estimated at 0.043 (Bacles

et al., 2004). High values of F_{ST} (average 0.169) were estimated in *Quercus suber* (Elena-Rossello and Cabrera, 1996) but low values of F_{ST} (average 0.010), similar to those found in this study, were recorded in *Betula maximowicziana* (Tsuda and Ide, 2005).

Although significant differentiation can occur in some species, it may be seen that the low among population differentiation in *Fraxinus excelsior* is not unusual in forest trees. The variation in degree of differentiation may be largely explained by differences in breeding system, pollination, seed dispersal and population density. Isolated small populations or dispersed trees may be expected to show greater differentiation, as may those with limited gene flow by pollination and seed dispersal. Wind-pollinated trees are expected to show least differentiation, especially in large interconnected populations.

8.2.4 Maintenance of genetic diversity between generations and breeding system

The comparisons among cohorts in this study, both between mature trees and regeneration in Bishopston Valley, and among mature trees, natural regeneration, nursery seedlings and seeds from Betws-y-Coed, show no change in genetic diversity from one generation to the next. The parentage analysis for the half-sib arrays of seeds also indicated high rates of outcrossing and large numbers of pollen donors for each seed tree.

Studies of breeding systems in forest trees have largely focussed on coniferous species. High outcrossing rates have been observed in a range of species, including for example *Pinus breweriana* ($t_m \leq 0.9$) (Ledig *et al.*, 2005), *Picea abies* ($t_m = 0.94$) (Burczyk *et al.*, 2004), *Pinus strobus* ($t_m = 0.92$) (Rajora *et al.*, 2002) and *Pinus sylvestris* ($t_m = 0.99$) (Burczyk, 1998). However, significant variation has also been observed, especially in disturbed or fragmented populations. For example, populations of *Larix decidua* and *Pinus cembra* close to the species' altitudinal limits in the Italian Alps had relatively low outcrossing rates of 0.68 and 0.81 (Lewandowski and Burczyk, 2000). Similarly, the severely fragmented remnant populations of *Picea martinezii* in Mexico had outcrossing rates between 0.40 and 0.69 (Ledig *et al.*, 2000).

A relatively small number of studies considering temperate broadleaved species have been carried out. In *Fagus sylvatica*, which is monoecious and wind-pollinated, high

outcrossing (t_m = 0.94 - 0.98) was found in natural Italian populations, despite the self-compatibility of this species (Rossi *et al.*, 1996). These results were similar to those found by Merzeau *et al* (1994) in French populations (t_m = 0.94 – 1.02). Similarly, selfing rates were found to be close to zero in *Quercus robur* and *Q. petraea* in a French forest (Bacilieri *et al.*, 1996) and in *Quercus rubra* in Pennsylvania (Schwarzmann and Gerhold, 1991).

Within the genus *Fraxinus* there is a wide range of breeding systems including polygamy, hermaphroditism, dioecy and androdioecy (Wallander, 2001). A number of studies have investigated the degree of outcrossing in several species. The breeding system of *Fraxinus lanuginosa* was studied in Japan (Ishida and Hiura, 2002). *Fraxinus lanuginose* is functionally androdioecious, as hermaphrodite individuals are capable of selfing. However, pollen from male trees has a higher fecundity and when high proportions of males were present outcrossing was found to be higher. Another species, *Fraxinus mandshurica*, is dioecious, with male and female individuals. In controlled crosses of *Fraxinus excelsior* selfing was found to be quite uncommon (Morand-Prieur *et al.*, 2003). In a detailed study of flowering, it was concluded that hermaphrodite flowers of *Fraxinus excelsior* could be self-compatible but are protogynous, with the female function occurring before pollen was fertile (Wallander, 2001). A study including mast and non-mast years found a slight increase in hermaphrodite fathering success and selfing in non-mast years (FRAXIGEN, 2005). The high levels of outcrossing recorded in this study are in agreement with these findings.

It has generally been found that inbreeding depression may be deleterious in forest trees (e.g. Sorensen, 1999), and so self-compatible species have evolved mechanisms to reduce self-fertilization (Rossi *et al.*, 1996). These mechanisms included protogyny (the differential timing of stigma receptivity and pollen dispersal in hermaphrodite flowers) and differential distribution of male and female flowers within the crown of the tree. In cases where selfing does occur, inbreeding depression may cause the selection of outbred individuals. For example, late life-stage individuals of *Fraxinus lanuginosa* were found not to be significantly inbred, in populations where a significant number of seeds were the result of selfing (Ishida and Hiura, 2002).

8.3 Recommendations from this study for the management of *Fraxinus excelsior*

Based on the findings of this study, there is no difference in genetic diversity between natural regeneration and seedlings grown in a nursery. This means that during the creation or replanting of woodlands and forests managers may choose the most convenient approach depending upon the site. The main situation in which genetic diversity is likely to be reduced is when an insufficient number of seed trees has been used. This may occur during natural regeneration, if very few mature trees exist on the site. In a study of clearcut regeneration of *Eucalyptus*, a slight reduction in genetic diversity was found in natural regeneration from four seed trees, compared to regeneration from aerial sowing (Glaubitz *et al.*, 2003a). Guidelines on seed collection, similarly, suggest collecting at least 500 seeds from ten trees, separated by at least 150 m, in order to conserve 95% of alleles with a frequency > 0.05, and from twenty trees to conserve 95% of alleles with a frequency > 0.025 (FRAXIGEN, 2005).

Due to the high levels of gene flow estimated in this study, as well as other studies of *Fraxinus excelsior*, it seems likely that in this species fragmentation and isolation of populations is unlikely to result in massive erosion of genetic diversity. Allelic richness and heterozygosity recorded in the present study are similar to those found in a study of fragmented populations in Scotland (Bacles *et al.*, 2005) and neither of these British studies have very different results from those for French populations (Morand *et al.*, 2002). France is characterised by much more extensive forests than Britain, where there is a long history of agriculturalisation and urbanisation, due to a more dense human population, causing deforestation and isolation of remaining woodlands.

8.4 Directions for future studies

In order to increase knowledge of the genetic diversity of *Fraxinus excelsior* it would be desirable to obtain a greater array of microsatellite loci. Whether the same trends, especially the excess homozygosity, remained when similar studies were carried out with 20 or more loci would provide evidence for or against the presence of null alleles at the loci used here. It would also be possible to investigate the present loci to find out what was causing the patterns observed. By sequencing amplified fragments and redesigning primers, it might be possible to amplify putative null alleles. A large controlled crossing experiment could also provide insights into the inheritance of

alleles. Another option would be to test the possibility that FEMSATL16 might actually be linked to a gene under selection, and therefore have potential as a marker for a quantitative trait locus (QTL). The use of QTLs in studies of environmental adaptation, provenance, and marker-assisted selection is an area of growth, and although somewhat beyond the remit of the current study, breeding superior genotypes may be of interest when *Fraxinus excelsior* is grown for timber.

Most of the findings of this study support earlier work and it may be that few advances remain to be made using the current microsatellite loci. Although areas have been outlined where increased or improved sampling might, retrospectively, have been better, it is clear that many studies have been published on *Fraxinus excelsior* based on the same microsatellite loci. For future studies, a fresh approach, such as integrating genetic techniques with environmental, phenotypical and silivicultural approaches, will be necessary.

8.5 Conclusions

This study found, in common with other studies of *Fraxinus excelsior*, a high degree of genetic diversity and low levels of among-population differentiation, revealed by six microsatellite loci. These findings are in agreement with general findings for long-lived woody species (Hamrick and Godt, 1996).

A less predictable finding was the excess of homozygotes at most loci in all populations. This may be due to the nature of the markers used, in terms of the presence of null alleles. However, the degree of small-scale spatial structure at FEMSATL16, the locus with the greatest excess of homozygotes, suggests that factors other than the presence of null alleles might be significant. It is suggested that FEMSATL16 might be linked to a gene under selection on a local scale.

The small-scale genetic structure recorded in one site remains under question, as the effects of the locus FEMSATL16 are significant.

Almost no differences in genetic diversity were found between mature trees, different types of regeneration, and seeds. This unexpected finding will be of interest to silvicultural practitioners, who may have considered the potential differences between

artificial and natural regeneration. According to this study, as long as good practices are followed, either can be equally effective in providing a genetically diverse population.

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Appendix I

Allelic data is listed by population. Sample ID is given, followed by two alleles at each locus, UK National Grid coordinates for relevant populations and diameter at breast height (DBH) for mature trees in Bishopston Valley.

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid		
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing	DBH
Bishopston Valley: mature trees															
1	174	200	194	194	146	170	187	187	234	236	218	278	57400.00	89000.00	27
2	164	174	184	194	188	198	187	187	208	228	188	266	57428.34	88982.97	21
4	174	192	186	198	188	194	185	185	264	272	260	260	57446.88	88960.88	34
6	164	192	184	198	170	188	185	201	194	220	218	238	57452.27	88931.34	30
8	174	182	184	184	180	196	185	187	202	258	216	240	57447.65	88909.59	38
10	166	172	190	202	187	187	185	185	236	264	198	262	57421.63	88872.21	9
12	164	174	192	194	188	211	185	185	220	266	258	268	57404.31	88855.49	33
14	180	192	184	200	175	189	185	185	220	290	218	234	57389.17	88846.39	29
16	164	174	186	194	180	201	177	177	236	258	234	250	57378.97	88837.84	21
18	164	196	198	198	180	188	177	185	198	198	198	242	57366.97	88827.76	16
20	180	184	182	202	146	180	177	201	262	262	198	204	57353.93	88802.58	28
22	162	174	198	206	180	197	183	185	208	254	198	216	57360.57	88784.36	16
24	174	182	204	204	180	188	177	187	224	260	198	256	57349.60	88769.27	18
26	162	192	202	202	180	188	201	201	224	224	204	242	57338.84	88757.72	8
28	162	176	184	184	155	188	185	187	204	260	206	266	57329.00	88746.40	8
30	164	198	184	210	170	185	187	201	220	220	198	206	57307.25	88748.69	40
32	164	168	184	194	170	180	199	201	260	260	198	234	57299.39	88724.50	17
34	160	170	184	212	171	195	185	187	208	246	202	210	57301.91	88700.51	26
36	160	164	184	184	185	195	185	185	196	234	222	232	57283.22	88688.83	20
38	160	162	186	198	186	187	185	185	222	244	212	250	57268.36	88673.44	15
40	170	192	186	198	170	187	179	205	224	262	234	256	57255.27	88648.82	24
42	160	162	186	198	180	188	185	185	188	210	206	254	57236.70	88635.33	19
44	164	188	186	198	187	195	185	185	214	260	212	254	57256.25	88627.43	8
46	174	188	192	192	171	188	177	185	224	260	242	264	57247.80	88601.44	16
48	162	174	192	192	170	180	185	185	248	258	234	254	57240.84	88573.51	15
50	188	188	192	198	170	196	185	185	248	264	198	254	57254.77	88547.30	29
52	172	186	192	194	193	197	185	201	240	256	202	212	57271.64	88533.15	30
54	164	186	184	184	180	197	195	197	272	272	214	216	57292.16	88511.90	11
56	180	196	186	194	184	188	185	187	236	262	232	238	57308.31	88499.29	17
58	164	164	192	192	180	188	187	201	260	260	218	258	57331.03	88481.53	25
60	168	220	184	192	186	194	185	185	240	270	202	202	57342.09	88460.72	12
62	190	236	218	218	187	187	177	185	226	260	202	252	57384.97	88406.01	26
64	182	200	192	204	188	197	187	187	220	258	220	240	57390.57	88389.76	33
66	192	196	194	204	187	197	185	185	246	246	198	220	57424.91	88344.94	48
68	160	174	192	198	188	188	185	185	256	256	206	264	57319.22	88306.88	41
70	162	166	184	198	188	188	201	201	222	260	254	264	57282.71	88289.08	17
72	180	196	184	184	180	185	185	199	272	272	198	212	57247.76	88257.79	60
74	158	162	0	0	188	196	185	185	204	250	214	232	57202.35	88268.14	23

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid		
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing	DBH
Bishopston Valley: mature trees															
76	174	192	192	204	193	196	183	189	202	224	222	254	57168.76	88270.41	24
78	166	166	186	202	186	188	183	183	268	268	196	204	57129.58	88261.73	15
80	164	174	192	204	170	188	185	185	224	268	198	266	57103.27	88216.73	20
82	164	164	192	192	187	207	185	201	226	266	188	220	57087.28	88193.03	24
84	164	186	184	186	180	185	185	187	262	272	198	210	57063.14	88172.01	28
86	182	226	194	194	146	196	199	199	204	260	194	258	57044.69	88157.60	24
88	164	164	192	204	187	187	185	185	270	270	202	202	57011.35	88126.71	30
90	160	164	194	204	180	188	185	185	208	224	206	238	56974.40	88124.69	29
92	164	166	192	198	168	186	185	185	256	272	202	268	56939.86	88115.45	16
94	166	168	184	192	178	216	187	187	276	276	200	234	56923.92	88094.11	28
96	166	190	204	204	188	188	185	185	218	258	202	240	56890.80	88079.47	14
98	164	168	192	200	180	188	177	187	190	220	202	264	56865.83	88071.55	8
100	162	164	198	200	188	188	177	177	256	256	202	238	56842.35	88067.41	17
102	166	182	184	202	146	180	187	187	258	264	218	262	56800.68	88075.09	47
104	166	230	192	192	185	187	185	185	218	218	198	198	56764.63	88080.57	28
106	166	170	184	184	187	195	187	187	262	296	198	212	56704.65	88056.36	29
108	162	164	196	206	187	187	183	185	188	236	246	258	56671.18	88029.40	60
110	164	174	192	192	190	192	185	185	260	272	230	242	56650.85	88003.87	56
112	164	164	184	198	187	187	185	199	212	248	232	232	56623.41	87955.58	26
114	164	192	204	204	187	187	177	185	234	256	220	220	56604.39	87900.40	10
116	164	192	192	216	168	184	185	185	240	252	194	244	56636.94	87901.78	20
118	164	198	188	192	165	185	185	201	220	220	198	220	56665.51	87854.39	31
120	164	164	204	206	172	187	185	185	262	262	220	240	56680.30	87841.72	60
122	180	180	204	204	192	206	185	185	224	256	198	242	56718.61	87826.76	20
124	170	180	186	204	194	194	183	203	220	254	198	242	56740.82	87832.71	13
126	170	178	184	186	188	196	185	185	250	254	198	242	56761.07	87821.02	17
128	174	190	192	192	180	194	185	185	242	242	198	202	56798.65	87836.19	47
130	164	170	184	186	196	196	183	183	254	288	224	224	56806.98	87857.88	55
132	164	196	206	206	188	188	199	199	254	272	202	228	56833.32	87852.76	53
134	170	182	192	192	187	187	183	183	260	296	202	244	56875.91	87817.96	39
136	174	174	192	192	169	196	183	183	202	260	218	256	56889.54	87796.15	27
138	194	196	184	192	187	196	175	183	220	256	202	254	56942.80	87791.17	14
140	166	174	184	192	196	197	183	183	224	224	196	240	56980.40	87768.68	10
142	168	178	184	196	193	195	185	185	202	254	196	238	57013.17	87744.04	60
144	166	172	192	194	180	211	197	203	206	246	204	256	57048.82	87715.99	30
146	172	194	186	198	178	191	183	183	248	260	212	220	57094.57	87686.19	40
148	170	176	202	202	185	196	183	199	244	256	196	260	57137.48	87636.22	
150	168	172	186	214	180	196	175	175	202	254	196	220	57152.64	87611.96	24

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid		
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing	DBH
Bishopston Valley: mature trees															
151	172	184	186	200	177	196	183	201	224	266	196	206	57050.38	87716.89	28
153	170	218	204	204	191	207	183	183	216	284	204	212	57105.66	87678.14	20
155	174	192	184	202	177	185	183	183	228	228	220	232	57134.54	87628.14	18
157	174	196	188	192	188	191	183	183	258	268	212	252	57138.15	87552.42	10
159	174	192	186	204	194	194	183	183	198	198	234	256	57148.94	87542.71	45
161	174	174	206	206	180	191	185	185	224	262	202	202	57160.69	87518.23	44
162	170	182	206	206	187	191	183	183	244	262	196	202	57211.24	87436.40	31
164	174	174	200	200	193	195	199	199	178	178	198	234	57218.96	87411.17	22
166	162	162	184	184	186	191	175	183	198	242	204	210	57225.33	87383.57	11
168	188	190	186	186	182	188	187	199	250	258	234	266	57224.68	87365.08	52
170	164	188	184	192	183	183	177	185	250	292	202	234	57222.06	87339.26	32
172	162	172	198	198	0	0	177	177	206	258	212	230	57239.60	87323.47	8
173	172	172	184	202	168	187	185	199	208	258	196	216	57261.46	87316.67	8
Bishopston Valley: natural regeneration															
3	158	164	194	194	170	188	185	187	212	252	198	234	57263.73	87294.98	
5	174	190	182	192	185	187	183	183	218	262	0	0	57260.75	87285.24	
7	174	174	182	184	188	197	185	201	240	262	202	230	57259.12	87275.98	
9	174	174	184	184	170	187	177	187	230	260	218	234	57423.14	88980.65	
11	164	174	184	202	180	187	177	185	246	246	206	206	57445.86	88957.32	
13	164	174	184	184	187	196	185	185	242	268	226	234	57451.55	88932.60	
15	168	192	194	202	184	188	185	185	230	248	198	262	57443.07	88907.36	
17	174	182	194	194	175	180	185	187	262	280	218	222	57421.14	88871.78	
19	164	164	186	186	146	188	185	201	262	270	212	244	57403.22	88856.80	
21	170	232	190	190	146	196	185	185	208	262	220	264	57385.85	88846.86	
23	174	174	214	214	188	196	187	187	220	220	212	240	57378.48	88840.12	
25	164	168	186	198	188	188	201	201	266	266	256	256	57361.61	88823.58	
27	164	200	206	206	188	188	177	185	222	222	220	238	57355.83	88796.36	
29	182	200	184	206	188	195	201	201	256	256	198	228	57365.21	88777.97	
31	160	164	184	184	180	195	185	185	200	200	198	202	57349.53	88770.36	
33	160	164	194	204	180	197	185	185	256	286	206	206	57336.06	88758.85	
35	164	182	214	214	187	187	185	185	244	256	210	218	57331.94	88748.10	
37	164	196	206	206	180	188	183	183	296	296	198	274	57308.69	88750.08	
39	174	182	204	204	146	191	185	185	260	260	204	230	57300.06	88723.90	
41	164	170	204	204	170	187	201	201	224	224	220	256	57301.66	88702.90	
43	190	190	204	204	185	193	177	185	234	246	234	256	57282.07	88687.87	
45	174	190	184	184	185	188	177	187	196	244	198	224	57274.50	88669.89	
47	164	188	182	192	170	180	177	185	246	258	234	242	57256.76	88651.88	
49	160	174	184	192	188	188	177	187	258	258	220	242	57240.17	88637.50	

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid	
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing
Bishopston Valley: natural regeneration														
51	164	174	184	188	180	185	179	179	240	270	198	208	57258.21	88628.43
53	162	180	194	206	170	197	179	187	208	208	202	202	57247.88	88599.34
55	164	190	186	198	185	196	179	187	224	224	220	246	57238.69	88579.11
57	168	172	188	198	170	196	185	185	260	280	202	256	57253.24	88545.87
59	160	182	186	204	194	195	187	201	224	244	198	244	57269.56	88531.28
61	168	170	184	196	170	188	185	185	224	258	218	264	57288.46	88511.84
63	164	170	184	204	170	170	185	201	260	272	264	264	57304.27	88498.13
65	186	196	204	204	180	188	189	189	208	254	216	220	57328.94	88484.41
67	162	174	192	210	186	193	183	199	190	190	196	218	57338.75	88464.71
69	160	164	192	198	188	188	185	201	222	262	204	236	57372.25	88413.25
71	174	188	182	194	168	188	183	183	208	240	0	0	57389.08	88389.97
73	174	192	192	198	188	196	187	187	222	258	240	258	57419.36	88335.33
75	164	170	204	210	188	188	185	185	214	224	198	240	57323.05	88314.07
77	164	164	194	206	169	194	183	183	224	262	196	232	57281.06	88290.68
79	164	166	192	204	188	196	201	201	220	268	180	198	57243.66	88254.92
81	174	182	194	194	170	196	185	185	202	256	232	232	57199.43	88266.39
83	164	164	194	194	183	195	185	185	224	266	198	232	57168.16	88266.16
85	174	180	196	198	188	196	183	183	222	248	196	260	57129.82	88259.45
87	164	174	186	196	187	196	185	185	224	224	226	238	57104.96	88215.49
89	164	164	200	204	170	188	185	185	252	270	198	278	57085.13	88195.26
91	164	164	186	192	187	190	187	187	224	266	180	264	57067.98	88174.81
93	164	174	198	198	168	186	185	185	206	254	198	202	57044.88	88163.10
95	164	172	198	204	169	186	201	201	250	250	216	240	57006.67	88130.93
97	168	178	186	198	191	195	185	185	250	264	256	264	56976.83	88127.48
99	178	186	198	200	188	192	185	185	224	256	218	218	56941.65	88118.10
101	162	194	192	200	184	186	185	185	220	300	232	232	56920.92	88091.77
103	164	194	184	194	186	193	177	177	220	220	238	258	56890.95	88083.66
105	164	174	186	204	185	191	177	177	222	236	244	266	56865.00	88074.43
107	164	188	192	206	190	194	201	201	178	178	198	198	56839.45	88067.41
109	164	164	204	204	187	187	185	187	170	204	198	268	56762.77	88097.40
111	168	196	192	204	192	192	177	177	220	220	196	196	56758.54	88080.99
113	164	164	184	198	187	187	185	185	212	248	232	232	56701.75	88054.40
115	174	192	192	204	146	187	177	177	260	268	210	210	56667.36	88028.59
117	164	196	184	192	146	185	183	183	256	256	198	216	56652.67	88009.48
119	164	164	192	216	187	193	185	201	244	276	204	242	56625.79	87956.35
121	164	190	186	206	0	0	185	201	296	296	194	194	56607.31	87910.59
123	164	174	186	192	187	196	185	185	290	290	206	262	56637.83	87903.79
125	164	194	186	204	179	179	183	199	220	228	0	0	56665.51	87854.39

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid	
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing
Bishopston Valley: natural regeneration														
127	166	182	190	204	197	197	185	185	224	224	212	212	56678.76	87848.96
129	164	190	198	204	170	196	177	185	248	260	196	202	56725.64	87829.89
131	170	182	186	186	168	197	183	183	292	292	224	274	56743.48	87830.14
133	174	188	192	192	188	193	183	183	252	252	196	240	56764.54	87824.04
135	166	166	192	206	148	188	199	199	254	254	256	256	56799.69	87835.25
137	198	198	192	202	168	188	175	183	224	224	218	218	56809.57	87854.57
139	166	174	192	204	188	193	183	183	224	234	228	228	56832.94	87858.25
141	178	206	202	202	182	193	197	203	202	262	246	266	56870.72	87826.27
143	162	172	188	192	185	187	175	183	192	254	196	202	56893.50	87795.59
145	180	198	184	196	184	194	183	185	202	250	196	204	56947.05	87783.81
147	162	190	192	192	180	180	183	199	244	262	196	242	57001.79	87749.34
149	164	190	184	192	185	196	177	183	198	254	0	0	57022.11	87746.94
152	174	190	184	198	187	187	199	201	224	264	194	210	57142.23	87567.68
154	164	174	186	204	185	187	175	183	216	252	204	206	57149.92	87542.53
156	164	192	186	198	178	187	177	183	224	224	218	220	57160.05	87517.47
158	186	192	184	194	188	191	183	183	228	250	216	264	57175.95	87484.44
160	172	188	208	208	187	193	183	183	250	250	196	210	57201.23	87452.43
163	166	170	184	206	187	196	183	199	244	244	196	224	57174.08	87479.80
165	164	170	198	198	146	146	175	185	0	0	194	194	57198.47	87451.94
167	164	174	196	204	186	193	187	187	200	200	196	196	57226.14	87407.02
169	162	188	186	192	183	193	185	185	222	260	196	204	57219.43	87378.62
171	160	194	198	202	168	186	185	185	256	268	202	228	57227.11	87363.32
174	178	194	204	204	168	184	185	185	224	256	196	202	57220.74	87337.14
Oxwich Wood														
200	166	230	186	188	188	192	183	183	250	258	214	240	250443.22	186050.68
201	164	172	186	186	146	188	185	201	214	254	254	254	250466.19	186041.01
202	164	188	184	204	146	180	183	199	210	218	234	258	250448.14	186049.81
203	164	174	188	204	194	230	185	203	204	222	210	222	250431.99	186030.56
204	164	166	192	198	188	188	185	201	266	274	196	206	250436.94	186016.18
205	196	216	190	198	180	188	187	187	254	296	198	218	250433.17	186002.11
206	166	222	204	206	190	198	185	185	254	262	198	224	250428.11	185996.08
207	164	194	192	198	188	188	185	185	222	266	198	220	250413.79	185958.27
208	164	164	184	206	180	188	185	201	174	224	198	206	250435.42	185949.09
209	180	190	184	194	168	196	185	201	242	252	198	238	250448.08	185936.88
210	180	182	184	184	188	192	185	187	206	232	216	226	250453.51	185932.31
211	174	188	184	204	180	196	185	185	224	254	196	196	250474.65	185910.43
212	168	180	194	198	188	194	187	201	232	248	198	216	250502.95	185899.90
213	174	174	204	204	194	198	201	201	204	204	196	220	250496.33	185885.05

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid	
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing
Oxwich Wood														
214	164	174	200	204	178	186	185	201	202	202	206	218	250526.11	185862.71
215	164	174	204	204	180	180	185	201	260	260	198	238	250576.05	185811.34
216	164	176	192	192	180	196	177	201	224	258	212	240	250542.97	185840.35
217	196	202	184	190	188	198	185	185	240	256	196	226	250555.56	185825.34
218	174	202	190	194	188	194	185	185	242	242	238	238	250564.99	185813.69
219	180	184	186	194	194	198	185	185	242	242	230	238	250592.73	185813.69
220	172	176	190	194	194	198	185	201	218	262	180	198	250610.76	185807.48
221	174	174	192	204	180	180	177	185	244	244	218	236	250606.09	185795.30
222	174	176	192	194	186	188	185	187	218	238	198	272	250621.02	185793.73
223	164	216	212	212	196	206	177	185	230	230	212	222	250613.20	185787.62
224	164	166	192	198	180	196	185	185	268	282	206	206	250613.45	185772.88
225	198	198	200	212	186	194	183	183	202	264	212	238	250601.24	185760.23
226	164	174	184	184	178	186	183	183	252	252	196	210	250604.19	185747.42
227	174	230	192	194	188	194	187	187	218	218	232	232	250600.89	185737.27
228	174	196	194	198	180	186	185	185	208	260	198	258	250613.28	185729.53
229	164	196	188	194	178	194	185	185	224	260	198	262	250622.65	185707.59
Nicholaston Woods														
301	166	174	194	194	182	184	177	177	208	254	238	238	252062.00	188175.00
302	174	174	192	192	170	188	185	201	178	252	226	230	252038.92	188163.24
303	166	174	194	194	170	170	185	201	272	272	226	228	252029.69	188152.99
304	162	162	200	206	170	170	187	187	224	240	206	206	252019.19	188161.19
305	174	180	184	192	170	170	177	187	224	224	0	0	252004.82	188161.19
306	166	222	188	188	182	190	187	187	268	268	206	206	251992.39	188149.95
307	164	222	190	206	170	170	185	187	224	264	214	226	251981.36	188144.33
308	170	174	190	194	170	170	177	177	262	262	198	214	251975.50	188153.71
309	186	224	184	190	170	170	183	199	258	286	196	230	251949.34	188148.63
310	164	174	190	190	170	170	177	201	262	282	198	216	251926.31	188148.63
311	164	220	192	192	170	170	177	185	244	244	196	234	251934.11	188129.32
312	164	174	206	206	170	170	185	185	262	262	226	230	251926.02	188123.00
313	196	208	184	192	170	170	185	187	212	222	240	240	251906.06	188106.24
314	164	180	186	206	170	170	201	201	208	264	202	268	251899.13	188099.31
315	164	202	188	188	170	170	177	185	258	258	198	226	251905.86	188086.67
316	182	196	184	202	170	170	185	187	220	264	214	220	251903.48	188075.48
317	180	196	184	190	148	188	177	177	254	254	238	252	251892.45	188064.06
318	164	164	192	192	170	188	177	185	218	224	198	202	251899.65	188056.60
319	164	220	192	192	188	194	177	199	246	264	198	198	251901.88	188043.99
320	164	240	192	206	180	188	195	211	262	272	214	214	251921.91	188040.46
321	164	164	184	186	188	188	185	201	218	244	238	238	251928.12	188022.43

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid	
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing
Nicholaston Woods														
322	164	222	184	192	170	198	177	185	234	256	266	266	251914.29	188008.59
323	164	182	204	204	180	186	185	201	238	238	212	216	251922.11	187998.21
324	164	198	184	204	188	198	187	187	244	262	228	242	251938.08	187997.10
325	164	164	184	192	180	186	185	201	222	266	232	236	251949.40	187994.69
326	160	180	184	206	178	188	177	185	206	224	210	216	251954.08	187959.20
327	174	198	184	184	180	198	187	187	222	266	182	182	251950.47	187948.09
328	166	196	192	192	188	194	177	185	300	300	198	198	251951.28	187936.47
329	164	164	184	206	194	196	177	185	224	262	198	220	251946.81	187934.10
330	174	174	192	192	180	185	177	201	224	246	220	232	251941.11	187932.25
Alyn Valley														
401	166	182	186	192	196	198	183	187	242	242	212	262	319796.55	362710.65
402	164	174	186	200	146	196	183	183	284	302	200	224	319782.92	362731.49
403	164	166	186	186	180	198	185	201	224	266	212	248	319778.49	362754.17
404	172	174	192	204	169	174	187	187	208	282	194	246	319777.70	362779.66
405	174	174	192	204	190	196	185	185	236	266	202	268	319754.75	362810.32
406	174	178	192	192	188	208	177	201	266	266	218	232	319701.75	362818.20
407	166	166	188	194	196	207	187	195	222	278	210	220	319693.00	362832.60
408	190	192	186	206	188	188	185	201	220	256	210	238	319631.82	362834.16
409	164	182	184	184	180	180	177	177	212	212	220	220	319616.73	362835.50
410	168	172	194	194	187	217	185	201	238	252	202	212	319597.58	362864.16
411	164	180	184	184	170	180	177	177	222	222	198	228	319603.67	362875.16
412	162	182	192	192	146	188	185	185	218	226	220	220	319603.28	362897.16
413	166	180	186	192	146	146	201	201	280	280	202	238	319587.30	362912.14
414	194	194	184	192	146	190	177	185	246	270	196	216	319557.89	362929.50
415	166	196	192	200	186	195	185	201	254	278	198	218	319550.17	362928.76
416	174	180	186	206	188	198	185	187	258	258	212	252	319529.08	362935.67
417	180	228	188	194	182	186	177	201	252	252	212	262	319499.16	362939.45
418	164	174	184	204	190	196	185	185	224	266	194	244	319481.36	362936.48
419	164	222	186	186	196	198	187	187	224	264	202	218	319453.02	362959.69
420	164	164	198	206	170	170	203	203	268	278	214	228	319427.23	362971.40
421	164	166	206	206	170	180	185	185	224	224	0	0	319414.23	362980.40
422	162	164	186	190	180	191	185	185	218	264	202	202	319410.59	362981.79
423	164	200	204	204	190	190	185	201	262	262	252	252	319364.50	363046.48
424	162	180	184	204	0	0	177	185	244	244	240	240	319358.61	363054.32
425	182	196	186	186	188	191	177	185	0	0	242	268	319356.21	363070.50
426	164	166	190	194	174	196	185	185	246	262	202	206	319352.48	363089.71
427	164	164	204	204	170	188	185	185	184	258	212	242	319355.18	363100.27
428	164	174	184	204	178	190	185	185	210	256	196	204	319346.93	363119.55

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30		UK National Grid	
	A	B	A	B	A	B	A	B	A	B	A	B	Easting	Northing
Alyn Valley														
429	164	218	184	204	188	190	185	201	234	288	264	264	319339.14	363140.89
430	160	164	186	190	180	188	177	185	234	288	232	262	319342.51	363135.99
431	164	174	184	194	188	194	187	201	224	224	216	286	319355.95	363144.36
432	164	174	200	200	188	209	201	201	290	300	204	254	319370.82	363156.44
Elwy Valley														
501	164	174	188	204	197	197	185	201	260	294	208	234	301900.00	368500.00
502	174	212	184	184	187	187	177	185	226	264	214	242	301897.36	368521.54
503	160	170	184	184	179	187	185	201	222	222	232	232	301902.73	368536.29
504	174	176	188	204	180	194	201	201	196	226	236	266	301915.06	368541.78
505	164	242	184	204	180	189	185	185	214	214	204	266	301919.93	368565.95
506	198	198	192	192	185	185	201	201	228	228	238	238	301938.72	368575.11
507	172	236	192	192	186	217	185	201	208	226	212	244	301958.22	368571.67
508	164	170	188	192	188	197	185	185	214	238	242	258	301956.62	368580.73
509	164	174	184	184	187	187	183	183	226	226	196	196	301965.01	368585.19
510	162	236	192	202	185	192	185	185	226	226	228	254	301966.18	368618.67
511	172	172	0	0	173	179	185	185	224	224	258	268	301969.55	368627.01
512	166	196	184	184	186	193	177	177	232	242	198	212	301976.88	368635.75
513	164	198	184	184	187	192	187	199	240	240	0	0	301960.49	368655.28
514	166	198	184	192	169	186	177	177	226	276	214	254	301964.81	368670.37
515	186	240	192	204	169	184	201	201	202	202	210	262	301961.92	368690.97
516	164	174	188	192	187	190	185	185	224	224	218	258	301963.84	368709.27
517	166	166	184	194	179	179	177	201	0	0	210	226	301982.29	368703.27
518	166	174	184	204	170	188	183	183	224	270	204	226	302007.16	368720.05
519	164	174	184	192	194	217	177	185	224	248	198	200	302014.77	368734.35
520	164	196	184	190	188	217	187	187	258	266	202	252	302015.99	368751.81
521	166	198	184	200	180	191	185	185	240	256	210	238	302025.84	368776.20
522	164	174	184	184	187	197	185	185	260	294	268	268	302052.71	368809.38
523	166	170	184	198	188	188	177	201	226	266	0	0	302069.94	368844.70
524	160	174	192	206	188	196	185	185	224	260	210	230	302073.30	368858.19
525	164	190	184	184	170	188	177	183	0	0	196	228	302081.57	368882.21
526	162	166	184	192	0	0	185	185	202	270	188	238	302102.24	368908.68
527	164	178	184	186	0	0	177	185	224	224	206	216	302095.14	368974.45
528	174	216	190	192	173	190	201	201	176	292	198	228	302097.09	368982.93
529	166	166	190	190	188	193	185	187	200	266	220	262	302089.18	368991.41
530	162	164	192	192	184	190	185	185	206	264	196	210	302092.16	369012.61
531	166	174	186	192	188	195	185	185	266	288	242	242	302096.15	369037.79

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: natural regeneration												
601	164	164	186	192	168	192	185	185	258	276	202	204
602	164	174	204	204	184	188	185	185	204	226	204	212
603	174	176	184	186	186	195	185	185	202	226	202	236
604	164	192	190	194	169	184	185	185	228	228	202	242
605	162	174	184	184	146	207	185	185	258	268	226	260
606	160	166	188	194	146	193	185	187	244	278	198	238
607	164	182	186	188	168	173	185	195	206	262	210	214
608	164	170	184	192	178	180	177	177	216	268	238	260
609	174	192	190	194	188	208	185	199	270	278	212	218
610	160	162	192	192	146	191	185	187	260	270	218	256
611	164	164	186	192	146	188	177	185	206	258	198	214
612	164	182	186	186	188	197	185	185	218	278	198	212
613	164	174	184	194	188	188	183	199	204	224	202	266
614	174	174	184	184	172	187	201	201	246	258	210	214
615	164	182	184	186	186	186	185	185	224	224	204	242
616	174	198	184	192	188	188	185	199	204	244	220	240
617	174	198	184	184	0	0	183	183	222	264	236	236
618	164	222	204	204	172	186	183	183	226	262	0	0
619	182	196	186	194	195	195	177	185	226	260	212	218
620	160	174	186	194	179	179	185	185	260	274	198	202
621	164	196	188	188	186	229	185	201	246	252	202	252
622	164	174	192	194	180	191	185	201	222	242	202	256
623	182	190	186	194	180	191	185	185	240	264	220	228
624	164	174	184	194	187	191	185	199	228	228	220	238
625	174	190	194	198	170	188	185	185	196	196	198	216
626	174	180	186	190	193	193	185	185	214	214	238	264
627	160	164	192	194	188	229	201	201	226	242	202	268
628	160	164	192	192	188	191	185	185	222	222	202	218
629	164	168	184	188	185	187	177	201	258	272	198	220
631	164	196	184	192	180	188	185	185	276	290	240	240
632	174	196	190	204	194	208	185	185	224	242	212	240
Betws-y-Coed: nursery seedlings												
701	164	166	184	206	186	186	187	201	244	274	0	0
702	170	196	202	202	180	188	185	185	258	258	228	230
703	174	182	204	204	178	190	187	187	222	222	184	280
704	164	174	204	204	146	180	185	201	174	224	202	220
705	164	174	184	206	188	191	185	185	184	262	198	216
706	164	188	194	204	188	198	185	185	216	262	206	206

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: nursery seedlings												
707	166	174	202	206	184	191	201	201	228	254	198	246
708	166	222	192	204	178	188	185	185	260	268	204	282
709	162	174	184	206	188	188	199	199	208	260	196	202
710	170	182	186	186	180	184	185	185	264	264	200	218
711	164	184	204	204	188	188	177	185	198	226	196	238
712	174	188	192	204	148	191	185	185	216	220	252	252
713	174	242	184	190	188	206	185	201	212	270	250	262
714	166	196	198	206	180	184	177	177	224	226	204	216
715	174	196	204	204	182	193	185	185	174	244	202	272
716	174	174	192	202	180	180	177	177	252	252	204	248
717	164	164	184	192	180	192	185	199	196	224	198	218
718	164	176	184	192	146	185	185	201	222	224	198	206
719	166	170	194	204	146	180	185	199	174	192	204	206
720	164	174	186	186	191	191	185	185	224	224	198	202
721	166	174	186	202	178	188	201	201	216	260	206	250
722	178	180	188	202	188	190	201	201	266	270	198	250
723	164	174	184	184	191	191	199	199	224	224	202	216
724	166	190	184	206	184	194	201	201	224	224	218	232
725	164	174	200	206	188	193	185	185	224	260	218	264
726	164	180	202	204	184	188	185	185	242	242	208	232
727	162	196	192	206	187	206	185	185	226	258	238	260
728	164	164	184	186	194	194	183	183	222	260	204	264
729	166	170	192	202	180	184	201	201	178	244	198	258
730	174	174	184	184	170	186	185	187	218	218	198	266
731	182	222	184	184	186	188	177	185	174	186	220	232
732	166	170	186	188	178	186	185	199	206	226	202	232
733	166	174	194	194	184	188	185	199	250	258	212	232
734	166	180	204	206	184	188	185	185	208	244	198	266
735	166	190	192	204	187	191	201	201	208	252	218	258
Betws-y-coed: mature trees												
801	170	190	186	194	146	185	177	177	204	204	0	0
802	172	226	188	190	196	196	183	199	256	260	212	260
803	164	164	186	192	146	180	177	201	204	206	198	204
804	164	170	198	198	170	180	177	177	256	284	214	256
805	164	164	186	186	146	184	177	185	204	258	204	240
806	176	190	190	204	178	184	185	185	238	260	198	218
807	160	160	186	190	146	188	185	185	268	282	0	0
808	164	166	186	186	180	180	177	185	194	268	214	238

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-coed: mature trees												
809	164	222	188	194	186	188	185	185	272	294	214	242
810	160	164	186	194	180	180	185	201	224	224	204	246
811	174	176	184	204	184	193	185	185	202	224	198	202
812	170	182	184	192	180	207	177	185	270	276	214	266
813	164	188	186	186	174	190	185	185	256	262	220	220
814	164	164	186	186	188	188	185	185	258	280	198	212
815	164	190	194	194	186	186	185	201	224	260	212	218
816	164	164	184	186	188	188	183	183	224	256	204	256
817	164	192	186	202	188	191	185	185	208	250	212	248
818	172	182	186	186	191	197	185	185	218	266	198	250
819	164	174	184	192	186	195	185	185	182	208	202	248
820	174	180	184	184	180	188	197	199	210	256	202	238
821	164	164	192	198	191	193	185	187	230	272	202	256
822	196	202	184	186	180	188	201	201	228	228	198	240
823	164	166	186	206	180	182	177	187	174	174	198	198
824	160	164	188	190	146	188	177	177	274	286	218	242
826	164	174	186	192	194	196	177	177	246	266	216	226
827	164	164	194	198	178	196	185	201	206	224	202	202
828	182	192	184	194	0	0	185	185	258	288	202	214
829	164	192	184	204	178	186	185	185	208	288	238	256
830	174	180	190	200	180	180	177	201	258	258	198	220
831	190	202	194	194	187	187	185	185	226	258	218	240
832	164	180	186	190	187	194	185	185	188	188	202	220
Betws-y-Coed: seeds												
901A	164	164	194	194	189	189	207	207	174	248	208	214
901B	166	178	186	194	189	199	199	199	174	174	206	268
901C	166	180	164	164	170	180	185	199	174	210	206	220
901D	162	166	188	194	180	190	185	199	262	262	214	234
901E	164	164	184	184	188	188	199	199	174	262	206	212
901F	166	168	194	194	170	189	199	201	174	238	206	230
901G	164	196	194	202	189	189	177	177	174	174	198	214
901H	166	192	184	206	146	189	185	185	174	236	206	240
901I	166	170	184	202	146	180	185	199	174	238	208	214
901J	164	166	194	194	180	189	185	199	224	250	206	238
901K	164	196	194	204	180	192	199	199	208	258	204	206
901L	166	166	184	184	180	188	199	199	174	206	198	206
901M	164	206	194	204	180	180	177	177	174	266	214	220
901N	164	168	184	204	189	189	185	199	192	280	208	214

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: seeds												
901C	166	174	194	202	189	196	185	185	174	224	194	214
901P	166	246	184	194	180	188	185	199	224	258	204	206
901Q	178	200	192	194	205	205	179	201	256	290	206	188
901R	164	164	192	194	186	189	199	199	174	212	198	206
901S	164	164	184	204	180	188	177	177	174	224	206	268
901T	164	192	184	206	170	180	185	199	174	238	208	214
903A	164	180	188	202	184	211	201	201	254	254	214	234
903B	174	180	192	202	188	192	177	201	224	252	220	242
903C	164	180	188	202	174	188	197	197	226	252	222	234
903D	166	192	206	206	188	208	177	201	208	242	206	234
903E	180	196	192	206	186	188	185	185	216	252	234	252
903F	166	170	202	202	188	188	201	201	220	252	198	234
903G	164	166	202	204	184	206	185	185	246	252	200	234
903H	182	198	188	202	184	193	201	201	174	244	234	234
903I	164	180	190	206	184	208	185	187	242	266	198	198
903J	170	180	194	202	188	192	185	201	220	252	198	198
903K	166	166	184	202	184	193	187	201	174	252	198	198
903L	164	180	184	206	184	193	201	201	242	272	198	234
903M	170	180	188	206	184	184	185	201	224	244	198	272
903N	164	180	184	202	184	184	201	201	252	272	198	234
903O	180	182	192	202	188	188	185	185	176	176	198	266
903P	174	180	194	206	184	195	187	201	224	224	198	212
903Q	166	190	186	202	188	188	193	193	224	252	202	208
903R	162	166	184	184	184	188	187	187	210	252	194	234
903S	170	180	206	206	184	192	185	185	220	244	198	198
903T	166	180	206	206	186	188	201	201	244	244	198	234
904A	164	174	184	184	148	192	185	185	260	294	198	198
904B	174	192	184	192	188	188	185	187	274	294	198	240
904C	166	174	186	204	180	189	185	185	226	226	198	204
904D	170	174	184	186	189	192	185	185	212	212	198	198
904E	174	192	184	204	190	192	185	185	212	212	198	266
904F	182	192	184	202	183	191	185	185	262	294	204	238
904G	164	198	186	194	170	192	185	185	212	260	198	216
904H	164	174	184	194	188	208	185	185	262	276	200	204
904I	164	192	184	186	188	208	187	187	226	226	202	214
904J	164	174	186	206	188	188	185	185	262	262	204	216
904K	170	192	184	184	188	188	185	185	262	262	204	216
904L	174	182	184	204	189	198	185	185	262	274	198	214

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: seeds												
904M	170	192	184	204	188	192	185	185	212	212	198	242
904N	166	174	186	208	192	192	185	201	224	262	204	208
904C	164	192	184	184	188	208	201	201	224	260	0	0
904P	174	184	184	184	192	192	189	189	224	262	210	210
904Q	164	174	184	184	192	192	185	185	224	224	204	204
904R	166	174	186	202	182	188	185	185	224	224	198	226
904S	164	174	184	206	188	208	185	199	262	262	198	216
904T	164	174	184	192	189	189	185	185	224	224	0	0
908A	170	222	184	204	192	192	189	189	188	238	224	224
908B	170	222	212	212	188	192	185	185	188	238	202	220
908C	190	222	184	184	192	193	185	185	224	244	220	220
908D	216	222	184	194	184	193	185	185	244	262	220	256
908E	174	222	186	192	189	189	177	177	224	244	214	222
908F	164	190	184	184	192	192	185	185	268	244	204	274
908G	174	196	184	184	188	188	185	185	188	222	204	274
908H	174	196	192	192	192	192	185	187	188	188	220	220
908I	196	196	184	204	188	192	177	177	188	224	222	222
908J	174	222	184	188	192	193	185	185	224	244	216	220
908K	166	166	204	204	176	189	185	185	188	256	220	248
908L	174	222	184	184	189	193	185	187	256	256	220	252
908M	164	196	206	206	193	193	185	185	188	224	252	274
908N	196	222	184	200	188	190	185	185	188	222	204	274
908O	170	222	210	210	188	198	185	187	214	244	224	274
908P	164	222	184	184	188	192	177	177	224	244	196	220
908Q	162	194	192	210	192	192	185	185	188	262	216	220
908R	164	194	210	210	188	192	185	185	188	228	260	274
908S	184	194	184	210	182	188	185	185	188	224	204	222
908T	174	222	196	210	180	188	185	201	188	188	238	274
911A	186	182	190	200	188	190	189	189	228	266	236	244
911B	164	196	200	204	188	188	177	185	254	268	204	242
911C	164	166	200	200	184	192	185	185	232	268	238	242
911D	164	196	186	192	180	188	185	185	234	264	208	242
911E	164	166	184	200	184	187	185	201	206	264	198	242
911F	174	196	200	210	188	188	185	185	264	274	214	222
911G	164	166	184	198	146	187	185	185	220	220	214	248
911H	166	180	186	194	184	194	185	185	194	266	204	242
911I	166	170	186	206	146	188	185	185	264	302	212	212
911J	164	196	186	188	188	190	185	201	268	278	242	268

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: seeds												
911K	166	166	200	200	187	188	185	185	210	268	242	262
911L	170	196	184	200	188	189	177	185	268	268	204	242
911M	164	196	200	200	170	184	185	185	240	264	214	248
911N	164	166	186	204	180	184	185	185	174	174	214	238
911C	164	166	186	194	184	190	185	185	224	264	198	242
911P	164	196	200	200	184	188	185	185	268	268	242	242
911Q	162	196	192	200	188	194	185	201	214	268	214	262
911R	164	196	183	192	182	184	177	185	224	268	212	242
911S	166	196	194	200	188	192	185	185	252	268	242	260
911T	186	196	200	204	188	188	185	185	232	264	198	214
912A	166	178	198	204	185	185	185	185	212	282	202	266
912B	174	196	188	204	170	188	187	187	204	212	216	222
912C	170	174	192	206	188	188	185	185	212	212	204	254
912D	164	170	204	204	188	193	185	185	262	266	204	250
912E	160	174	206	206	188	190	177	177	224	224	222	234
912F	164	170	206	210	188	188	185	185	262	262	222	268
912G	174	176	192	206	188	188	185	187	212	244	222	222
912H	164	196	188	206	188	191	185	187	224	262	198	222
912I	164	174	196	204	185	185	185	185	212	260	190	200
912J	164	174	204	206	189	189	185	185	212	212	222	228
912K	174	176	192	206	188	188	185	185	212	218	222	222
912L	164	174	204	206	188	188	185	185	262	274	222	222
912M	170	174	184	204	188	208	185	185	212	212	204	214
912N	164	174	186	204	180	188	177	185	254	262	222	238
912O	174	196	184	204	188	191	185	185	224	262	198	222
912P	164	164	206	206	188	196	201	201	212	212	204	242
912Q	174	188	194	206	188	188	185	185	212	216	204	208
912R	170	174	184	206	188	188	185	185	212	294	198	222
912S	166	174	204	206	188	188	185	185	192	212	198	204
912T	174	196	204	206	179	187	187	187	204	212	204	242
913A	170	196	184	204	182	207	189	189	252	304	210	264
913B	196	218	204	204	180	196	185	185	240	252	206	268
913C	174	196	184	206	180	206	185	185	244	244	204	226
913D	164	196	198	204	180	196	177	177	212	244	204	216
913E	164	174	206	206	206	206	185	185	240	252	204	246
913F	164	174	184	204	180	206	185	185	202	244	200	244
913G	174	170	192	204	204	204	185	185	244	252	200	200
913H	170	196	192	204	180	184	185	185	216	244	200	262

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: seeds												
913I	170	174	184	204	184	204	177	185	0	0	200	204
913J	174	196	194	206	189	206	177	201	252	256	200	238
913K	164	174	204	204	180	180	185	185	244	244	204	258
913L	164	166	184	204	184	206	185	185	216	244	204	262
913M	174	192	184	206	180	180	185	185	224	244	200	204
913N	166	196	204	206	181	188	177	185	252	266	206	214
913C	178	180	204	204	0	0	185	185	244	264	204	238
913P	174	174	190	204	180	186	177	177	220	244	198	200
913Q	162	196	186	206	206	217	177	177	244	244	196	196
913R	170	196	186	204	180	190	185	185	230	244	206	224
913S	164	196	186	206	180	190	177	177	222	252	206	264
913T	166	174	192	206	180	188	177	185	244	244	206	240
914A	164	174	184	206	146	180	185	201	244	256	198	198
914B	174	190	184	198	172	189	185	201	224	256	198	198
914C	164	164	184	186	146	181	201	201	214	224	222	264
914D	164	174	192	192	172	190	177	201	224	224	198	222
914E	174	200	206	206	172	212	0	0	224	252	198	244
914F	164	174	184	188	146	188	0	0	216	224	220	242
914G	164	180	202	202	172	180	0	0	224	230	222	252
914H	174	190	192	192	172	208	0	0	256	256	200	236
914I	164	164	184	188	146	187	185	185	224	262	220	234
914J	164	190	184	194	172	193	185	201	256	256	198	234
914K	184	190	184	198	166	208	185	185	206	256	198	198
914L	164	174	184	192	172	190	177	201	224	256	220	240
914M	174	186	184	184	172	189	185	201	160	256	198	226
914N	164	166	184	200	146	188	185	185	224	224	198	198
914O	174	192	202	202	146	180	177	201	224	224	222	226
914P	164	174	184	206	172	190	185	201	242	256	198	252
914Q	164	174	184	192	146	190	201	201	258	258	198	222
914R	164	170	204	204	146	188	185	201	220	224	198	222
914S	174	240	192	192	172	188	185	185	208	258	188	248
914T	164	166	184	206	146	188	185	185	224	224	214	222
917A	164	174	186	186	0	0	185	185	228	228	202	202
917B	170	174	184	286	185	198	185	185	254	302	196	198
917C	170	174	186	186	178	192	185	185	224	256	194	198
917D	170	170	186	186	178	185	201	201	224	256	198	212
917E	164	212	186	202	185	191	177	201	228	228	198	272
917F	170	214	186	186	185	185	185	185	228	228	198	212

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: seeds												
917C	170	214	186	206	172	185	187	201	268	256	198	204
917H	170	174	184	186	185	188	201	201	202	202	198	222
917I	164	170	188	194	181	193	185	199	224	256	200	240
917J	170	186	186	200	185	185	185	201	224	224	204	204
917K	164	170	186	188	185	188	199	201	256	276	198	242
917L	172	214	184	186	185	208	185	201	214	228	198	248
917M	162	170	186	186	180	180	177	185	220	220	208	208
917N	162	212	186	192	180	185	185	201	222	256	198	264
917C	170	212	186	186	178	185	185	201	224	224	204	204
917P	170	190	184	186	185	188	185	185	254	284	204	236
917Q	164	212	186	186	189	194	201	201	224	228	204	240
917R	170	174	186	186	170	185	185	201	256	270	198	204
917S	170	214	186	186	178	178	185	185	224	228	204	204
917T	164	170	184	186	178	208	185	201	226	256	198	226
920A	164	180	184	206	183	185	185	201	222	270	200	248
920B	174	196	184	184	188	208	185	201	246	270	216	216
920C	164	174	184	188	189	190	177	201	266	266	200	216
920D	174	180	184	186	188	192	185	201	224	266	240	250
920E	180	196	184	194	178	188	185	201	252	270	250	270
920F	174	174	184	184	182	188	201	201	224	266	198	250
920G	178	180	188	202	188	190	177	201	224	266	216	262
920H	170	174	184	184	184	188	185	201	206	206	216	238
920I	0	0	200	202	186	186	185	201	0	0	210	246
920J	166	180	184	204	188	198	185	201	244	244	216	238
920K	178	180	188	202	186	188	177	201	266	266	216	260
920L	180	212	184	184	175	188	185	201	266	288	216	234
920M	166	180	184	184	188	188	185	201	244	288	228	236
920N	162	180	184	184	188	188	185	201	206	266	204	250
920O	164	180	192	202	146	188	185	201	224	266	216	274
920P	166	180	202	204	188	192	185	201	260	266	216	234
920Q	176	180	202	202	188	189	201	201	224	270	192	248
920R	174	182	184	184	184	188	187	201	248	266	250	272
920S	174	174	204	208	188	188	185	201	228	270	216	218
920T	174	188	202	202	188	211	201	201	268	270	216	236
902A	174	184	208	208	183	183	177	177	258	266	198	268
902B	164	246	294	294	189	189	185	185	224	224	204	260
905A	164	176	184	206	146	187	185	185	250	262	196	252
905B	166	174	184	204	187	187	187	187	264	264	198	226

ID	FEMS4		FEMS11		FEMS19		FEMS16		FEMS2		M2.30	
	A	B	A	B	A	B	A	B	A	B	A	B
Betws-y-Coed: seeds												
907B	164	182	204	204	180	190	185	185	230	264	214	222
909A	164	174	194	204	146	180	187	187	250	250	198	234
909B	164	164	188	204	146	197	201	201	196	285	234	268
910A	174	174	186	192	178	188	185	185	260	266	196	240
910B	164	180	184	186	178	189	185	185	260	288	208	258
915A	170	182	184	184	189	190	185	201	224	256	234	266
915B	170	196	204	204	180	192	177	185	256	292	204	226
916A	170	178	186	206	182	196	185	185	236	236	262	262
916B	160	162	188	194	185	194	185	185	236	274	0	0
918A	160	170	198	204	188	188	201	201	196	246	204	220
918B	174	196	184	204	180	188	185	201	246	256	208	270
919A	174	238	192	194	194	208	185	185	234	260	222	240
919B	202	238	194	204	188	194	177	177	224	254	198	198

Appendix II

Large scale maps of the study sites Oxwich, Nicholaston, Alyn and Elwy. Maps of the remaining two sites are included in Chapter 4 (Bishopston) and Chapter 5 (Betws-y-Coed).

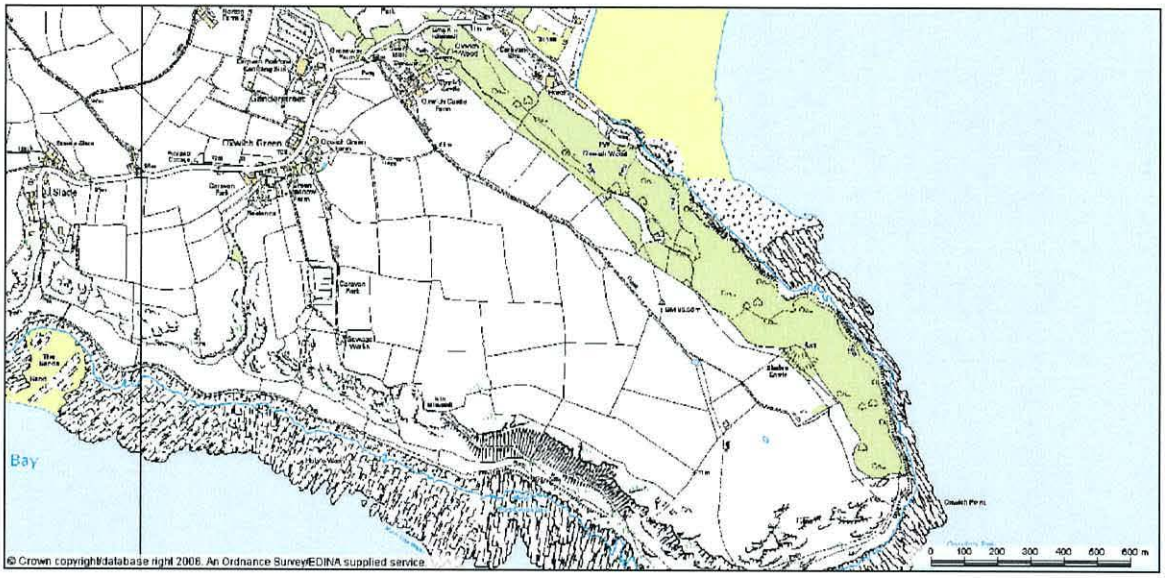

Figure AII.1 Large scale map of Oxwich Wood, Gower Peninsula, South Wales (NGR SS504860).

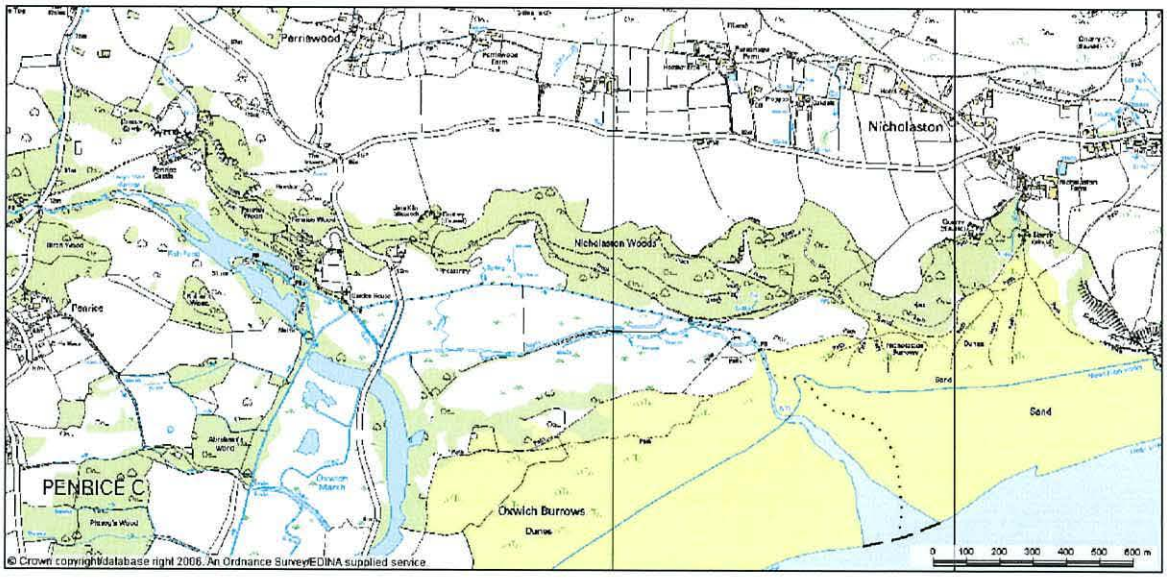

Figure AII.2 Large scale map of Nicholaston Woods, Gower Peninsula, South Wales (NGR SS520881).

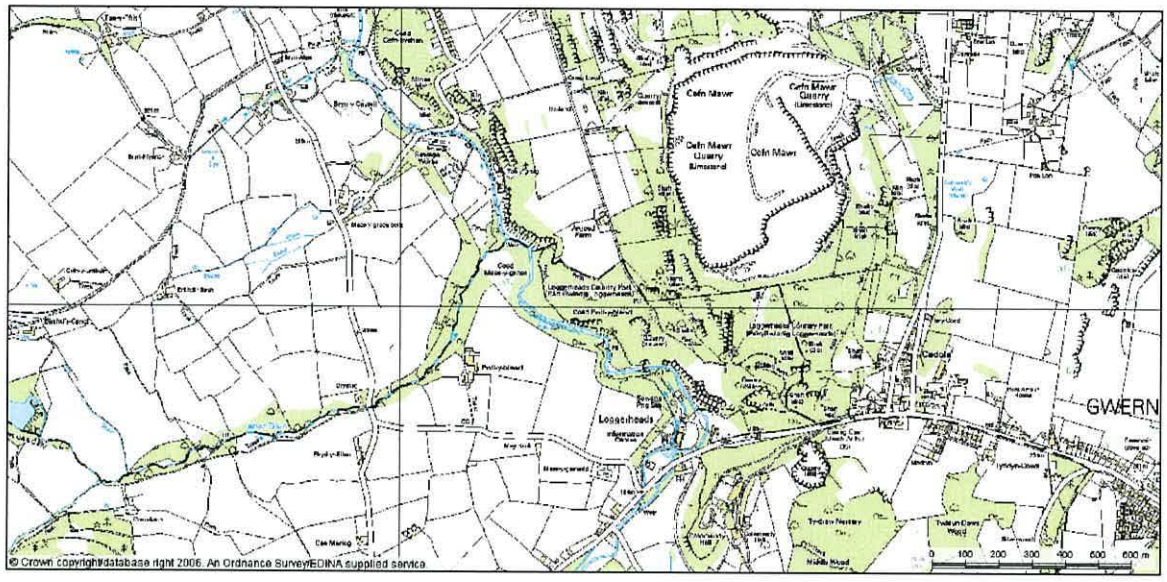

Figure AII.3 Large scale map of Alyn Valley, Clwyd, North Wales (NGR SJ197627).

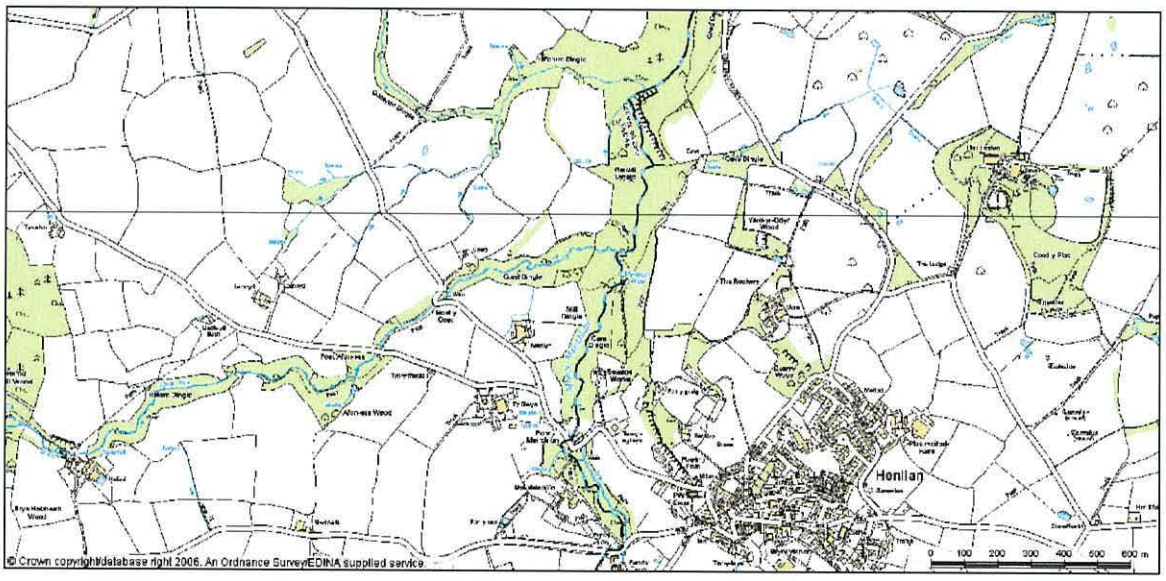

Figure AII.4 Large scale map of Elwy Valley, Clwyd, North Wales (NGR SJ019685).

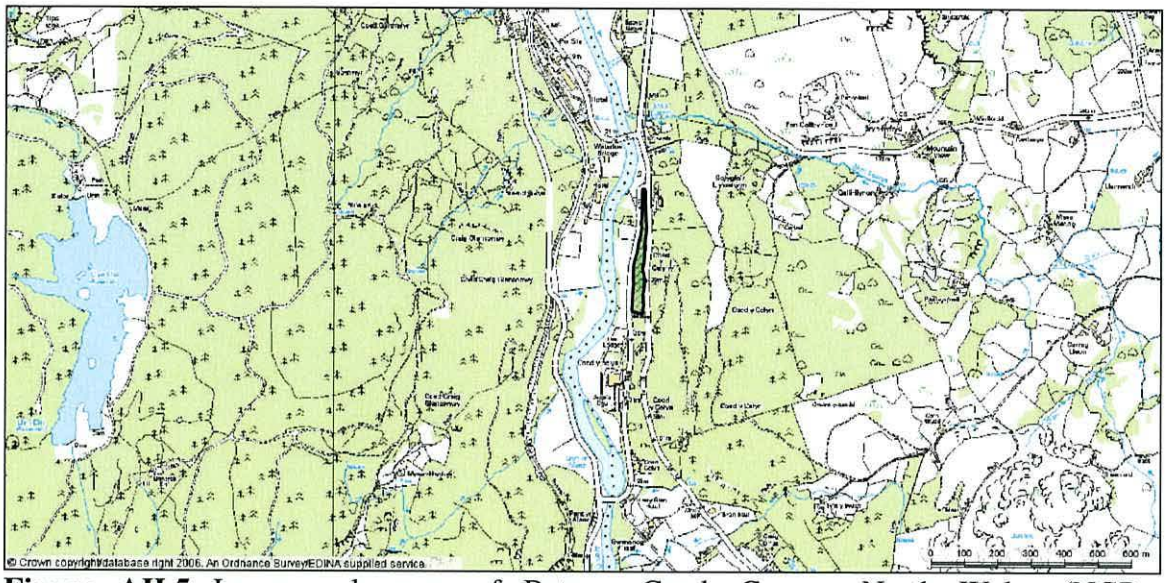

Figure AII.5 Large scale map of Betws-y-Coed, Conwy, North Wales (NGR SH799555).

Appendix III

Addresses for laboratory suppliers.

Abgene Limited (UK Head Office)

Abgene House
Blenheim Road
Epsom
KT19 9AP
UK
Telephone: 01372 723456
www.abgene.com

Beckman Coulter (U.K.) Limited – Biomedical Research

Oakley Court
Kingsmead Business Park
London Road
High Wycombe
Buckinghamshire
HP11 1JU
UK
Telephone: 01494 441181
www.beckmancoulter.com

Qiagen Ltd. – United Kingdom

Qiagen House
Fleming Way
Crawley
West Sussex
RH10 9NQ
Telephone: 01293 422911
www.qiagen.com

Sigma-Proligo

Telephone: 0810 400982
Fax 04 74 99 10 12
oligosupport-eu@proligo.com
www.proligo.com