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Introduced hybrid ash: *Fraxinus excelsior* × *F. angustifolia* in Ireland and its potential for interbreeding with native ash

Thesis submitted to the University of Dublin, Trinity College, for the
Degree of Doctor of Philosophy (Ph.D)

By

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based on research carried out under the supervision of
Dr. Trevor Hodkinson
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Declaration

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Muriel Thomasset

Summary

Fraxinus excelsior L. is the only native ash in Ireland. It has important economic value for timber exploitation. After the introduction of grants by Irish government in the 1990s for planting native broadleaf trees, with an extra premium of hardwood species including ash, provenances of supposed *F. excelsior* were imported from continental Europe to fulfil the needs of foresters and farmers. At the time of planting, the trees appeared to be of excellent quality but after some years, the trees started to show very bad stem form, poor vigour and growth, and unusual morphology. Management authorities, farmers and foresters have questioned the authenticity and origin of the imported trees. Indeed very pale and brown buds observed on introduced trees are more characteristic of another species *F. angustifolia* Vahl present in continental Europe, with usually a more southern distribution than *F. excelsior*. Moreover, natural hybrids between these two species have been observed in two different zones where the species grow in sympatry. Reciprocal artificial crosses have also been made.

The broad aims of this thesis were to try to resolve the origin of the introduced trees, using molecular and morphological markers, and to understand and examine the risk of introgression of genes from plantation trees into the Irish environment. By doing this, I hoped to improve general knowledge about hybridization, introgression and invasiveness potential in *Fraxinus* that would also be of value to the wider scientific community beyond Ireland.

I first determined the morphology of reciprocal artificial F_1 hybrids between the two species to help understand the morphology of trees found in introduced Irish plantations. Studies were mainly focussed on two plantations, Clonee and Kildalkey, in County Meath, that both contained suspect material. By using six microsatellite markers developed in previous studies and morphological markers that provide useful information with F_1 generation plants, I showed that on both plantations there was a substantial number of hybrids between the two species (between 28-58% depending on the threshold chosen for genetic analysis) with a large range of morphological variation. Hybrids were in general found to be advanced generations of backcrosses, often difficult to recognize on the basis of morphological characters because they were highly introgressed with *F.*

excelsior. Trees that were classified as *F. angstifolia* (up to 12%) were also collected, however some trees were identified as *F. excelsior* that were ill adapted to Irish conditions.

I undertook studies of flowering phenology over two years and observed considerable synchrony between native and introduced ash in terms of flowering time. Over the two years of observations, native and plantation trees were releasing pollen at the same time for over a month. Hence, this pre-zygotic barrier to prevent gene flow between introduced and native trees was weak. The paternity analysis results using the logarithm of odds (LOD) score method showed that hybrids and *F. angustifolia* are able to reproduce with native trees. This occurred even though there was a delay in flowering time in native *F. excelsior* that would reduce the risk of gene flow and hence structure the pollen cloud. However, the structure of the pollen cloud was mainly found to be determined by the different flowering time of trees and not geographic distance.

I then undertook comparisons of the germination rate, dormancy requirement, and quality of seeds from plantation mother trees and seeds from native trees. The results showed that seeds from introduced trees mainly expressed *F. excelsior* characteristics; meaning that there was no germination without stratification and that they had similar seed size. These results indicate that they could compete for the same niche as seeds produced from native trees. Seeds from introduced trees were viable, however they showed a large amount of insect infection that could in the long term reduce the fitness of the hybrid populations.

Overall, the results indicated a risk of gene transfer via gene flow between introduced plantations and native ashes that will depend on the source of the introduced material, the weather of each year and the size of plantation. In order to preserve Irish resources, management of sites with alien ash is needed. It will also be necessary to determine how ash species and their hybrids will respond to climate change. Preliminary results presented in this thesis indicate that future climate of Ireland under global warming scenario will not be suitable for *F. angustifolia* or hybrid trees.

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List of content

Declaration III

Summary V

Acknowledgments VII

List of content IX

List of tables XV

List of figures XVII

List of appendices XX

Chapter I 1
General introduction 1
1.1 Hybridization 3
1.1.1 Definition and natural hybridization 3
1.1.2 Process and barriers to geneflow 4
1.1.2.a Pre-reproductive isolating mechanisms 5
1.1.2.b Post-reproductive isolating mechanisms: genomic incompatibility, hybrid inviability or sterility 6
1.1.3 Consequences of hybridization 7
1.1.3.a Creation of hybrid zones 7
1.1.3.b Hybrid speciation and species divergence 8
1.1.3.c Introgression 9
1.1.4 Hybridization in forest tree species 10
1.2 Species introduction and consequences 11
1.2.1 Human activity 11
1.2.2 From introduced to invasive: the barriers to overcome 12
1.2.3 Consequences of hybridization and introgression after species introduction 13
1.2.3.a Genetic effects and genetic pollution 14
1.2.3.b Ecological effects 15
1.2.3.c Benefits of hybridization 15
1.2.3.d Exotic pollen flow among tree species 15
1.3 Study species 16
1.3.1 Oleaceae and Fraxinus 16
1.3.2 European ash: Fraxinus excelsior and F. angustifolia 17
1.3.2.a Distribution 17
1.3.2.b Morphology, flowering phenology and breeding system 18
1.3.2.c Biogeography and genetic diversity 20
1.3.2.d Hybridization between F. angustifolia and F. excelsior 21
1.3.3 History of ash trees in Ireland 21
1.3.3.a Colonization 21
1.3.3.b Use of ash 22

1.3.4 Recent importation and problems 22
1.4 Aim of the thesis and outline 23

Chapter II 25
Molecular and morphological characterization of reciprocal F_1 hybrid ash (*Fraxinus excelsior* × *F. angustifolia*, Oleaceae) and parental species reveals asymmetric character inheritance 25
Abstract 27
2.1 Introduction 28
2.2 Materials and methods 30
2.2.1 Plant material 30
2.2.2 Nuclear microsatellite marker assessment 31
2.2.3 Morphological assessment 32
2.2.4 Analysis 33
2.2.4.a Morphometric characters 33
2.2.4.b Molecular data 34
2.2.4. c Combined molecular and morphological data analyses 34
2.3 Results 34
2.3.1 F_1 identification 34
2.3.2 Morphological differentiation 35
2.3.3 Molecular markers 41
2.4 Discussion 42
2.4.1 Classification success 42
2.4.2 Leaf shape patterns and character inheritance 44
2.4.3 Asymmetry in character inheritance 45
2.4.4 Conclusions 46
Acknowledgments 46

Chapter III 47
Characterization of introduced ash in Ireland reveals cryptic hybrids (*Fraxinus excelsior* x *F. angustifolia*) and possible multiple source populations. 47
Abstract 49
3.1 Introduction 50
3.1 Introduction 50
3.2 Materials and methods 53
3.2.1 Sampling of suspected alien ash sites 53
3.2.2 Native control population 54
3.2.3 Morphology measurements 55
3.2.4 DNA extraction and microsatellite amplification 55
3.2.5 Data analyses 56
3.2.5.a Genetic diversity 56
3.2.5.b Morphological analysis 57
3.2.5.c Assignment and individual classification 57
3.3 Results 64
3.3.1 Genetic diversity and molecular differentiation 64
3.3.2 Molecular differentiation among populations and geographical groups. 65
3.3.3 Morphological differentiation 66
3.3.4 Multivariate analysis 67
3.3.5 Bayesian clustering of reference populations 71

3.3.6 Performance of the hybrid assignment based on Bayesian methods assignment 71
3.3.7 Genetic admixture analysis and assignment testing 72
3.3.7 Characterization of hybrids types 75
3.4 Discussion 76
3.4.1 Power of the admixture analysis and molecular detection of the hybrids 77
3.4.2 Morphological variation 78
3.4.3 Advanced generation hybrids and cryptic hybrids 78
3.4.4 High genetic diversity of natural populations and introduced plants 80
3.4.5 Conclusions and implications for conservation 81
Acknowledgements 83
Supplementary material 84

Chapter IV 85
Flowering and bud flushing patterns in native and introduced ash trees (*Fraxinus* spp.) highlights potential for hybridization 85
Abstract 87
4.1 Introduction 88
4.2 Materials and methods 90
4.2.1 Study species 90
4.2.2 Sampling site 91
4.2.3 Phenological survey 92
4.2.4 Statistical analysis 94
4.3 Results 96
4.3.1 Climatic differences between years 96
4.3.2 Proportion of flowering trees 96
4.3.3 Gender of the trees 97
4.3.4 Flowering phenology 97
4.3.4.a Clonee site 97
4.3.4.b Kildalkey site 102
4.3.5 Leaf flushing phenology 105
4.3.6 Phenology and hybrid recognition 106
4.4 Discussion 108
4.4.1 Overlapping flowering time and introgression 109
4.4.2 Intensity of flowering 110
4.4.3 Possible sources of variation 110
4.4.4 Vegetative bud flushing and leaf unfolding 112
4.4.5 Conclusions and perspectives 113
Acknowledgements 114

Chapter V 115
Paternity analysis and pollen mediated gene flow between native and non-native populations of *Fraxinus* (Oleaceae) in Ireland 115
Abstract 117
5.1 Introduction 118
5.2 Materials and methods 122
5.2.1 Study material and sites 122
5.2.2 Sampling 122
5.2.3 Phenological data 123

5.2.4 DNA extraction and microsatellite marker genotyping 124
5.2.5 Data analysis 125
5.2.5.a Genetic diversity 125
5.2.5.b Mating system 125
5.2.5.c Paternity assignment 126
5.2.5.d Genetic structure of pollen pools 127
5.2.5.e Gametic assay 128
5.3 Results 129
5.3.1 Genetic diversity 129
5.3.2 Mating system 131
5.3.3 Gene flow 133
5.3.3.a Paternity assignment 133
5.3.3.b Pollen pool genetic structure 135
5.4 Discussion 139
5.4.1 Mating system 139
5.4.2 Pollen dispersal 141
5.4.3 Pollen pool genetic structure 142
5.4.4 Consequences of non-native gene flow into native Fraxinus 144
5.4.5 Conclusion 145
Acknowledgments 145
Supplementary material 147

Chapter VI 149
Fitness and seed characteristics of native ash (*Fraxinus excelsior*) and introduced population of hybrid ash (*F. excelsior* x *F. angustifolia*) 149
Abstract 151
6.1 Introduction 152
6.2 Materials and methods 155
6.2.1 Plant material 155
6.2.2 Stratification treatment and germination 156
6.2.3 Seed characteristics 157
6.2.4 Seed production 158
6.2.5 Data analysis 158
6.2.5.a Germination 158
6.2.5.b Quality of the seeds 159
6.2.5.c Seed and embryo size 159
6.3 Results 160
6.3.1 Experiment 1: Species comparison and validation of experimental conditions. 160
6.3.2 Experiment 2: Irish plantation seed compared to native seed 163
6.3.2.a Germination without and with stratification treatment 163
6.3.2.b Seed quality 164
6.3.2.c Seed characteristics 166
6.3.2.d Seed production 167
6.4 Discussion 170
6.4.1 Dormancy breakdown 170
6.4.2 Germination of plantation populations 171
6.4.3 Seed production 172
6.4.4 Seed and embryo size 172
6.4.6 Conclusions and consequences 173

Acknowledgements **174**
Supplementary material **175**
Chapter VII **177**

General discussion **177**
7.1 An overview of the findings **179**
7.1.1 Morphology of artificial F_1 hybrids and inheritance patterns 179
7.1.2 Characterisation of hybrid alien ash (Fraxinus excelsior x F. angustifolia) 180
7.1.3 Overlapping flowering period 181
7.1.4 Vigour and growth of introduced trees 183
7.1.5 Pollen mediated gene flow between native and alien populations of F. excelsior and paternity analysis 184
7.1.6 Fitness of introduced plantation trees as determined by seed germination capacity, dormancy requirement and quality 185
7.2 Implications for control and management of alien plants **186**
7.2.1 Risk assessments 186
7.2.2 Management strategies 187
7.2.2.a Management of introduced plantation trees 187
7.2.2.b Policy for future breeding programmes 189
7.2.3 Future climate change and consequences for Irish plantations 190
7.3 Conclusions and further directions **193**

References **195**

Appendix 1 ..... **217**

Appendix 2 ..... **241**

List of tables

Table 2.1 Source of analysed individuals, geographical coordinates and sample size. 31

Table 2.2 Results of the stepwise discriminant analysis on morphological characters. * indicates the variables with $P<0.05$, which were included in the model. 39

Table 2.3 Morphological traits and the standardized coefficients (Can1, Can2, and Can3) from the canonical discriminant analysis based on the morphological characters used to differentiate the two parental species and their hybrids. 39

Table 2.4 Genetic diversity statistics for *F. angustifolia* (ANG), *F. excelsior* (EXC) and reciprocal F_1 hybrids. 41

Table 2.5 Correlation of the canonical function (Can1, Can2, Can3) with the variables of the discriminant analysis based on molecular and morphological characters. 43

Table 3.1 Details of sampled ash populations. 54

Table 3.2 Within-population diversity at all loci for all populations and plantations. 62

Table 3.3 Summary of the genetic diversity statistics for the 6 different geographical/species groups. 64

Table 3.4 Population differentiation calculated from nuclear microsatellite data. Above diagonal, pairwise population estimates of R_{ST}; below diagonal, pairwise population F_{ST} values; all statistics were significant at the $P<0.05$.65

Table 3.5 Analysis of molecular variance (AMOVA) within and among the populations grouped according to geographic region. 66

Table 3.6 Summary of morphological variation for leaf characters within geographical groups. 70

Table 3.7 Results of STRUCTURE and NEWHYBRIDS analyses with simulated samples 72

Table S3.1 Analysis of molecular variance (AMOVA) within and among the geographic region grouped according to the species level (*F. excelsior, F. angustifolia* and plantation trees). 84

Table 4.1 Mean temperature and rainfall values for Dublin airport during the two years of the study (2009 and 2010), and the monthly averages between 1961 and 1990. 92

Table 4.2 Pairwise comparisons between populations for flowering phenology. Significance of the *P*-values for Mann-Witney U test. 99

Table 4.3 Spearman rank correlations between the different variables in 2009 and 2010. Correlations significant at $P<0.05$ are above the diagonal. 102

Table 4.4 Percentage of individuals classified as early, intermediate, and late flowering according their origin and the year. The classification of individual trees was made according their flowering bud burst date. 103

Table 5.1 Parameters of genetic diversity for the adult *F. excelsior* trees and their progeny. 130

Table 5.2 Genetic differentiation measured by pairwise F_{ST} (Weir and Cockerham, 1984) (below diagonal) and significance (above diagonal) among the four adult populations 131

Table 5.3 Estimated mating system parameters for the progeny from Clonee and Kildalkey. 132

Table 5.4 Estimated mating system parameters for the progeny of the different phenological groups (Early, Intermediate or Late) from the Clonee and Kildalkey sites. 133

Table 5.5 Results from paternity assignment (using the LOD score method implemented in CERVUS). Number of seedlings assigned with confidence interval of 80%, number of detected fathers and pollen dispersal distances for seed sired from pollen sources and observed standard deviation (sd) within the two sites, Clonee and Kildalkey. 134

Table 5.6 Φ_{ft} per phenological group estimated using TwoGener 137

Table 6.1 Study populations for Experiments 1 and 2 (sample size, origin, latitude, longitude). 156

Table 6.2 Effect of stratification treatment (Treatment), species or population (Species, Population) and trees within population (Trees(Population)) on seed germination. Data were modelled using a generalized linear model with binomial error and logit link. Interaction (Treatment*Species) has been considered. 162

Table 6.3 Significance of the pairwise comparisons following the generalized linear model for Experiment 1(Control). 163

Table 6.4 Analysis of variance table for seed variables partitioned inot Populations (Pop) and trees within population (tree (Pop)) 167

Table 6.5 Intensity of seed production by native and plantation trees, % of trees by category. 168

Table S6.1 Means and standard error of the nine different variables regarding the seed characteristics for trees from the two different plantations classified as *F. excelsior* or hybrid type. 175

Table 1 Eleven candidate genes potentially involved in adaptive variation in *F. excelsior* and *F. angustifolia.* 245

Table 2 Details of the different populations of *F. excelsior* and *F. angustifolia* used for sequencing 246

List of figures

Figure 1.1 Schema of barriers for invasion from Richardson *et al.* (2000)..... 13

Figure 1.2 Map from Thomasset *et al.* (2011b). **A.** General distribution of *F. excelsior.* **B.** General distribution of *F. angustifolia* 18

Figure 1.3 Morphology of *F. angustifolia* and *F. excelsior.* **A.** on the left- typical leaf of *F. angustifolia,* and on the right -leaf of *F. excelsior.* **B.** shows the differences in bud colour between the two species, *F. excelsior* (left) and *F. angustifolia* (right). C. shows the verticilisation by three -often encountered in *F. angustifolia.* 19

Figure 1.4 Examples of bad shape trees observed on plantations of introduced trees from continental Europe. 23

Figure 2.1 Means and standard deviations of morphological characteristics. Comparisons include *F. angustifolia* (ANG), *F. excelsior* (EXC), their F_1 hybrid with *F. angustifolia* as mother (F_{1ANG}) and their F_1 with *F. excelsior* as mother (F_{1EXC}). 36

Figure 2.2 Means and standard deviations of morphological characteristics. Comparisons include *F. angustifolia* (ANG), *F. excelsior* (EXC), their F_1 hybrid with *F. angustifolia* as mother (F_{1ANG}) and their F_1 with *F. excelsior* as mother (F_{1EXC}). 37

Figure 2.3 Plot of canonical scores of the seven morphological traits for each of the individuals of *F. excelsior*, *F. angustifolia* and their two types of hybrids. The plot indicates the percentage of variation of each canonical variate axis and the canonical score of each species..... 40

Figure 2.4 Plot of canonical scores of the morphological traits and PC factors for each individual of *F. excelsior*, *F. angustifolia* and their two types of hybrids. The plot indicates the percentage of variation of each canonical variate axis and the canonical score of each species..... 42

Figure 3.1A. Canonical discriminant analysis of six microsatellites markers and all groups of 808 samples. **B.** Canonical discriminant analysis of 10 phenotypic characters and all groups of 744 samples. **C.** CDA based on morphological variables and molecular data for the Irish, French and plantation populations only. 69

Figure 3.2 Histogram of estimated membership proportions for each parental species. Each vertical bar represented a single individual. 74

Figure 3.3 CDA based on morphological variables..... 76

Figure 4.1 Description of the flowering stages and score notation. 93

Figure 4.2 Distribution of flowering intensity measured as number of flowering trees in 2009 and 2010 for native and introduced trees at Clonee and Kildalkey sites. 97

Figure 4.3 Means and standard deviations of the different phenological variables among populations (native or introduced), site (Clonee or Kildalkey), and year (2009 or 2010). 101

Figure 4.4 Frequency distribution for flowering phenology of the different sites monitored in 2009 and 2010. 104

Figure 4.5 Representation of the individual trees on the first two axes of the principal component analysis (PCA) based on the ten phenological variables collected over the two years of the study..... 108

Figure 5.1 Map of the two study sites. 123

Figure 5.2 Distribution of detected pollen dispersal distance classes when mother trees are either native (in black) or from the plantation (in white) in relation to the position of pollen donor within the sites. **A.** Clonee site and **B.** Kildalkey site. 135

Figure 5.3 Pairwise Φ_{ft} in relation to the distance between mother trees at Native-Clonee (a), Native-Kildalkey (b), Plantation-Clonee (c) and Plantation-Kildalkey (d). 136

Figure 5.4 Plot of the two first canonical scores of the CDA analysis. The pollen cloud differentiation for each mother tree is shown. **A.** Clonee site and **B.** Kildalkey site. Trees were grouped according to their time of flowering phenology (Int for intermediate dates of flowering period; Early and Late for the two other flowering groups) and their sampling location..... 138

Figure S5.1 Posterior density curves for the inbreeding coefficient for the progenies and the adults of the native trees at the two sites. The tables show the mean of the inbreeding coefficient and the confident interval... 142

Figure 6.1 Germination rates (means ± SE if >5%) and effects of stratification, for Experiment 1 ('control'), of seeds from natural population of *F. angustifolia* (in red), natural population of *F. excelsior* (in blue) and putative hybrid population (in grey) without a stratification treatment (no stratification) and after a stratification treatment. 161

Figure 6.2 Germination rates (means ± SE if >5%), for Experiment 2 (Irish native and non-native material), of seeds from native Irish *F. excelsior* (in blue) and the two plantation populations (in grey) without stratification treatment (no stratification) or after stratification treatment. Different letters above the bars indicate significant difference ($P<0.05$) in final germination percentages. 164

Figure 6.3 Proportion (means ± SE if >1%) of empty seeds (on the left part) and infected seeds (on the right part) of seeds from native Irish *F. excelsior* (in blue) and the two plantation populations (in grey) from Experiment 2 (Irish native and non-native material). Different letters above the bars indicate significant difference ($P<0.05$) in final germination percentages..... 166

Figure 6.4 Seed characteristics (means ± SE) for the different variables (SeedL, SeedW, EmbryoL, SeedL/SeedW, EmbryoL/SeedL) of the two native

populations and the two plantations. Different letters above the error bars indicate significant (P<0.05) differences among populations (Tukey HSD).169

Figure 7.1 Potential niche distributions of *Fraxinus* hybrid zones under current conditions (grey scale) and a future climate scenario (mustard colours). A. Maxent simulation of the potential niche distribution of *F. angustifolia* x *F. excelsior* hybrid populations under current climate conditions (average 1950-2000, see text for details), AUC of the simulation=0.997; B. Maxent distribution of the potential niche of the hybrid zones under a global warming scenario of doubled CO_2 (from Thomasset *et al.*, 2011b)..... 192

List of appendices

Appendix 1: Thomasset M, Fernández-Manjarrés JF, Douglas GC, Frascaria-Lacoste N, Hodkinson TR (2011) Hybridisation, introgression and climate change: a case study for the tree genus *Fraxinus* (Oleaceae). In: *Climate change, ecology and systematics* (eds. Hodkinson TR, Jones MB, Waldren S, Parnell JAN). Cambridge University Press, Cambridge

Appendix 2: A set of primers for candidate genes potentially involved in ecotypic variation in *Fraxinus excelsior*, *F. angustifolia*.

Chapter I

General introduction

This thesis investigates the introduction of an exotic ash species *Fraxinus angustifolia* Vahl and its hybrids, in the Irish environment and the potential consequences of genetic crosses with native ash, *Fraxinus excelsior* L. Consequences of gene flow between the two species for forestry management and conservation are also examined. This chapter introduces the context of the project and provides background information on ash in Ireland and in Europe. I first present a literature review on natural hybridization, and the impact of introduced species on native systems.

1.1 Hybridization

1.1.1 Definition and natural hybridization

"Species are the fundamental units of natural diversity" (Harrison, 1998). The species concept and definition is one in the most former and the most persistent sources of debates in biology. Mayden (1997) provides a review and hierarchical classification of 22 species concepts. To summarize briefly, two types of concepts gradually appeared, some very close in the traditional Linnaeus taxonomy, such as the Morphological Concept (MSC, Cronquist, 1978), the others making reference to evolutionary processes. Among them, the most widely used remained the Biological Species Concept (BSC, Dobzhansky, 1937; Mayr, 1942) based on reproductive isolation, the Cohesion Species Concept (CSC, Templeton on 1989) based on the most inclusive population of organisms having the potential for cohesion, and the Phylogenetic Species Concept (PSC, diagnosable version, Cracraft, 1983) based on divergence and monophyly. At the moment, one of the solutions of the problem of species concept involves widening the previous definition by demonstrating that they are not incompatible with most of the other concepts (for the BSC, see for example Mayr, 2001; Orr , 2001; Coyne and Orr, 2004).

Because of incomplete isolation barriers, many plants species do not fit the BSC species definition. Arnold (1997) estimates that between 30-70% of the world angiosperm flora originates from hybridization events. In a broad sense,

hybridization can be defined as the reproduction between individuals from populations that are genetically or phenotypically distinguishable regardless of the taxonomic status of the two taxa (Harrison, 1990; 1992). This broad definition has the advantage that it does not require the acceptance of any species concept. The definition has been restricted by some users to include only interbreeding between individuals from different species or genera. To avoid confusion, in this thesis, hybridization is defined as the crosses between two characteristic species or taxa, or the offspring of such crosses with each other or the parental species.

The offspring of crosses between two species are hybrids of the first generation (F_1) and can be viable or not, fertile or sterile. If hybrids are viable and fertile, they can produce hybrids, second and third generations and so on...(F_2...F_n); they can also cross with parental species in a phenomenon called backcrossing (Arnold, 1992; Seehausen, 2004). If hybrid progenies backcross with their parents, the phenomenon is termed introgression. Thus, introgression can be defined as "the infiltration of germplasm from one species into another, through repeated backcrossing of hybrids with parental species". (Anderson & Hubricht 1938; Arnold 1997). Hybridization and introgression can be directional (e.g. more breeding events between male from a species with the female of the other species) or reciprocal (Wirtz, 1999; Bettles *et al.*, 2005). The term 'hybrid swarm' is used for populations of individuals with varying degrees of introgression due to the presence of F_1, F_2...F_n and backcrosses from different generations.

Natural hybridisation is now recognised as an important evolutionary phenomenon and has been particularly well documented in plants (Stace, 1975; Ellstrand *et al.*, 1999; Ellstrand and Schierenbeck, 2000; Rieseberg *et al.*, 2003; Arnold, 2004a; Rieseberg and Willis, 2007).

1.1.2 Process and barriers to geneflow

Hybridization will occur when a breakdown of the geographical isolation and the reproductive isolation between two taxa is achieved, i.e. when mechanisms which prevent gene flow have disappeared (Arnold, 1997; Orr and Smith, 1998; Mallet,

2005). Geographical isolation refers to physical barriers or distance separating two populations (Seehausen, 2004), while reproductive isolation is based on the existence of barriers before or after the reproductive event (Levin, 1978; Rathcke and Lacey, 1985; Arnold, 1997). These barriers are in general divided into two categories: pre-zygotic and post-zygotic (for review see: Coyne and Orr, 2004).

1.1.2.a Pre-reproductive isolating mechanisms.

Intrinsic isolation

Isolation can be behavioural. In animals, isolation behaviour seems particularly important for the isolation between species. Many species use elaborate behaviours and physical characteristics to attract mates. Recognition between male and female is essential for breeding. For example, Grant and Grant (1997) showed that different songs isolate two bird species (*Geospiza fortis* and *Geospiza scandens*) that are otherwise morphologically indistinguishable.

An incompatibility of reproductive organs (mechanical isolation) can also act as a barrier. Within many insects, the male and female copulatory organs of closely related species do not fit together, preventing sperm transfer (Sota and Volger, 2001). Finally, intrinsic isolation may be due to gametic isolation: either by gametic incompatibility (e.g. intrinsic defects causing physiological or biochemical recognition of gametes during fertilization (Lewis and Crowe, 1958), or by gametic competition, also called pollen competition (for review see: Howard, 1999). This may be due to a lesser mobility or viability of heterospecific gametes compared to conspecific gametes. Several studies (Rieseberg *et al.*, 1995; Carney and Arnold, 1997) have shown that the rate of pollen tube growth plays an important role as a barrier to gene flow in plants.

Ecological isolation

Sympatric species may occupy different niches locally. Two organisms that use different habitats, even within the same geographic area, are unlikely to encounter each other and will therefore not reproduce. The isolation is often related to a time lag of reproductive events (Schluter, 2001). Moreover, in plant systems, pollinator

specificity (such as insects, birds or bats) may also act as a barrier. For example, specialization for a particular species of pollinator (pollinator syndrome) has been shown to isolate species among tropical trees (Kay, 2006) and in orchids (Schiestl and Schlüter, 2009).

Another mechanism that can lead to reproductive isolation is known as temporal isolation. Temporal isolation is related to a time delay of reproductive events. It may be triggered by reproductive seasons as well as the timing of the release of gametes. In plant species, this mechanism involves different flowering periods (Vanden Broeck *et al.*, 2003a; Gérard *et al.*, 2006b; Gérard *et al.*, 2006c; Pascarella, 2007) or a delay in the different steps of flowering: flowering peak or period of pollination (Weis and Kossler, 2004). Species with overlapping flowering times have a greater opportunity to interact. The timing of reproductive events is thus an important mechanism for maintaining species co-existence in diverse plant communities (Rathcke and Lacey, 1985).

1.1.2.b Post-reproductive isolating mechanisms: genomic incompatibility, hybrid inviability or sterility

Zygotic

Zygotic isolation is caused by genomic or physiological incompatibilities. During chromosomal recombination, a variable number of chromosomes between species will prevent normal meiosis (Rieseberg and Carney, 1998); zygotic mortality may occur.

Post-zygotic

Post-zygotic barriers act after zygote formation by decreasing viability or the fertility of the hybrids. Extrinsic post-zygotic isolation, or exogenous selection, is characterized by hybrids showing intermediate phenotypes which are ill-adapted to the parental environment. Coyne and Orr (2004) argue that exogenous selection can be ecological (normal development of the hybrid but showing decreased viability because they are not adapted to their environment) or behavioural (hybrids have reduced fertility due to difficulties in encountering a partner). Intrinsic

post-zygotic mechanisms are the consequence of genetic incompatibility or negative epistasis between alleles from different species (Dobzhansky, 1937).

Intrinsic post-zygotic isolation can occur at different levels: non-viability of the first generation hybrids (F_1), sterility of F_1 hybrids and hybrid depression that can affect viability and fertility beyond the first hybrid generation (Coyne and Orr, 2004).

It is noteworthy that not all hybrids individuals are necessarily less fit than their parents (Arnold and Hodges, 1995). Indeed some individuals might express hybrid vigour with increased germination rates, growth characteristics and survival, and decreased susceptibility to pathogens (Arnold and Hodges, 1995).

1.1.3 Consequences of hybridization

1.1.3.a Creation of hybrid zones

Hybrid zones may occur when previously allopatric taxa or populations come into contact (Lowe *et al.*, 2004). A hybrid zone is defined as a narrow zone in which admixed individuals co-occur (Barton and Hewitt, 1985; Harrison, 1990). Hybrid zones may be short lived (ephemeral) when the hybrids and their genes are removed by selection and have no lasting effect on the parental populations through introgression. However, hybrid zones may be long lasting and of more consequence to the evolution of the plant populations because there is permanent introgression of genes among species (Anderson and Hubricht, 1938). Hybrid zones that are not short lived have been studied and different models regarding the fitness of hybrids have been developed (see Lowe *et al.* 2004 for a review of these models).

First, there are models which consider that the reproductive success of hybrids does not depend on the environment and the 'Tension Zone Model' is one of the best known of these (Barton and Hewitt, 1985). According to this model, the endogenous selection pressures, such as incompatibilities, and genetic negative epistasis, ensure that the reproductive success of hybrids is less than the parental taxa. The maintenance of the hybrid zone is possible only when there is

equilibrium between the low reproductive fitness of hybrids and the dispersal into the hybrid zone (Barton and Hewitt, 1985). However, Arnold (1997) found that the genetic and ecological patterns observed in hybrid zones do not always coincide with the model. Often, data indicate that environmental selection may play an important role in the reproductive success of hybrids (Arnold and Hodges, 1995). In this case, there is a second class of hybrid zones, in which the reproductive success of hybrids is dependent on the environment. In this category, some models, such as the 'Mosaic Hybrid Zone Model' consider that the reproductive success of hybrids is lower than that of the parental individuals (Harrison, 1986; Howard, 1986). Others consider that the reproductive success of hybrids may be lower or higher depending on the habitat in which hybrids are found such as the 'Bounded Hybrid Superiority Model' (Moore, 1977). Combining elements of both of these types of models, the 'Evolutionary Novelty Model' (Arnold, 1997) considers both genetic and environmental selection pressures for and against certain hybrid genotypes and generations and the possibility of invasion of novel hybrid recombinants into new habitats.

The hybrid zones might be stable, however Barton and Hewitt (1989) demonstrated that hybrid zones can move. For example, if the hybrids show a better adaptation for the parental environment than the 'pure' species, the hybrid can expand to the detriment of the parental species. The expansion might be unidirectional if one of the parental species is less competitive. A directional expansion is also possible when hybridization is asymmetric, i.e. backcrosses only or more frequently with one of the parental species (Rieseberg and Carney, 1998). In hybrid plants such asymmetry can be revealed by comparing the genome inherited only from one parent such as the chloroplast or the mitochondrial genome.

1.1.3.b Hybrid speciation and species divergence

The hybrid origin of several species has been shown (Rieseberg, 1997; Seehausen, 2004). Hybrid speciation might result from the reproductive isolation of the hybrids from the parental species and/or to their higher competitive fitness

of hybrids than parental species. A common mechanism of hybrid speciation involves polyploidy (Levin, 2002; Ramsey and Schemske, 2002). However, the existence of homoploid speciation i.e. the creation of new species without change in the ploidy level has also been observed (Rieseberg, 1997; Rieseberg and Carney, 1998; Rieseberg and Burke, 2001). If parental species differ by small chromosomal differences, the recombination after a few hybrid generations might lead to genomic incompatibility with the parental species (Rieseberg, 1997). The factors that promote such a scenario are: 1) a strong selection in favour of the recombinant; 2) a small number of chromosomal differences (McCarthy *et al.*, 1995); 3) a fast chromosomal evolution; and 4) the access to new habitat (Buerkle *et al.*, 2000). By generating individuals that exceed parental phenotypic values, transgressive segregation could give hybrid individuals the opportunity to colonize new ecological niches, including those that may be created by changing climatic conditions. To my knowledge, one of the only examples of hybrid speciation in tree species concerns a pine, *Pinus densata* Mast. After successive bottlenecks and a few hybrid generations, hybrid species colonized a new niche in higher altitudes than that occupied by the parents (Song *et al.*, 2002; Song *et al.*, 2003).

Another potential outcome of hybridization is parental species divergence or parapatric speciation (Coyne and Orr, 2004). If the two species in contact are in fact adapted to very different environments, the low fitness of hybrids and the geographic isolation might lead to ecological divergence and increased reproductive isolation until gene flow ceases. Reinforcement of the reproductive barriers, as described first by Dobzhansky (1937) may also result in parental species divergence, as individuals with stronger fitness will be favoured by selection.

1.1.3.c Introgression

As mentioned above, introgression involves repeated backcrosses between hybrids and parental species which can lead to the exchange of genetic material from one species to another (Anderson and Hubricht, 1938). The introgression of useful agronomic traits from wild to domesticated species has often been used in

agriculture for variety improvement and for increasing genetic vigour. The ease of genetic transfer from a species to another will mainly depend on the reproductive barriers (Rieseberg and Carney, 1998). The process of introgression might have different consequences that I will detail further.

1.1.4 Hybridization in forest tree species

Large zones of contact of potentially hybridizing tree species often occur due to their wide distribution and long distance pollen dispersal that is characteristic of many species (Streiff *et al.*, 1999; Dick *et al.*, 2003; Bâcles *et al.*, 2005; Valbuena-Carabanã *et al.*, 2005; Goto *et al.*, 2006; Byrne *et al.*, 2008). Because of this natural hybridization occurs in a large number of sympatric forest species and the frequency of hybridization in forest trees is amongst the highest of angiosperms. (Craft *et al.*, 2002; González-Martínez *et al.*, 2005; Lexer *et al.*, 2005).

However, natural hybridization, if often documented, has been observed to have different consequences on populations including asymmetrical introgression or persistence of the two parental species. For example, gene flow between *Quercus robur* and *Q. petrea* has been well documented (Bacilieri *et al.*, 1996; Streiff *et al.*, 1998). Morphological analysis of European populations revealed a contrast in differentiation between the two species (Kremer *et al.*, 2002), and genetic differentiation of the two parental species remained with very few individuals not being unassignable to one or the other species (Muir *et al.*, 2000). Muir and Schlotterer (2005) suggested that the polymorphism between the two species was ancestral and a strong post-zygotic selection can explain the low occurrence of hybrids in natural populations. Similar results indicating that hybrids are rare in natural populations have been found for other oak species (Craft *et al.*, 2002; Tovar-Sanchez and Oyama, 2004; Valbuena-Carabanã *et al.*, 2005). The degree of introgression in contact zones appeared to be highly variable. Introgression might be very low or non existent in some species, for example in the complex *Salix alba-Salix fragilis* (Triest *et al.*, 2000). It may also occur with high frequency as reported for *Picea sitchensis* x *glauca* (Bennuah *et al.*, 2004). Sometimes introgression is asymmetric with respect to parental species. For example, Lexer

et al. (2005) showed that gene flow between *Populus alba* and *P. tremula* was more important through the species which had the most favorable ecological condition.

1.2 Species introduction and consequences

1.2.1 Human activity

Human migrations have long aided species transfer from one continent to another. With the development of international exchanges in the 15th century, the introduction of species from different taxa outside of their natural distribution increased in consequence (di Castri, 1989; Hodkinson and Thompson, 1997; Mack *et al.*, 2000; Reichard and White, 2001). During the last decade, this phenomenon has accelerated due to increased human activity related to transport, commerce, and leisure. Wright (1976) noted that between 90 and 95% of the species used by humans are grown outside of their natural range. The deliberate or accidental introduction of novel species by humans have severe consequences on local landscapes, ecosystems and economies (see Anderson and Hubricht, 1938; Thompson *et al.*, 2010). Species introductions are, at present, seen as one of the major causes of biodiversity loss (Vitousek *et al.*, 1997; Sala *et al.*, 2000; Sakai *et al.*, 2001).

After introduction, not all species acclimatize via phenotypic plasticity or genetic adaptation (Williamson, 1996; Jeschke and Strayer, 2005). On one hand, introduced species may become established and part of the local community. On the other hand they can become a severe economic or ecological problem by modifying the ecosystem in which they have became acclimatized; such species are regarded as invasive (Mack *et al.*, 2000; Rosenzweig, 2001). According to Williamson and Fitter (1996), only 10% of introduced species become established and only 10% of these become invasive.

At the global scale, more than 2000 introduced woody species have been inventoried (Richardson, 1998). Forestry plantations at a large scale are

responsible for most woody species introductions (Zobel *et al.*, 1987; Richardson, 1998). The principal reasons for the introduction of new tree species include the economic value of increasing wood production, improving areas with weak natural resources, the search for new products and the 'restoration' of degraded environments (Zobel *et al.*, 1987). The long generation time in trees (in some case over 50 years) means that the consequences and ecological impacts of introduced tree species are difficult to evaluate. Peterken *et al.* (2001) analysed introductions made during the last 20 centuries in England and concluded that not all the ecological consequences can yet be determined due to the low numbers of generations since the original introduction.

1.2.2 From introduced to invasive: the barriers to overcome

Several studies have tried to define the different steps that can lead to species invasion (Williamson, 1996; Richardson *et al.*, 2000). The process is generally described in terms of a series of barriers to overcome. Richardson *et al.* (2000) differentiate three phases: introduction, naturalization and invasion (Figure 1.1). The authors in this case use the term invasion based only on the species capacity to colonize a new environment.

The introduction phase is the first in the process (Figure 1.1), in which a species overcomes the geographical barriers that usually separate its original environment and the one it is introduced to. After the initial introduction phase, naturalization may occur. The individuals face new biotic and abiotic conditions and have to establish a viable population. Once these barriers are overcome, the organisms go through an expansion phase, during which organisms disperse and colonize the new environment. The established species will be regarded as invasive if changes are observed in the host environment or if the presence of the new species leads to economic or ecological problems during the process. The schema are however difficult to generalize due to the lack of knowledge on biological invasive mechanisms (Richardson *et al.* 2000).

There have been several reports in which exotic trees have become invasive outside of their original distribution. Indeed, a few *Eucalyptus* species from Australia planted overseas became invasive in their introduced environment (Rejmánek, 2000; Le Maitre *et al.*, 2002). In a review, Richardson (2006) reported that among the 111 species of pines, 30 are recognized as naturalized in non-native locations and 21 are reported as invasive.

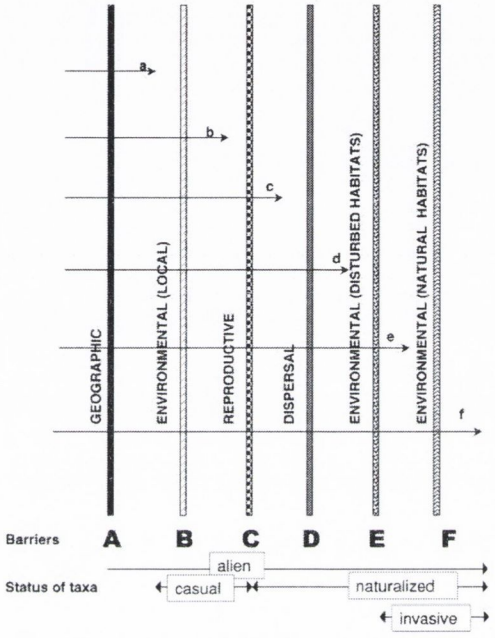

Figure 1.1 Schema of barriers for invasion reproduced from Richardson *et al.* (2000).
The different capital letters (A to F) represent the different barriers: (A) Major geographical barrier(s); (B) Environmental barriers (abiotic and biotic) at the site of introduction; (C) Reproduction barriers; (D) Local/regional dispersal barriers; (E) Environmental barrier(s) in human-modified or alien-dominated vegetation; and (F) Environmental barriers in natural or semi-natural vegetation. The arrows (a through f) indicate the paths followed by taxa to reach different states from introduced to invasive in natural vegetation. Crossing of the barriers is not irreversible.

1.2.3 Consequences of hybridization and introgression after species introduction

Exotic species if inter-fertile with native ones, with overlapping phenology and introduced at proximity of the native community may produce viable hybrids. The consequences of hybridization between introduced species and native species can be divided in two categories (Vila *et al.*, 2000): 1) indirect genetic effects and 2) direct ecological factors. Two types of hybridization are possible following the

introduction of alien species: 1) hybridization between several introduced species and 2) hybridization between native and alien species.

1.2.3.a Genetic effects and genetic pollution

The term 'genetic pollution' is often used (Anttila *et al.*, 1998; Daniels and Sheail, 1999; DiFazio *et al.*, 1999; Muller *et al.*, 2002) and can be defined as 'the movement or introgression of exotic genes from domesticated or other introduced species into native populations following any human disturbances in an environment' (Ellstrand and Hoffman, 1990). If hybrids are fertile, repeated backcrosses with either one of the parental species or both can lead to the introgression of genetic material from a population to another (Anderson and Stebbins, 1954; Rhymer and Simberloff, 1996; Schierenbeck *et al.*, 2005).

If hybridization and introgression are natural phenomena in plants (Arnold, 1997), genetic pollution refers to the introduction of foreign genes under the influence of human intervention. Some factors related to human activity have been found to be able to influence introgression and hybridization, including habitat fragmentation (Vila *et al.*, 2000), climate change, and species introduction.

Several events will facilitate or preserve introgression and invasion by hybridization (Arnold, 1997). The first hybridization event (production of F_1 hybrids) is a key event for pollen-mediated gene flow from exotic plant species. The fertility and fitness of such hybrids is also important as too is the probability of hybrid formation, and the scale in which the exotic plant was introduced.

Exotic gene flow and introgression threatens the integrity of native gene pools (Levin *et al.*, 1996; Rhymer and Simberloff, 1996; Anttila *et al.*, 1998) and in extreme cases results in the complete replacement of native species (Ellstrand, 1992; Ellstrand and Elam, 1993; Rhymer and Simberloff, 1996). Rare or endangered species have been found to be even more sensitive to extinction by hybridization after the introduction of an exotic species by means of demographic swamping or genetic swamping (Ellstrand and Elam, 1993; Levin *et al.*, 1996;

Anttila *et al.*, 1998). Moreover, if hybridization can lead to out breeding depression (Ellstrand and Elam, 1993) it can also lead to hybrids with higher fitness that may have higher competivity than their parents (Arnold and Hodges, 1995).

1.2.3.b Ecological effects

Hybridization has been found to enhance invasion and colonization in some cases (Potts and Reid, 1988; Petit et al. 2004). Major changes in native ecosystems occur when hybrids are able to spread quickly and the alien hybrids can threaten the native species by limiting access to resources or by modifying important component of the community (pollinators, pathogens). Abbott (1992) states that interspecific gene flow can result in the expansion of the range of the hybrids that might then quickly become weeds. Ellstrand and Schierenbeck (2000) reviewed several cases where hybridization had preceded the emergence of successful invasive populations.

1.2.3.c Benefits of hybridization

If hybridization is often seen as a source of genetic contamination (Levin *et al.*, 1996; Rhymer and Simberloff, 1996; Schierenbeck *et al.*, 2005), interbreeding between distinct species or populations can also result in enhanced genetic diversity (Abbott, 1992; Rieseberg *et al.*, 1999a) and provide beneficial novel genetic combinations to populations (Anderson, 1954; Arnold, 2004; Seehausen, 2004). Introduction of novel genes into small, inbred, populations may be beneficial to the future success of that population (Byrne and Macdonald, 2000)

1.2.3.d Exotic pollen flow among tree species

The fact that trees have long life span and are often able to hybridize with other species might have an important impact on determining patterns of introgression (González-Martínez *et al.*, 2005). Several studies have reported introduced tree species hybridizing with native species because of gene flow between planted stands and natural populations (Barbour *et al.*, 2002; Potts *et al.*, 2003a; Vanden

Broeck *et al.*, 2003b; Ziegenhagen *et al.*, 2008). In Europe, the introgression of *Populus deltoides* Bartr. genes into native black polar (*Populus nigra* L.) via gene flow between *P. nigra* and cultivated hybrids *Populus x canadensis* (also known as *Populus x euramericana*) is considered as a real threat for the native species (Vanden Broeck *et al.*, 2003b; Vanden Broeck *et al.*, 2005; Smulders *et al.*, 2008).

1.3 Study species

1.3.1 Oleaceae and *Fraxinus*

The genus *Fraxinus* includes 43 species (Wallander, 2008) and belongs to the Oleaceae family. Oleaceae consists of 25 genera and 600 species distributed across the world from tropical to temperate zones (Wallander and Albert, 2000). Species are either shrubs or trees and are either deciduous or evergreen. *Fraxinus* species are often deciduous trees and appear unique in the family with pinnate, compound, opposite or whorled leaves and dry indehiscent fruit known as samaras, which contain only one seed (Wardle, 1961). Flowers, in general, have one pistil and two stamens, however, flowers can vary from unisexual to bisexual or polygamous (Wallander, 2001). The corolla is usually sympetalous with four lobes ranging in colour from white to pale yellow.

Fraxinus is present across the Northern hemisphere (Hinsinger, 2010) from temperate regions (i.e. *F. excelsior, F. mandshurica*, *F. nigra, F. pennsylvanica*) to subtropical regions (i.e. *F. chinensis*). The genus is divided in five to six sections and three species are treated as *incertae sedis* (Wallander, 2008; Hinsinger, 2010). In Europe, only three species of *Fraxinus* are native: *F. excelsior* L., *F. angustifolia* (Vahl) and *F. ornus* L. In this thesis, the focus is on the two first species.

1.3.2 European ash: *Fraxinus excelsior* and *F. angustifolia*

1.3.2.a Distribution

Fraxinus excelsior and *F. angustifolia* have a widespread distribution across Europe. *Fraxinus excelsior* occurs from Ireland in the west to Iran in the east and from southern Norway in the north to southern Italy in the south (Figure 1.2A). In the mountains, *F. excelsior* can be found up to an elevation of 1600-1800m (Pliûra and Heuertz, 2003; FRAXIGEN, 2005). *Fraxinus excelsior* may occur along a hydric gradient from dry to humid but it generally thrives better on moist, well drained soils and on slopes (Kerr and Boswell, 2004; FRAXIGEN, 2005).

Fraxinus angustifolia has a Mediterranean distribution; it occurs from Spain and Portugal in the west to Turkey, Syria, and Iran in the east (Figure 1.2B). *Fraxinus angustifolia* forests are often found along waterways (river or lakes) implying that *F. angustifolia* is resistant to flooding (Jaeger *et al.*, 2009). However, in Mediterranean regions, it can also be found on drier sites (FRAXIGEN, 2005). Mild climate and precipitation of between 400 to 800mm per year are in general required for adequate growth of *F. angustifolia*. Physiological and biochemical studies (Patonnier *et al.*, 1999; Marigo *et al.*, 2000; Oddo *et al.*, 2002) have highlighted that the two species responded in different ways to water stress during summer: *F. excelsior* relying more on malate, and *F. angustifolia* on mannitol accumulation, for drought tolerance.

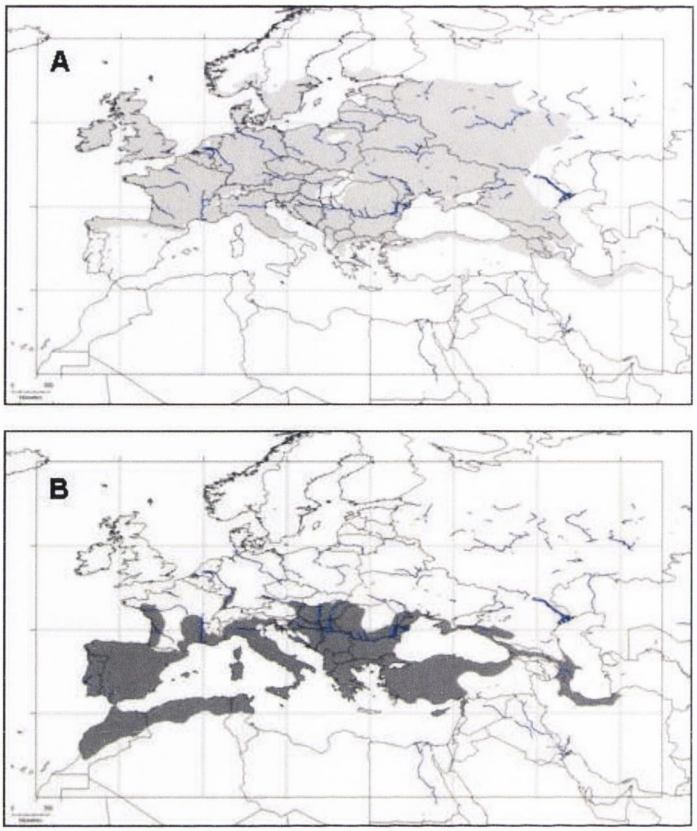

Figure 1.2 Map from Thomasset *et al.* (2011b). **A.** General distribution of *F. excelsior.* **B.** General distribution of *F. angustifolia*

1.3.2.b Morphology, flowering phenology and breeding system

The two species have compound leaves. However, they differ morphologically mainly by the number of leaflets (Rameau *et al.*, 1989; Fernández-Manjarrés *et al.*, 2006). The number of leaflets is reduced in *F. angustifolia* and they are narrower compared to *F. excelsior* which has large leaves generally divided into four to eight pairs of lanceolate leaflets (Figure 1.3A). The buds are in general very dark and are larger in *F. excelsior* compared to the small, brown buds observed in *F. angustifolia* (Figure 1.3B). Moreover, *F. angustifolia* is known for altering its phyllotaxy status during its early years of growth changing from opposite to whorled leaves (Figure 1.3C) which have increased branching and forking (Ramirez de Arellano *et al.*, 2004).

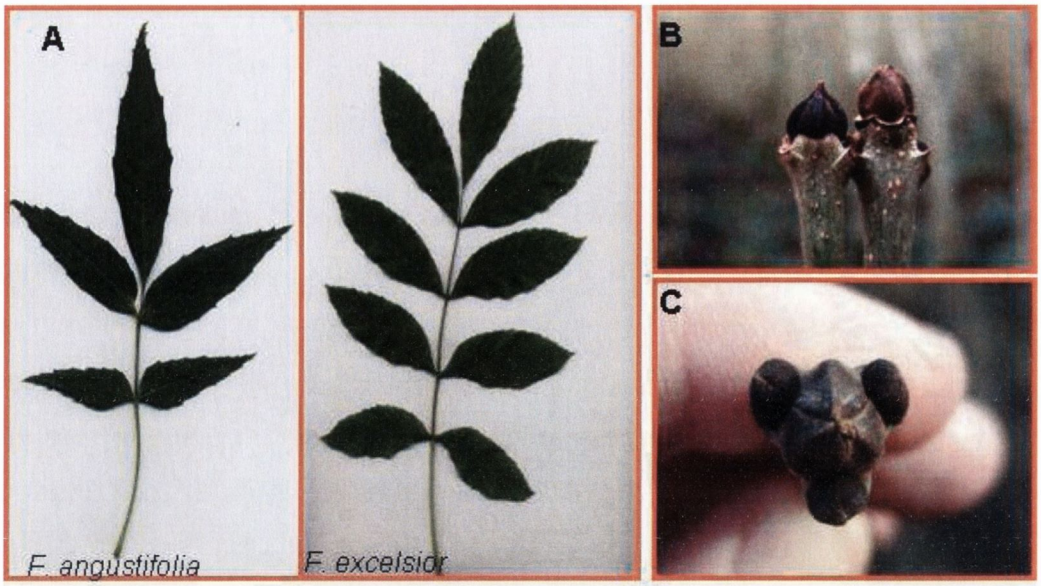

Figure 1.3 Morphology of *F. angustifolia* and *F. excelsior*. **A.** on the left- typical leaf of *F. angustifolia,* and on the right -leaf of *F. excelsior.* **B.** shows the differences in bud colour between the two species, *F. excelsior* (left) and *F. angustifolia* (right). **C.** shows the verticilisation by three - often encountered in *F. angustifolia*.
Source: photos Juan Fernández-Manjarrés and Gerry Douglas

The pollen and seeds of the two species are wind dispersed, both produce winged samaras, usually containing one seed. The flowers cluster in inflorescences and both species are polygamous. Wallander (2001) observed that, for *F. excelsior,* flowers can range from pure male with rudimentary or aborted pistil to pure female with rudimentary of aborted stamens, with any intermediate between this being possible. The same author suggests that the expression of the gender might vary at different levels: i) floral level, ii) inflorescence level, iii) tree level, and iv) a temporal level (gender might vary between years). *Fraxinus angustifolia* is less well documented; however, Wallander (2001) and Rameau *et al.* (1989) reported a polygamous flowering system as for *F. excelsior.* In the Fraxigen project (FRAXIGEN, 2005), a reduced number of flowers per inflorescence was reported for *F. angustifolia* compared to *F. excelsior.* Moreover, the inflorescence of *F. angustifolia* was described as a simple, unbranched raceme while in *F. excelsior* it is a compound inflorescence (panicle). In general, *F. excelsior* flowers later in the season than *F. angustifolia*, from March to the end of April (Gérard *et al.*, 2006b; Gérard *et al.*, 2006c). The flowering time for *F. angustifolia* has been observed between mid December until the end of January in France by Raquin *et al.* (2002a) and in Spain by Jato *et al.* (2004).

1.3.2.c Biogeography and genetic diversity

The first Holocene pollen record of *Fraxinus* in northwest Europe dates to 10000 years ago (Huntley and Birks, 1983) The use of molecular markers, chloroplast microsatellite markers (Heuertz *et al.*, 2004b) and nuclear microsatellites (Heuertz *et al.*, 2004a) revealed the presence of four possible refuges: the eastern Alps, the Iberian Peninsula, Italy, and the Balkans area. The results obtained from the two previous studies with different molecular markers are contradictory. Indeed, the chloroplast markers showed a strong differentiation across Europe (Heuertz *et al.*, 2004a) suggesting a rapid expansion from the refuges and thus a reduction of the gene flow among newly colonised sites. In contrast, the nuclear markers (Heuertz *et al.*, 2004b) showed high genetic homogenization between North and Central European populations, suggesting a high frequency of gene flow via pollen. By combining data from chloroplast markers from *F. angustifolia* and *F. excelsior* (Heuertz *et al.*, 2006), the existence of the four refuges previously mentioned has been confirmed, and two new refuges were also detected (one in southern Italy and one in Turkey). Ferrazzini *et al.* (2007) confirmed the possible existence of a huge refuge in Italy during the last glaciation event due to the high genetic diversity observed.

Long distance pollen dispersal has been observed in *F. excelsior* (Bâcles *et al.*, 2005; Gérard *et al.*, 2006c; Bâcles and Ennos, 2008) as expected for tree species that are wind pollinated. The distances for pollen dispersal are in general higher than the distances for seed dispersal (Heuertz *et al.*, 2003; Morand, 2003). The use of nuclear microsatellite markers revealed, at a local scale, a low differentiation among populations, high allelic polymorphism, and consistent, inbreeding within populations (Heuertz *et al.*, 2001; Morand *et al.*, 2002; Ferrazzini *et al.*, 2007; Sutherland *et al.*, 2010).

1.3.2.d Hybridization between F. angustifolia and F. excelsior

Given the present day distribution of the two species (Figure 2A and Figure 2B), a widespread contact zone occurs between them, running from France to Turkey. The existence of natural hybrids has long been suspected on the basis of individuals with intermediate morphology (Rameau *et al.*, 1989). Artificial crosses with either *F. angustifolia* as pollen donor or *F. excelsior* were found to be possible (Raquin *et al.*, 2002b). Studies of natural populations using molecular markers have demonstrated the existence of natural hybrids (Fernández-Manjarrés *et al.*, 2006; Gérard *et al.*, 2006b; Heuertz *et al.*, 2006). However, hybridization appeared to be restricted to some areas with special climatic conditions, such as river valleys (Fernández-Manjarrés *et al.*, 2006) but may also be restricted at a temporal level (Gérard *et al.*, 2006c). Indeed, different patterns of hybridization have been observed in two different valleys in France (Fernández-Manjarrés *et al.*, 2006). In the Saône Valley (east of France), hybrid individuals did not show morphological introgression as was observed for hybrid individuals from the Loire Valley (Southwest of France). Moreover, individuals from the Loire valley were genetically more similar to *F. angustifolia.* A negative correlation between *F. angustifolia* genotypes and winter temperatures suggested the weather could be a limiting factor for the expansion of *F. angustifolia* to the north.

1.3.3 History of ash trees in Ireland

1.3.3.a Colonization

There have been several theories proposed on the colonization routes of Irish tree species after the last glaciation. Some authors suggest that colonization could have occured trough a east-west route across the Irish sea (Dumolin-Lapegue *et al.*, 1997; Petit *et al.*, 2002). However, some studies (Mitchell, 2003; Mitchell, 2006) proposed a temporal variation in pollen data suggesting that tree invasions did not cross the Irish sea but came from the south. The genetic diversity and the distribution of plastid haplotypes of Irish oak compared to European oak supported this route (Kelleher *et al.*, 2004; Harbourne, 2005).

Ash was present in Ireland about 9,000 years ago when the first humans reached the country (Mitchell, 2003). Neolithic farmers arrived in Ireland around 6,000 years ago and started to alter the landscape by opening woodland for agriculture, using the wood of trees for housing. The overexploitation of woodland led to the destruction and the decline of the forest cover of Ireland over the last 4,000 years. Today almost all woods in Britain and Ireland are managed and less than 8% of Irish land is forest and only 1.6% is represented by ash (Lefort *et al.*, 2000).

1.3.3.b Use of ash

Fraxinus excelsior is a noble hardwood with important economic value in Ireland. Commercially, common ash is attractive to farmers due to its short rotation time compared to oak. Its wood has many properties that make it suitable for furniture, fences, veneer and boat buildings. It is strong and robust but with pliable and elastic properties that make it difficult to break. In the past, it was used in the production of spears, axe-handles walking sticks. More recently, it has been used for sport equipment as hockey sticks and in Ireland especially for the manufacture of the caman, the stick used in the popular Irish game, hurling (Harbourne, 2005).

1.3.4 Recent importation and problems

Due to the development of forestry activities in the nineteen nineties, farmers were encouraged by Irish government grants to plant native broadleaves. At this time, nursery stocks were too low to be sufficient to meet the demands of farmers. There was no legislation regarding seed provenances and therefore seeds and seedlings from *F. excelsior* were bought on the continent from different providers. At the time of planting, trees showed normal growth and usual vigour. Seeds and seedlings were imported from continental Europe over a period of several years (from 1992 to 2000). After planting, a few trees started to show unusual morphology with very small, pale buds. Growth was very slow and trees were small compared to *F. excelsior*. Moreover, trees demonstrated unusually high rates of forking and curved stems (Figure 1.4); they were also more susceptible to gale attacks. Foresters began to wonder if the problem was due to the use of *F.*

excelsior provenances ill adapted to the Irish environment or if the source of seeds was not pure *F. excelsior* but rather hybrid individuals between *F. angustifolia* and *F. excelsior* that have been found on the continent. Today, there are more than 100 plantations in Ireland, with introduced material that show poor vigour and poor stem growth.

Figure 1.4 Examples of poorly shaped trees observed on plantations of introduced trees from continental Europe.
Source: Gerry Douglas

1.4 Aim of the thesis and outline

In this thesis, I investigated the nature of introduced ash material in Irish plantations and examined the risk of introgression of the introduced provenances into the native environment. For this purpose, I mainly worked on two plantations of introduced ash, which have now reached maturity age. This thesis also aimed to improve knowledge and understanding of hybridization in ash trees in general.

In the **second** chapter, I attempted to elucidate the morphology of hybrid individuals by working on trees raised from artificial crosses and grown on a common garden at Orsay University, France. Chapter II was important in helping to understand basic patterns of inheritance in F_1 hybrid trees and to help me select morphological characters to use in the subsequent study of Irish plantations.

In the **third** chapter, I used six nuclear microsatellite markers and morphological markers to identify the introduced trees i two different Irish plantations. To classify the trees, I used a combination of multivariate analyses and different assignment approaches, implemented in the software STRUCTURE and NEWHYBRIDS.

The aim of the **fourth** chapter was to assess the phenology of introduced and native ash to help determine the potential for gene flow between alien and native ash. I also investigated the vegetative phenology to determine whether or not it could be a contributing factor for the poor growth and bad stem form observed in plantation trees under current Irish conditions.

In the **fifth** chapter using direct and indirect methods to measure pollen dispersal, I attempted to assess whether or not geneflow is occurring between introduced trees and native trees and tried to elucidate the direction of pollen flow. I measured potential gene flow by studying progeny arrays collected from native and introduced mother ash trees with six nuclear microsatellite markers.

In the **sixth** chapter, I compared the seed production, viability, germination and morphological traits of introduced plantation trees with seeds from native Irish *F. excelsior.* I investigated the fitness of introduced trees to help determine the survival probability of seeds from introduced trees in the Irish landscape.

The last chapter (**seventh**) is a general discussion about the major findings of this thesis, with recommendations on management strategies when hybrid individuals are suspected. I also discuss the influence of climate change (mainly temperature and precipitation) on hybridization and introgression in Ireland by presenting climate niche modelling results for a projected future climate scenario in Europe.

Chapter II

Molecular and morphological characterization of reciprocal F_1 hybrid ash (*Fraxinus excelsior* × *F. angustifolia*, Oleaceae) and parental species reveals asymmetric character inheritance

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Abstract

Hybridization between *Fraxinus excelsior* and *F. angustifolia* commonly occurs. However, identifying hybrids in natural populations is difficult because the closely related parental species share many morphological characters and the inheritance pattern of these characters in hybrids is unknown. We evaluated how morphological characters are inherited and whether morphological and molecular markers can efficiently discriminate artificial first generation hybrids. Reciprocal F_1 hybrids of *F. excelsior* with *F. angustifolia* were examined using six microsatellite DNA marker loci and 14 morphological characters. Plants were divided into four groups (*F. angustifolia*; *F. excelsior*; F_1 hybrid with *F. angustifolia* as maternal parent; and F_1 hybrid with *F. excelsior* as maternal parent). The F_1 hybrids showed intermediate morphology in most characters and the range of variation overlapped with the parental species. Canonical discriminant analysis using only the morphological traits separated the four groups without any overlap between the two parental species. F_1 hybrids from different maternal parent species could therefore be distinguished. A further analysis that combined molecular and morphological traits allowed clear separation of the four groups and strongly confirmed the *a priori* defined groups. Our results suggest that intermediate characters can be expected in F_1 hybrids of ash but differences may be observed due to maternal / paternal affects.

2.1 Introduction

Natural interspecific hybridization has been recognized as an important process in evolution (Stebbins, 1959; Rieseberg, 1997; Barton, 2001) and is common in many plant species (Mallet, 2005). Hybridization may have several consequences including hybrid zone formation, hybrid speciation, and genetic introgression (Arnold, 1997; Rieseberg *et al.*, 1999b; Rieseberg and Willis, 2007). The identification of parents and progeny of interspecific hybrids can be a difficult process, particularly if gene flow occurs between parental species and hybrid progeny. After a hybridization event, individuals generally display a mosaic of morphology from their parental species (Rieseberg and Ellstrand, 1993). In some cases, intermediate morphology between the two parental species is observed, while the hybrid resembles one parent more than the other or the hybrid may look unlike either parent (Ponton *et al.*, 2004; Ito, 2009). Because of this, the use of single or multiple morphological characters may sometimes be insufficient for hybrid recognition, especially after several generations of backcrossing (Rhymer and Simberloff, 1996).

The development of molecular markers has therefore facilitated the detection and study of hybrids, provided that alleles specific or quasi-specific to either species can be found. Molecular markers have been shown to be useful to differentiate and identify hybrids (Ishida *et al.*, 2003; Ebina and Otho, 2006; de Conceição *et al.*, 2008). However, in closely related species a large number of molecular markers is often required for efficient distinction (Kelleher *et al.*, 2005). A simulation study by Boecklen and Howard (1997) suggested that the number of molecular markers needed for hybrid detection / differentiation increases exponentially with the number of cycles of backcross. They showed that 4 to 5 codominant molecular markers provided a coarse classification of individuals in hybrid zones but that the number of markers needed to distinguish between advanced backcrosses from parental populations might be as high as 70. Combining morphological and genetic datasets into a multivariate analysis could increase the discriminatory power of the analysis.

The shape and architecture of the leaf can influence the interaction of the species with the environment, which is particularly clear with water use related traits. Sun leaves are often smaller than shade leaves (Fitter and Hay, 2002). For example, studies by Bragg and Westboy (2002) on sclerophyll woodland trees and Poorter and Rozendaal (2008) on 38 tropical trees species showed that sun species had smaller leaves than shade species. Moreover, reducing the transpirational surfaces of leaves is an important drought adaptation (Rieger and Duemmel, 1992; Van Splunder *et al.*, 1996). At a microscopic level, adaptation to drought is also influenced by number and size of stomata. Nevertheless, stomatal density is a key character often overlooked in systematic and population biology studies of hybridizing plants that include species from wet and dry areas. Several studies have shown that stomatal density has a heritable basis (Martin and Stimart, 2005; Rae *et al.*, 2006). Variation in stomata can be related to different local adaptations to water levels. The closure of large stomata is slower than smaller ones and this increases the potential for hydraulic dysfunction. There is often a negative relationship between the size of the stomata and the stomatal density in several species (Aasamaa and Sõber, 2001). Furthermore, stomatal number and size can also be highly dependent on age of the individuals (Dai *et al.*, 1995; Elias, 1995; Coupe *et al.*, 2006). Hence, stomatal characters should be used with caution and ideally with evenly aged common garden material.

Two closely related species, *Fraxinus excelsior* L. (common ash) and *Fraxinus angustifolia* Vahl (narrow-leaved ash), are known to hybridize profusely (Rameau *et al.*, 1989; Jeandroz *et al.*, 1996; Heuertz *et al.*, 2001; Heuertz *et al.*, 2004b; Gérard *et al.*, 2006b). *Fraxinus excelsior* has a more northern and Atlantic distribution (Pliûra and Heuertz, 2003) and *F. angustifolia* a more southerly distribution (Thomasset *et al.*, 2011b) that replaces *F. excelsior* towards the Mediterranean. However, they have a sympatric zone, running at intermediate latitudes from Turkey to Northern Spain including France. *Fraxinus angustifolia* generally differs from *F.* excelsior in a number of morphological characters (Fukarek, 1960; Fernández-Manjarrés *et al.*, 2006) such as the number of leaflets and the colour of the leaf buds. The two species have compound leaves but in *F. angustifolia,* they are divided into fewer and narrower leaflets than *F. excelsior. Fraxinus excelsior* has large leaves generally divided into four to eight pairs of

lanceolate leaflets, compared to *F. angustifolia* that has one to four pairs of leaflets. Fernández-Manjarrés et al. (2006) were able to detect natural hybridization using leaf morphological characters and molecular markers for naturally growing hybrid zones in France. The differentiation between the two species and their putative hybrids was mainly due to a reduction in leaflet number and size in *F. angustifolia* and consequently a reduction in total leaf area. It is unknown if *F. angustifolia* compensated for leaf surface area in branches that exhibited three-whorled leaves in contrast to *F. excelsior* that always bears opposite leaves. However, despite that a micromorphological study in stomatal density by (Kremer *et al.*, 2008) confirmed significant differences between *F. excelsior* and *F. angustifolia*, no investigation have been undertaken yet in hybrid populations.

This study characterizes a collection of artificially produced F_1 hybrids between *F. excelsior* and *F. angustifolia* that was originally used for verification of Mendelian inheritance of nuclear microsatellite markers by Raquin *et al.* (2002b). The main objective of our study was to examine whether discrimination between the two parental species and artificial F_1 hybrids is possible with macro and micro-leaf morphological characters, determine how they relate to microsatellite marker segregation, and evaluate whether asymmetric character inheritance is observed.

2.2 Materials and methods

2.2.1 Plant material

The samples included in this study are described in table 2.1. Five *F. excelsior* (three hermaphrodites: EXC_{H1}, EXC_{H2}, EXC_{H3}, and two males: EXC_{M1}, EXC_{M2}) and two hermaphrodite *F. angustifolia* (ANG_{H1}, ANG_{H2}) were chosen as parents in controlled crosses. Artificial crosses and *in vitro* culture from the seeds were performed as described in Raquin (2002a). Potential F_1 hybrids were planted in a common garden at Paris-Sud 11 University, Orsay, France, and were, at the time of writing, 12 to 14 years old. A total of 19 wild collected 'pure' *F. excelsior* and 20 'pure' *F. angustifolia* plants were also raised from seed and grown in the same

common garden (seed source is provided in table 2.1) and used as 'reference' material.

Table 2.1 Source of analysed individuals, geographical coordinates and sample size.

Species and source of material	Population	Origin location (longitude / latitude)	*n*
Fraxinus excelsior	Saint Paul de Salers	0°11' / 49°21'	9
	Saint Gobain	3°22' / 49°35'	10
Fraxinus angustifolia	La Môle	6°27' / 43°12	10
	Cogolin	6°31' / 43°15'	10
Individuals from common garden plot			
Fraxinus excelsior	EXC_{H1}, EXC_{H2}, EXC_{H3} EXC_{M1}, EXC_{M2}	2°11' / 48°41'	5
Fraxinus angustifolia	ANG_{H1}, ANG_{H2}	2°11' / 48°41'	2
F_1 Hybrids between *F. excelsior and F. angustifolia*			
Code: female x male			
F. angustifolia x *F. excelsior*	ANG_{H2} x EXC_{M2}; ANG_{H2} x EXC_{M1}	2°11' / 48°41'	19
F. excelsior x *F. angustifolia*	EXC_{H1} x ANG_{H2}; EXC_{H2} x ANG_{H2}; EXC_{H2} x ANG_{H1}; EXC_{H3} x ANG_{H1}	2°11' / 48°41'	19

2.2.2 Nuclear microsatellite marker assessment

Genetic analysis on 80 potential hybrids was conducted to confirm interspecific hybridization in the F_1 material. F_1 trees were raised from 1996 to 1998 from *F. angustifolia* or *F. excelsior* as the maternal parent, and named respectively F_{1ANG} and F_{1EXC}. DNA analysis was also used on the 19 *F. excelsior* (EXC) and the 20 *F. angustifolia* (ANG) reference plants. DNA was extracted from fresh leaf material using Qiagen Plant Mini Kits ® (QIAGEN) following the manufacturer's instructions. All samples were genotyped using six microsatellite markers, namely FEMSATL 4, FEMSATL 11, FEMSATL 12, FEMSATL 16, FEMSATL 19 (Lefort *et al.*, 1999) and M2–30 (Brachet *et al.*, 1999) which have previously been used successfully to study the population genetics of common ash (Heuertz *et al.*, 2001; Morand *et al.*, 2002). PCR reaction methodology followed Morand et al. (2002). Verification of putative hybrids was based on the comparison between expected

and observed multilocus genotypes. Allele sharing was evaluated between potential parents using the likelihood based software Cervus Version 3.03 (Kalinowski *et al.*, 2007) and paternity was assigned with a threshold of 95% (a strict confidence level; Marshall *et al.* 1998). Null allele frequencies and the paternity exclusion probability for the second parent across all loci were estimated using the same program.

2.2.3 Morphological assessment

Fully expanded leaves from genetically verified F_1 hybrids were sampled from the south side of the upper crown (sun leaves) to minimize variation in morphology resulting from position on the tree. Leaf samples from the 19 wild-collected *F. excelsior* and 20 *F. angustifolia* were also taken (Table 2.1). For each tree, three leaves were collected, air dried and pressed. Analysis of morphology was carried out using 14 continuous quantitative variables on each leaf. Nine traits were dimensional: leaflet length (LeafletL), leaflet width (LeafletW), leaflet area (LeafletA), apex angle of the terminal leaflet (AA), internode length between the second and third leaflet (IL), total leaf area (LA), total leaf length (LL), total leaf width (LW), and total dry leaf weight (W). Leaflet measurements were always taken on the second leaflet pair (in the direction from the petiole towards the leaf apex). LW and LeafletW were measured on the widest part of the leaf and the leaflet, respectively. The apex angle (AA) measurement was a direct output included automatically in the WinDIAS Image Analysis System (Delta-T Devices, Cambridge, UK). Three variables were discrete: number of leaflets per leaf (LeafletN), number of teeth present on the second leaflet (TeethN), and stomatal density on the second leaflet (LeafletS). Two continuous transformed characters were included: leaflet shape, which is the ratio between leaflet length and leaflet width (LftL.LftW), and leaf shape, which is the ratio between total leaf length and total leaf width (LL.LW). The dimensional measurements were made with the WinDIAS Image Analysis System (Delta-T Devices, Cambridge, UK).

Stomata were counted on epidermal peelings taken from the abaxial leaflet surfaces. The impression was obtained by applying a layer of nail varnish to the

dried leaf and then peeling it off. The epidermal peelings were mounted on microscope slides and observed at 400-x magnification with an Olympus BH-2 microscope and electronic image analysis equipment (Camera: UI – 1545LE and Quick Photo Camera 2.3, Software, Promicra). Stomatal counts were conducted for each of 10 different fields of view for each leaf sample. The number of stomata was counted per field of view and their densities calculated as number of stomata per mm^2.

2.2.4 Analysis

2.2.4.a Morphometric characters

Statistical analysis included both univariate and multivariate methods. Data analysis for basic statistics and data transformation were performed using R statistical computing software (2009, R Foundation for statistical software) and Statistica v.6.0 (Statsoft, 2004). All variables were tested for normality using the Shapiro-Wilk W-test (Shapiro and Wilk, 1965) and the Bartlett test for variance homogeneity (Bartlett, 1937). In order to meet the normality criterion, the variables LA, W, LeafletS and TeethN were log transformed. One-way analysis of variance and Tukey HSD post-hoc tests (at 5% probability) were used to make comparisons among the different groups. All variables were standardized using the following formula to avoid bias in the analysis due to scale differences in the measurements: Std. Score=(raw score - mean) / std. deviation.

A multivariate stepwise discriminant analysis (Legendre and Legendre, 1998) was carried out with four categories (parental species and the two different hybrid types) to determine which traits would be most useful to distinguish between each species. By using a stepwise method, the variables were added one at the time on the basis of a Wilks' lambda test. The characters that showed no significance for the *F* test ($P>0.05$) were considered as contributing the least to the discriminatory power of the model and were removed. In consequence, the final CDA (canonical discriminant analysis) was performed with a subset of the original variables. The resultant discriminant functions were also used to assign individuals into each group based on their posterior probability.

2.2.4.b Molecular data

Genotype data and allele frequencies were used to quantify genetic polymorphism among and within parental species and their hybrids. We calculated the mean number of alleles per population (A), the mean number of effective allele (A_e), the observed heterozygosity (H_o), the expected heterozygosity (H_e) and the mean number of private alleles per locus (GenAlEx 6.3; Peakall and Smouse, 2006). A kinship estimator between pairs of sibling within an artificial family (F_{ij} of Loiselle et al. 1995) was computed using SPAGEDI version 1.2 software (Hardy and Vekemans 2002). Means were compared using a Kruskal-Wallis test implemented in the software Statistica v.6.0 (Statsoft, 2004).

2.2.4. c Combined molecular and morphological data analyses

A principal coordinates analysis (PCoA) on the matrix of pairwise genetic distance (Nei 1978) was performed using GenAlEx 6.3 (Peakall and Smouse, 2006). A second canonical discriminant analysis using the first six PCoA factors as input variables and the standardized morphological data was performed using Statistica v.6.0 (Statsoft 2004). The canonical scores for individuals from the different groups were retained to plot the two first canonical variates.

2.3 Results

2.3.1 F_1 identification

The four controlled cross groups (the two parental species, the F_{1ANG} and the F_{1EXC}) were genotyped. Of 48 analysed seedlings with *F. angustifolia* as pollen donor, 19 were identified as F_1 hybrids (39.6%). For the two controlled crosses involving *F. excelsior* as a pollen donor, 19 plants were recognized as F_1 hybrids among 32 seedlings (59.4%). For 46.2% of the individuals paternity was unresolved at the 95% confidence level, five individuals (i.e. 6.2%) were assigned to another potential father present in the study. Non-assigned individuals or

individuals which were assigned to the expected father were possibly the results of pollen contamination (Raquin et al. 2002a) and discarded in further analyses. We did not detect the presence of null alleles.

2.3.2 Morphological differentiation

The values (mean ± standard deviation) for the 14 morphological variables in the parental and hybrid groups are shown in Figure 2.1 and Figure 2.2. *Fraxinus angustifolia* and *F. excelsior* had morphologically distinct features in most leaf dimensions. The results of the Tukey HSD post-hoc between the two parent species indicated that 13 of 14 quantitative characters were significantly different between *F. angustifolia* and *F. excelsior* ($P<0.05$). Most of the dimensional measurements of the leaf and leaflets (Figure 2.1 and Figure 2.2) were larger in *F. excelsior* than in *F. angustifolia*. The only variable not significantly different between the parental species was internode length. The mean number of leaflets was significantly ($P<0.05$) smaller in *F. angustifolia* (7.9 ± 2.37) than in *F. excelsior* (11.95 ± 1.82) (Figure 2.2). The mean number of teeth was significantly ($P<0.05$) smaller in *F. angustifolia* (11.95 ± 3.1) than in *F. excelsior* (25.27 ± 6.1). Stomatal density (LeafletS) was the only variable found to show significantly higher values in *F. angustifolia* than *F. excelsior* (Figure 2.1).

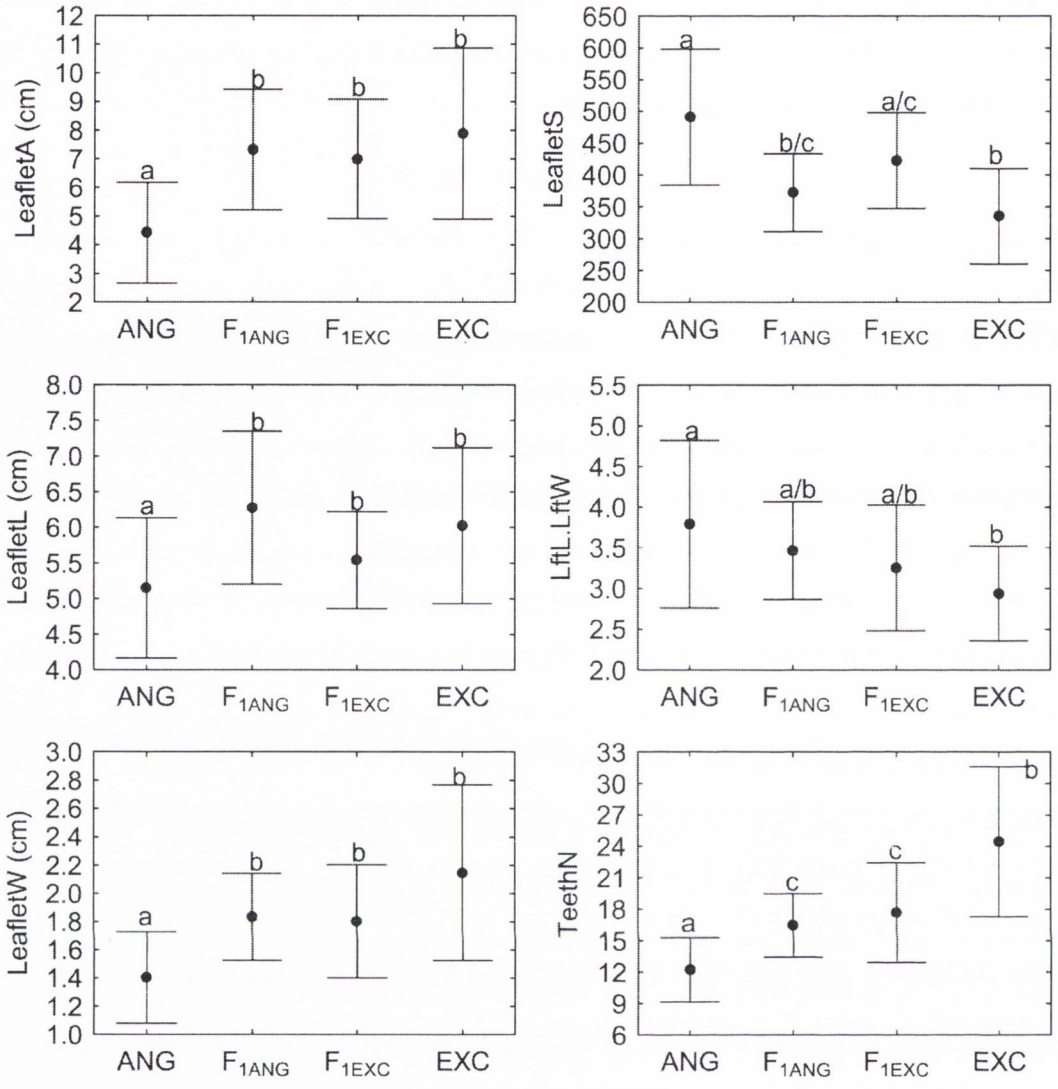

Figure 2.1 Means and standard deviations of morphological characteristics. Comparisons include *F. angustifolia* (ANG), *F. excelsior* (EXC), their F_1 hybrid with *F. angustifolia* as mother (F_{1ANG}) and their F_1 with *F. excelsior* as mother (F_{1EXC}).

One-way analysis of variance and multiple comparisons (HSD Tukey Test, unequal n) were carried out. Differences in letters above the bars indicate groups that differ significantly at $P<0.05$. For the variables LeafletS and TeethN, the ANOVA tests were carried out on the log-transformed variables. LeafletL: leaflet length, LeafletA: leaflet area, LeafletN: number of leaflets per leaf, TeethN number of teeth present on the second leaflet, LeafletS: stomatal density on the second leaflet, LftL.LftW: ratio between leaflet length and leaflet width .

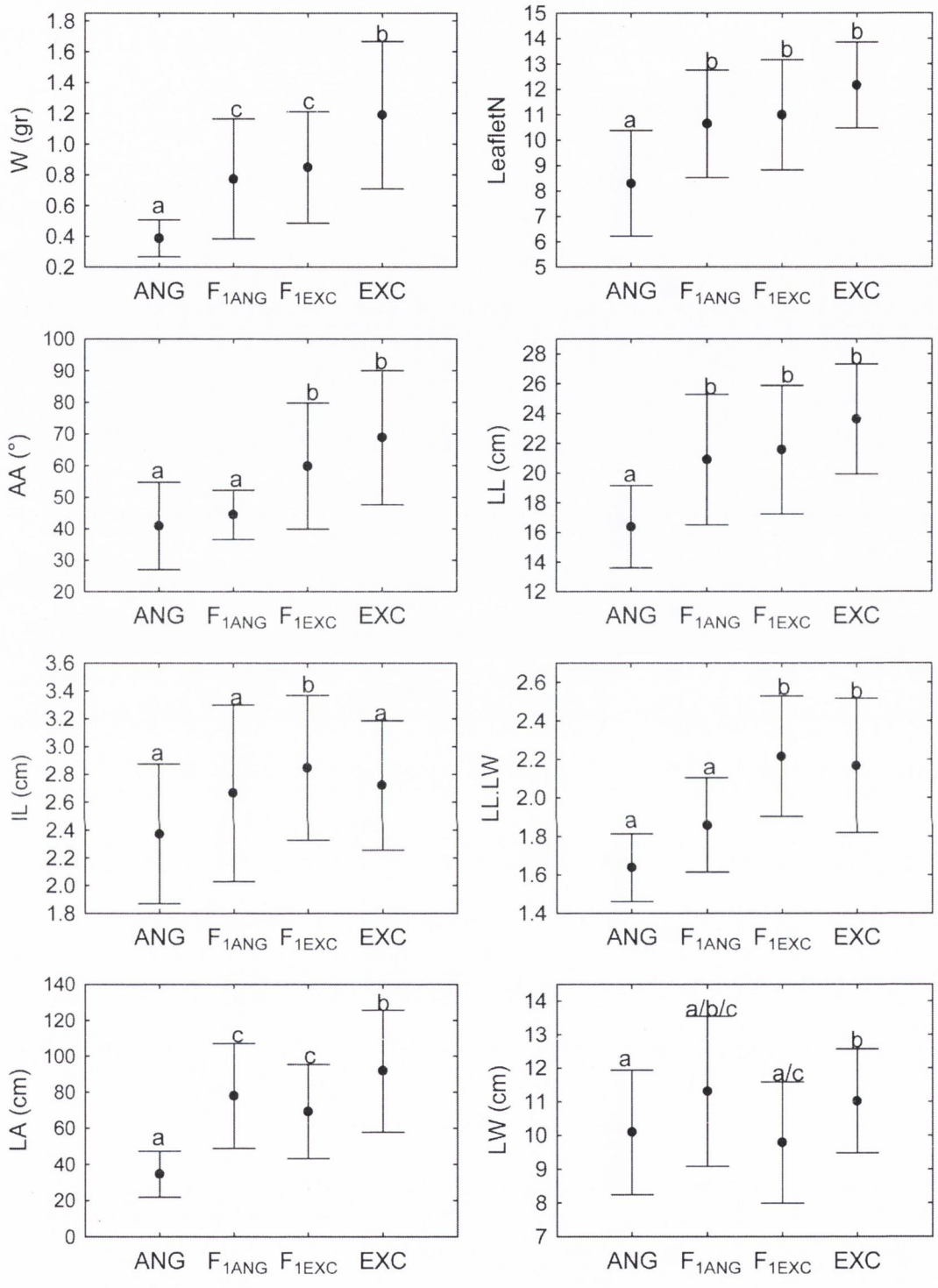

Figure 2.2 Means and standard deviations of morphological characteristics. Comparisons include *F. angustifolia* (ANG), *F. excelsior* (EXC), their F_1 hybrid with *F. angustifolia* as mother (F_{1ANG}) and their F_1 with *F. excelsior* as mother (F_{1EXC}).

One-way analysis of variance and multiple comparisons (HSD, Tukey Test, unequal n) were carried out. Differences in letters above the bars indicate groups that differ significantly at $P<0.05$. For the variables Leaf Area (LA) and weight (W), tests were carried out on the log-transformed

variables. LL.LW: ratio between total leaf length and total leaf width. LeafletW: leaflet width AA: apex angle, IL: internode length, LA : total leaf area, LL : total leaf length, LW : total leaf width, W: total dry leaf weight.

The two different types of F_1 hybrid, with either *F. excelsior* (F_{1EXC}) or *F. angustifolia* (F_{1ANG}) as maternal parent, were morphologically intermediate between the two parental species, although the ranges of values for hybrids generally overlapped those of one parental species. Moreover, the two types of hybrids did not completely cluster together. Only three of the variables were significantly different ($P<0.05$) between the two types of F_1 and both parental species. These were number of teeth (TeethN) (Figure 2.1), leaf area (LA) and leaf weight (W) (Figure 2.2). For five of the characters (LeafletL, LeafletW, LeafletA, LL, and LeafletN) there was no significant difference between *F. excelsior* and the F_1 progeny from reciprocal crosses. However, for these characters the F_1 hybrids were significantly different from *F. angustifolia*. For the characters apex angle of the terminal leaflet and the ratio of leaflet length to width (LftL.LftW), the F_{1ANG} were not significantly different from *F. angustifolia*; in the same way the F_{1EXC} were not different from *F. excelsior* (Figure 2.2). For the variable internode length (IL) only the F_{1EXC} was different from the three other cross groups. The ratio of leaflet length to width (Figure2.1) and the total leaf width (Figure2.2) were similar in each of the reciprocal hybrid types. *Fraxinus excelsior* had a lower density of leaf stomata than *F. angustifolia* (Figure2.1). The two hybrid types were also not significantly different from each other for stomatal density and also *F. angustifolia* and the F_{1EXC} were not significantly different from each other. However, F_{1ANG} was significantly different from *F. angustifolia*, and F_{1EXC} was significantly different from *F. excelsior*.

Table 2.2 Results of the stepwise discriminant analysis on morphological characters. * indicates the variables with $P<0.05$, which were included in the model.

	Wilks' Lambda	Partial Lambda	F-remove (3,62)	*P*-level
LeafletL	0.0636	0.948	1.1341	0.3423
LeafletW	0.0691	0.8727	3.0156	0.0365*
LeafletA	0.0729	0.8267	4.331	0.0078*
AA	0.075	0.8043	5.0275	0.0035*
LL	0.0625	0.9654	0.7404	0.532
LW	0.0617	0.9779	0.4678	0.7058
IL	0.068	0.887	2.6319	0.0578
LeafletN	0.0623	0.9675	0.6939	0.5592
LftL.LftW	0.0619	0.9742	0.5479	0.6514
LL.LW	0.0653	0.9229	1.7277	0.1705
lnLA	0.0872	0.6918	9.2084	0.0000*
lnW	0.0744	0.8103	4.8398	0.0043*
lnTeethN	0.079	0.7629	6.4245	0.0007*
lnLeafletS	0.0767	0.7859	5.6296	0.0018*

The stepwise discriminant analysis was used to select morphological variables which best discriminated among the four groups (Table 2.2). Seven variables (LeafletL, LL, LW, IL, LeafletN, LftL.LftW, and LL.LW) were removed in the stepwise canonical discriminant analysis as they contributed very little to the model ($P>0.05$). The first canonical correlation explained over 82% of the variation, the second explained 14% and the third explained the remaining 4%. The standardized canonical coefficients showed that, for the first canonical variable, leaf width (lnW) and number of teeth (lnTeethN) had the most discriminatory power to separate the four groups. For the second canonical variable, the total leaf area (lnLA) was the most powerful variable to discriminate between the two species (Table 2.3).

Table 2.3 Morphological traits and the standardized coefficients (Can1, Can2, and Can3) from the canonical discriminant analysis based on the morphological characters used to differentiate the two parental species and their hybrids.

	Can1	Can2	Can3
LeafletW	0.4414	0.1195	1.6626
LeafletA	-0.5682	0.1228	-1.1945
AA	0.4683	0.428	-0.7289
lnLA	-0.068	-2.1169	-0.6101
lnW	0.666	1.481	0.2233
lnTeethN	0.5935	0.3161	0.1027
lnLeafletS	-0.5027	0.1961	-0.6154

This discriminant analysis based only on the morphological characters clearly separated the two species, *F. excelsior* and *F. angustifolia*, with no overlap (Figure 2.3). The two types of hybrids were positioned in between these groups but overlap with both parental species and with each other was observed. Multivariate classification of individuals, according to their canonical scores, placed 86% of individuals sampled in *F. angustifolia* group within the predicted control group. The misplaced individuals were put into the F_1 hybrid group and never into the *F. excelsior* control group. For the *F. excelsior* group, 86% were also placed in the predicted group; the other 14% were put into the F_1 hybrids. 82% of the F_{1ANG} group and 78% of the F_{1EXC} group were placed in their predicted category. In sum, morphological markers provided a good classification of the individuals although for some cases it was not possible to distinguish between the parental species and the hybrids.

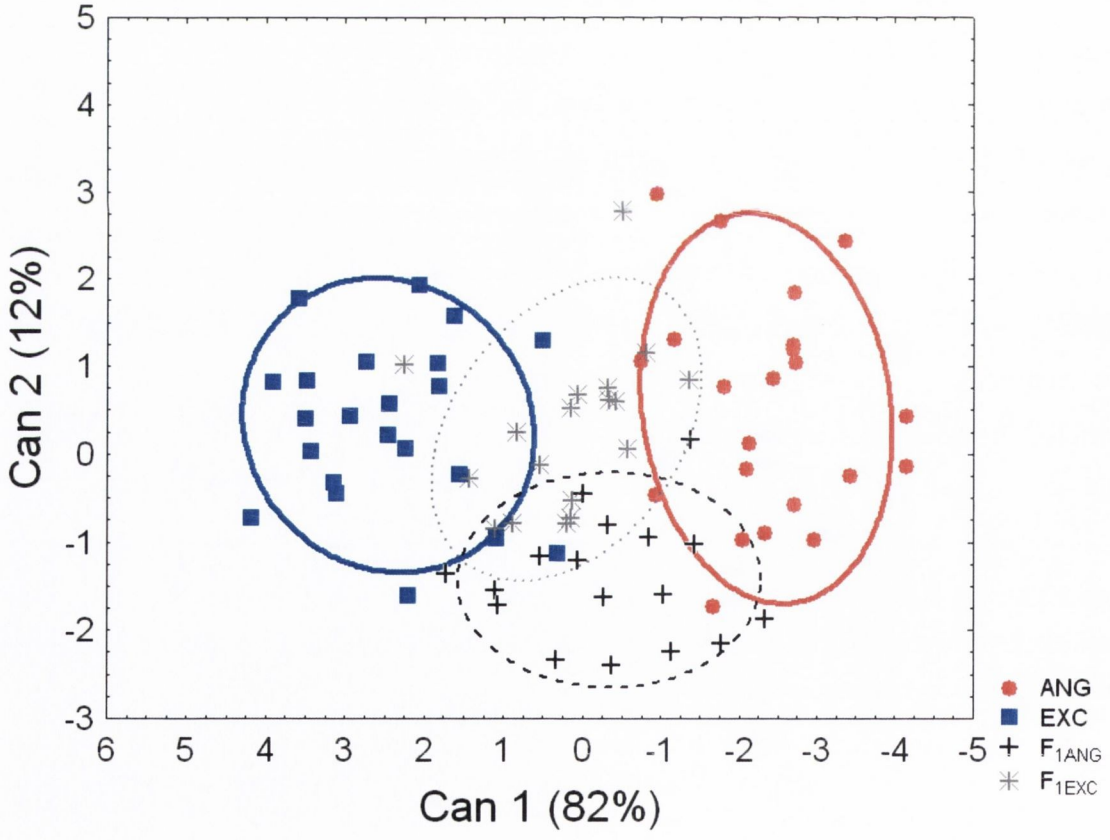

Figure 2.3 Plot of canonical scores of the seven morphological traits for each of the individuals of *F. excelsior*, *F. angustifolia* and their two types of hybrids. The plot indicates the percentage of variation of each canonical variate axis and the canonical score of each species. 95% confidence intervals around the mean canonical score for *F. excelsior*, *F. angustifolia* and the two different types of F_1 hybrid are indicated with ellipsoids.

2.3.3 Molecular markers

A total of 107 alleles were scored from the six nuclear loci across the four different groups. The locus M2-30 was the most polymorphic with a total of 29 alleles; in contrast the locus FEMSATL16 exhibited the lowest level of diversity with only 5 alleles. The mean genetic diversity summary statistic values per group are shown in table 2.4. The total number of alleles was lower in the F_1 hybrids than parental species because of the number of parental individuals involved in the crosses was low. Relatedness was similar for all progenies as the average within family kinship was non significant (Kruskal-Wallis test; H (5, N=106)=4.63, *P*=0.327). We thus assumed that the subsequent analyses based on genetic distances were not biased in a significant way.

Table 2.4 Genetic diversity statistics for *F. angustifolia* (ANG), *F. excelsior* (EXC) and reciprocal F_1 hybrids.

Species	*A*	A_e	No. private alleles	H_o	H_e
ANG	10.667	6.04	4.167	0.614	0.676
EXC	12.333	7.301	4	0.654	0.757
F_{1ANG}	4.333	2.977	0	0.716	0.572
F_{1EXC}	5.5	3.643	0	0.741	0.611

A, mean number of alleles per locus; A_e, effective number of alleles; No. private alleles, Number of alleles unique to a single population per locus; H_o, observed heterozygosity; H_e, gene diversity, or expected heterozygosity

Analysis combining molecular and morphological data allowed a correct classification of the individuals in more than 99% of the cases and increased the power of the analysis. First, no overlap between the two parental species and F_1 hybrids was observed with the PCoA from the molecular data (data not shown). The principal coordinates analysis based on the genetic distance matrix extracted six coordinates; the first six coordinates had Eigenvalues superior to one. The first three factors in combination possess the highest cumulative variance of nearly 67% (data not shown). Next, the second CDA was carried out on a standardized data matrix including the first six factors of the PCoA on molecular marekers and 14 morphological characters. The first two canonical scores explained over 82% of the variation (Figure 2.4). The first canonical discriminant function had an Eigenvalue of 5.453 and a canonical correlation of 0.919 (Wilks' lambda=0. 0124,

Chi-square 289.70, df=60, $P< 0.001$). The canonical discriminant function has the highest correlation to the variable 'number of teeth' followed by the PC1 variable (Table 2.5). Multivariate classification of individuals according to their canonical scores placed 100% of *F. excelsior* and F_1 hybrids within the same group from which they were sampled. Only one *F. angustifolia* individual was misclassified according to its posterior probability and was placed with F_1 hybrids, having *F. excelsior* as a mother.

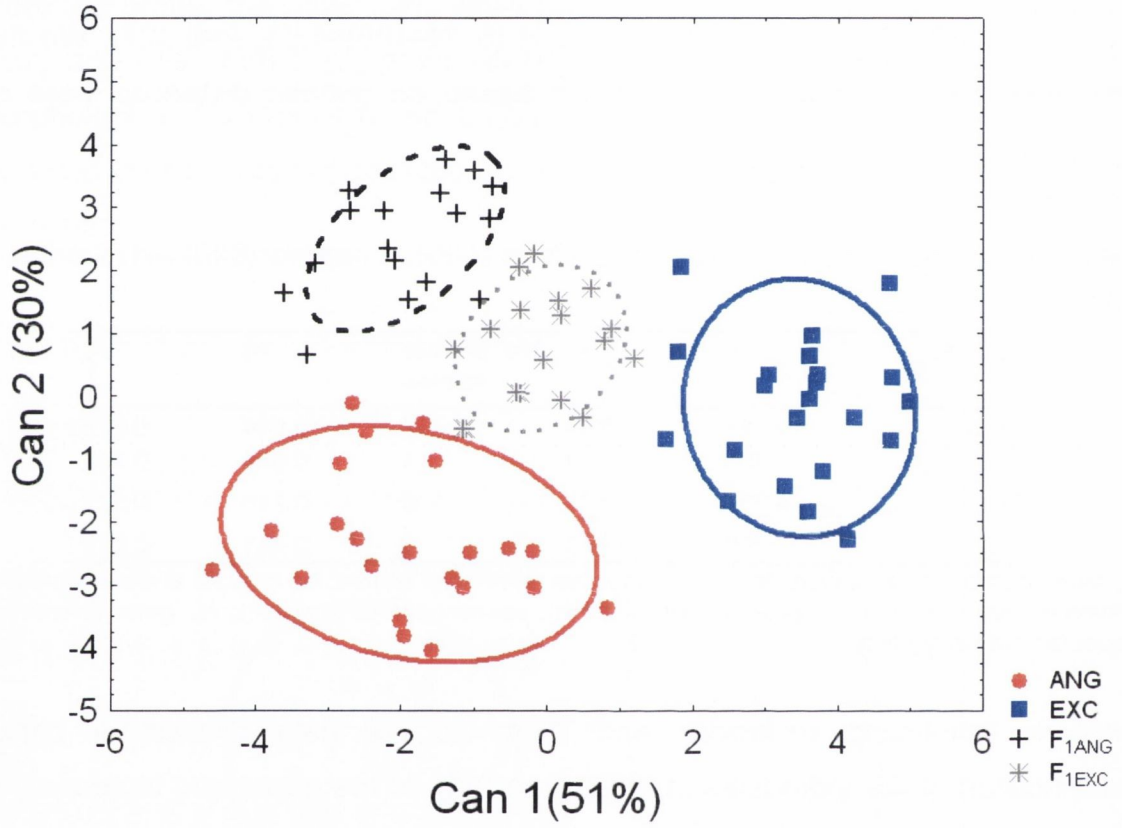

Figure 2.4 Plot of canonical scores of the morphological traits and PC factors for each individual of *F. excelsior*, *F. angustifolia* and their two types of hybrids. The plot indicates the percentage of variation of each canonical variate axis and the canonical score of each species. 95% confidence intervals around the mean canonical score for *F. excelsior*, *F. angustifolia* and the two different type of F_1 hybrid are indicated with ellipsoids.

2.4 Discussion

2.4.1 Classification success

The discriminant analysis of morphological characteristics confirmed that *F. excelsior* and *F. angustifolia* could be clearly distinguished using multivariate

statistics. However, greater resolution is attained when morphological data is combined with a set of nuclear molecular markers. Leaf characters are often applied with success for morphological classification in other trees species such as *Qercus petraea* and *Q. robur* (Kremer *et al.*, 2002; Kelleher *et al.*, 2005; Gugerli *et al.*, 2007). Although CDA, based only on leaf morphology, has enabled the separation of the two parental species (*F. excelsior*, *F. angustifolia*) and their F_1 hybrids without any overlap, genotypes were classified with a success of between 78% and 87% by this first discriminant analysis. Morphometric analyses are therefore useful by providing a preliminary detection of hybrid individuals. However, working with older hybrid generations and introgressed individuals could turn out to be a difficult challenge based only on morphology. At present, we do not know if the reduced success in separating individuals is due to the use of characters that are too general or to the fact that, with backcrosses, hybrid phenotypes are more difficult to distinguish. Hence, future applications of these markers to natural populations will need to address this issue.

Table 2.5 Correlation of the canonical function (Can1, Can2, Can3) with the variables of the discriminant analysis based on molecular and morphological characters.

	Can1	Can2	Can3
LeafletL	0.1351	0.1915	-0.1319
LeafletW	0.2334	0.1996	-0.0435
LeafletA	0.1984	0.2416	-0.022
AA	0.2786	0.0836	0.1557
LL	0.2778	0.2637	0.0313
LW	0.0966	0.1017	-0.2111
IL	0.0851	0.1299	0.11
LeafletN	0.2458	0.2777	0.0517
LftL.LftW	-0.1148	-0.0859	-0.0556
LL.LW	0.241	0.2245	0.3202
lnLA	0.327	0.4472	-0.0449
lnW	0.3731	0.3036	0.0357
lnTeethN	0.4351	0.2698	-0.0372
lnLeafletS	-0.2413	-0.1812	0.189
PC1	0.4245	-0.2297	-0.3766
PC2	-0.1064	-0.5672	0.1561
PC3	-0.1614	0.0438	-0.41
PC4	0.0613	0.0449	0.2527
PC5	-0.077	0.025	-0.0088
PC6	0.0636	-0.0316	0.1029

2.4.2 Leaf shape patterns and character inheritance

To our knowledge, this is the first detailed morphological analysis of first generation hybrids resulting from interspecific crosses between *F. excelsior* and *F. angustifolia*. The F_1 hybrids either with *F. angustifolia* or *F. excelsior* as mother, were clearly intermediate in morphology to the parental species but did not totally overlap. The morphological patterns of variation recorded in *F. angustifolia* and *F. excelsior* were consistent with previous studies in ashes and oaks, respectively (Fernández-Manjarrés *et al.*, 2006; Kremer *et al.*, 2008). Thirteen of our variables were statistically different between the two species but main differences were observed for reduction in leaf area, increased stomatal density and increased number of teeth in *F. angustifolia* compared to *F. excelsior.* For future studies, all significant variables could be measured but with special emphasis on the total leaf area and the number of teeth. Measuring stomatal density should be done with caution since age might have an influence on it. The sampled trees were raised in the same common garden, which would have minimized the influence of environmental variation. Therefore, it seems most likely that there is a genetic basis for smaller leaves and higher stomatal density in *F. angustifolia* than *F. excelsior*. Limited offspring size precludes, however, an appropriate heritability analysis. Future research should measure more offspring from different parents and from different types of crosses including backcrosses to further explore the interaction of these characters.

Finally, these results are in accordance with the ecology and the distribution of the species. The two species have a large sympatric zone but are usually found in different micro-habitats: *F. angustifolia* shows more affinity for seasonally flooded zones that may explain the increased number of stomata that are useful during wet periods compared to *F. excelsior*. Stomatal size and closure time certainly play a role in the micro-habitat adaptation of both species but our present data does not allow us to test this.

The extent of introgression and the history of the hybrid zone plants were unknown in previous studies on hybridization in ash, as these studies focussed on natural hybrid zones (Fernández-Manjarrés et al. 2006). It is very likely that the hybrid

plants included in previous studies were mostly introgressed individuals as the known hybrid zones appear to be stable (P. Gérard, personal communication). The results from the F_1 plants in our study are important as they confirm the inheritance patterns of morphological characters with controlled crosses. These results can therefore be used to help interpret patterns of hybridization and introgression in natural populations.

2.4.3 Asymmetry in character inheritance

Among the F_1 hybrids, three variables, leaf weight (W), total leaf area (LA), and the number of teeth per leaflet (teethN), showed significant differences with each of the two parental species. For five variables, the F_1 groups were statistically different from *F. angustifolia* but not from *F. excelsior*. The expression of parental or intermediate characters in hybrids depends of the genetic control of the characters and the influence of the environment (Rieseberg, 1995). The Paris-Sud University common garden at Orsay is situated inside the natural distribution of *F. excelsior* but is further north (over 200 kilometres) than the northern limit of *F. angustifolia*. Environmental conditions at Orsay are more suitable for *F. excelsior* and may have affected gene expression. However, such asymmetry might also be explained by a parental affect since the morphology of F_1 hybrids differed depending on the paternal or maternal parent species. For example, the F_1 progeny derived from reciprocal crosses showed a stomatal density pattern similar to the pollen parent.

A second set of characters that was sensitive to cross direction was leaf size and leaf shape. The progeny F_{1ANG} had significantly bigger leaves than their mothers in terms of length, area, and weight. In contrast, leaf size and leaf area of the hybrid with *F. excelsior* as mother showed no differences or were marginally smaller than observed values in *F. excelsior*. There was a bias toward the range of values measured in the mother for the apex angle in the terminal leaflet (leafletN) and the ratio of leaf length to width (LL.LW). Studies have shown that the fitness of hybrids, as well as morphology, can be influenced by parental effects (Campbell

and Waser, 2001; Kirk *et al.*, 2005) and these differences in ash could be verified in the field in future studies.

Overall, these results suggest that F_1 hybrids can be subject to strong selection pressures in natural conditions. Gerard *et al.* (2006) found a directional preference of hybridization in the Loire region in France where *F. angustifolia* flowers earlier than *F. excelsior* and in consequence, is the most frequent pollen donor for the early offspring *F. excelsior*. Hence, *F. angustifolia* offspring that are sired in the hybrid zones away from high water level areas may be strongly counter selected, as high stomatal density may allow for more water loss.

2.4.4 Conclusions

In this study, the combined analysis of molecular and morphological markers showed that it was possible to separate the two parental species and their F_1 hybrids with almost 100% success. In the future, more molecular markers may be needed for the recognition of hybrids in natural populations because of the confounding effects of backcrossing. It is clear from our analysis that intermediate characters can be expected in F_1 hybrids of ash but asymmetry in the inheritance may be observed due to maternal or paternal affects.

Acknowledgments

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Chapter III

Characterization of introduced ash in Ireland reveals cryptic hybrids (*Fraxinus excelsior* x *F. angustifolia*) and possible multiple source populations.

To be submitted as Muriel Thomasset, Juan F. Fernández-Manjarrés, Gerry C. Douglas , Paola Bertolino, Natalie Frascaria-Lacoste and Trevor R. Hodkinson

Abstract

Large-scale reforestation programmes are a major source of unwarranted gene flow and can have profound consequences on local genetic diversity. *Fraxinus excelsior* is the only native ash in Ireland. Recently, ash has been introduced from continental Europe to stock many plantations but has often exhibited unusual growth. It is not known if these trees are *F. excelsior*, *F. angustifolia,* or interspecific hybrids. Using morphological characters and six microsatellite markers, we investigated the source of the introduced trees for two plantations (Clonee and Kildalkey). Samples from continental Europe were included for comparison. Plantations exhibited higher genetic diversity than control populations probably because they contain a mixture of several provenances. There was a small but significant differentiation between plantations and control populations (Φ_{CT}=0.0211). Bayesian analysis to infer population structure and to assign introduced individuals to reference populations clearly demonstrated admixture and the presence of hybrid individuals within the plantations. The percentage of hybrids detected ranged from 28-58% depending on the plantation and the threshold level chosen. Most hybrids could be considered as cryptic because there was a lack of intermediate morphology for hybrid individuals that mainly clustered with *F. excelsior*. Hence, most hybrid individuals may have originated from sources that were highly introgressed with *F. excelsior*. Moreover, the results indicate that the source of material at the two sites differed. Implications for conservation and forestry are discussed.

3.1 Introduction

During the last few centuries, dispersal of many species has increased considerably because of deliberate or involuntary human mediated introduction. Newly introduced species have many consequences on landscapes, ecosystems, and sometimes the economic value of our environment (Richardson *et al.*, 2000). Exotic trees are often planted because of their economic value (Zobel *et al.*, 1987). In certain conditions, and after overcoming several ecological barriers (Richardson *et al.*, 2000), introduced species can become naturalized or invasive. Natural invasions are considered to be the second greatest cause, after habitat loss, of biodiversity loss in the world (Sala *et al.*, 2000; Sakai *et al.*, 2001). Alien plants can also have other negative consequences on native species via hybridization and introgression (Rhymer and Simberloff, 1996). Peterken (2001) analyzed the introduction of alien tree species in Britain during the last 200 years and mentioned three genetic consequences following introduction: "i) disturbance to the natural genetic patterns of native trees, (ii) genetic differentiation of introduced trees and iii) hybridization with local species." In general, large-scale reforestation programmes can become the source of undesired genes, even if close attention has been paid to the origins of the material.

Pre- and postzygotic barriers (Rieseberg and Carney, 1998) usually prevent species hybridization. However, reproductive barriers are often weak in closely related plants so that hybridization is a common phenomenon and is well recognized as a strong evolutionary force (Stace, 1975; Ellstrand *et al.*, 1999; Ellstrand and Schierenbeck, 2000; Rieseberg *et al.*, 2003; Arnold, 2004a; Rieseberg and Willis, 2007). In addition to widespread natural hybridization processes, new hybridization and hybrid zone formation can increase because of human activities like species introduction and habitat perturbation that allow migration of formerly separated species (Rhymer and Simberloff, 1996; Field *et al.*, 2009).

In Ireland *Fraxinus excelsior* L. is the only native ash species and has high economic value. It grows fast and has pliable timber, that is suitable for various uses including hurleys (Harbourne, 2005). In the 1990s, the Irish government

provided grants to foresters to encourage the planting of native broadleaf trees. At that time, stocks of ash in nurseries were not sufficient to cover demand and seeds and seedlings were imported from continental Europe. After a few years, the trees started to show unusual morphology, often with very small and pale buds and sometimes with highly crooked stems (Douglas *et al.*, 2009). It was not known if the morphology was: 1) only due to poorly adapted alien provenance of the *F. excelsior* saplings or 2) if the imported material was not "pure" *F. excelsior*. Indeed, in continental Europe, two other species of *Fraxinus* occur: *Fraxinus ornus* L. and *Fraxinus angustifolia* Vahl (Wallander, 2008). *Fraxinus angustifolia* has been found at its northernmost distribution to grow in sympatry with *F. excelsior* and to hybridize with it (Jeandroz *et al.*, 1996; Raquin *et al.*, 2002a; Fernández-Manjarrés *et al.*, 2006). The two ash species have different climatic and edaphic requirements (Marigo *et al.*, 2000). They have different leaf and bud morphology (Fernández-Manjarrés *et al.*, 2006; Thomasset *et al.*, 2011a), and also have different flowering times: *Fraxinus excelsior* flowers later (between March and May) than *F. angustifolia*, which starts to flush in December until February (Raquin *et al.*, 2002a; Jato *et al.*, 2004). In fact, Gerard *et al.* (2006b) showed that a hybrid zone population is not only structured by distance but also by flowering time.

One challenge of hybrid research, and especially with backcrossed hybrids, is to identify and categorize individuals, as there is not one "hybrid type." Backcrossed individuals might express a mosaic of phenotypes (Rieseberg and Ellstrand, 1993). The case of European ashes is not different. Studies of large-scale hybridization patterns between *F. excelsior* and *F. angustifolia* showed that, at two different natural hybrid zones, hybrids exhibited different morphology and hybrids were identified through the use of molecular markers (Fernández-Manjarrés *et al.*, 2006). Indeed, the development of molecular markers during the last few decades has greatly facilitated hybrid recognition (Arnold, 1992; Rieseberg *et al.*, 1996; Allendorf *et al.*, 2001), because studies based only on morphological markers often failed to recognize hybrids, especially after several generations of backcrosses (Rhymer and Simberloff, 1996).

With the increased availability of genetic markers, new methods have been developed to identify and categorize hybrids. Multivariate analyses that represent

the individual in a factorial space have proved useful to distinguish species (Kremer *et al.*, 2002) and also allow the incorporation of morphological and molecular data in the same analysis (Chapter II). Nevertheless, success of taxon assignment often depends on the type of distance statistic used. Assignment methods based on population genetic models (linkage disequilibrium and Hardy Weinberg equilibrium) classify individuals using alternative approaches from those based only on multivariate statistics. The presence of different species or populations of source material in a sample tends to increase the departure from Hardy-Weinberg equilibrium (Lowe *et al.*, 2004). Individuals are assigned to one of the populations based on minimization of departure of Hardy-Weinberg equilibrium and of linkage disequilibrium. Two different approaches are commonly performed: i) supervised classification which needs reference populations to assign individuals to specific groups (Paetkau *et al.*, 1995; Rannala and Mountain, 1997; Paetkau *et al.*, 2004), by using, for example, the software GeneClass2 (Piry *et al.*, 2004); or ii) clustering which uses only the genetic data without reference populations. Powerful methods based on Bayesian statistics implemented in the software STUCTURE (Pritchard *et al.*, 2000) and NEWHYBIRDS (Anderson and Thompson, 2002) are now available for this last approach.

We used a combination of multivariate analyses and different assignment approaches, implemented in the software STRUCTURE and NEWHYBRIDS, to genetically identify introduced alien trees in two different Irish plantations. We also examined the utility of these methods for hybrid recognition (including cryptic hybrids) by comparing results from morphological and molecular markers. We concluded our analysis with recommendations on difficult seed management strategies when hybrid individuals are suspected.

3.2 Materials and methods

3.2.1 Sampling of suspected alien ash sites

Two plantations, which were established using imported trees and which also showed very bad stem form, were selected for this study. Kildalkey (53° 34' 17.80"N; 6° 54' 54.21"W), is a site of 20 ha with provenances, according to the provenance certificate, mainly from Alsace France. Planted in 1999, the trees had initially grown vigorously but began to show crooked growth after 4-5 years. Some trees, which showed the poorest quality, were removed and inter-planted with oak (*Quercus robur*) and alder (*Alnus glutinosa*). Clonee (53° 28' 03.09" N; 6° 28' 58.90"W) is a site of over 3.5 ha planted in 1999. Provenance certificate was not available for this site. Again, trees at this site performed poorly, the poorest trees were cut back, and stumps treated with Roundup (Monsanto) weed killer. The plantation was reconstituted with native ash sources approximately 3-4 years after the initial planting. Some original trees remained and were distinguishable from the new re-planted ash based on their size (age). Trees at these sites are now sufficiently mature for flowering and seed-setting. At each plantation, we selected ash trees at random among the trees of original plantings. The starting tree was selected at a corner of the plantation and then sampling continued along a transect over the entire plantation. Sampling points along the transect were separated by 25 to 30 m in Kildalkey and by 10 to 15 m for Clonee depending on the size of the plantation. 192 trees were sampled at each plantation (Table 3.1).

Table 3.1 Details of sampled ash populations.

Geographic region/species	Population name	*n*	Population Identity	X	Y	Morphological data
Ireland/ *F. excelsior*	Clonee	96	Exc-Cl	53° 28'	6°28'	√
	Kildalkey	96	Exc-Kild	53° 34'	6°54'	√
Ireland/ Plantation	Plantation-Clonee	192	Cl-Plantation	53° 28'	6°28'	√
	Plantation-Kildalkey	192	Kild-Plantation	53° 34'	6°54'	√
Balkan/ *F. angustifolia*	Bulgaria-Elhovo	32	Ang-Bu	42° 19'	26° 56'	no
	Croatia-Lonjsko-Polje	32	Ang-Cr	45° 41'	16° 70'	no
France/ *F.angustifolia*	Mas Larrieu	24	Ang-M	42° 35'	3°01'	√
	Ortafa	24	Ang-Or	42° 35'	2°56'	√
	Nidoleres	24	Ang-Ni	42° 54'	2°85'	√
	Saint Jean	24	Ang-StJ	42° 51'	2°79'	√
	La Forge	24	Exc-LF	42° 48'	2°69'	√
France/ *F. excelsior*	Parcigoule	24	Exc-Pa	42°40'	2°47'	√
	La preste	24	ExcLP	42°24'	2°41'	√
Total sample size		808				744

3.2.2 Native control population

96 random native ash trees were sampled from around each plantation and these served as a control for *F. excelsior* in Ireland. Ash trees were located along the hedgerows of the arable fields around each plantation. In addition, *F. excelsior* and *F. angustifolia* samples collected in France were chosen as 'reference trees' based on the absence of putative hybrids among them (Special care was taken to avoid the presence of hybrids: Fernández-Manjarrés, personal observation). These populations were situated along an altitudinal cline in the Pyrenees Orientales from sea level (Mas Larrieu 42°35 ', 3°01 ') to an elevation of 1,100 meters at La Preste 42°24 ', 2°41'). Seven populations from this transect were chosen, among which three were *F. angustifolia* (Mas Larrieu, Ortafa, Nidolerès, at low altitude), and four *F. excelsior* (La Forge, La Preste and Parcigoule at respectively 200, 850 and 1100 metres elevation) (Table 3.1). *Fraxinus*

angustifolia samples were also used from two more populations, one in Croatia and one in Bulgaria to account for Eastern Europe genotypes (Table 3.1).

3.2.3 Morphology measurements

In order to assess morphological differences between the introduced trees and the two parental species, we examined leaves from 744 adult trees present in the populations specified in Table 3.1. Leaf samples were not available from the two populations in Croatia and Bulgaria. The minimum (Min) and maximum (Max) numbers of leaflets were estimated for more than 20 leaves per tree. The number of leaflets (LeafletN) was estimated in the field, from a sample of 20 leaves, by taking the average of leaflets. One mature leaf from a sun-exposed part of the tree (crown) was then collected and pressed for further measurements. Analysis of morphology was carried out using seven quantitative variables on each leaf. Six traits were dimensional: leaflet length (LeafletL), leaflet width (LeafletW), leaflet area (LeafletA), total leaf area (LA), total leaf length (LL), and total leaf width (LW). Measurements on the leaflet were always taken on the distal and first leaflet pair from the terminal leaflet. Teeth number on the second leaflet (TeethN) was also recorded. For the samples collected in Ireland, trait measurements were made with the WinDIAS Image Analysis System (Delta-T Devices, Cambridge, UK). The data from the reference population from France are derived from unpublished previous work by members of our group. These morphological characters have been validated as highly discriminant using controlled hybrids and common garden conditions (Thomasset *et al.*, 2011a, Chapter II)

3.2.4 DNA extraction and microsatellite amplification

Total genomic DNA was extracted from dried leaf samples using a DNeasy 96 Plant Kit (Qiagen). The quality and the quantity of DNA (from 10 to 50 $ng.\mu l^{-1}$) were checked using NanoDrop® ND-1000. Samples were genotyped using six polymorphic microsatellite loci: FEMSATL 4, FEMSATL 11, FEMSATL 12, FEMSATL 16, FEMSATL 19 (Lefort *et al.*, 1999) and M2–30 (Brachet *et al.*, 1999), which have successfully characterized common ash populations (Heuertz *et al.*,

2001; Morand *et al.*, 2002). PCR reaction methodology followed Morand *et al.* (2002). Allele size was determined using an ABI PrismTM 3130 Genetic Analyzer and the software Genemapper version 4.0 (Applied Biosystems).

3.2.5 Data analyses

3.2.5.a Genetic diversity

Genetic diversity in each population, and at each locus, was estimated with several statistics including: number of alleles per locus (*A*), allelic richness (*Rs*: measure of the number of alleles independent of sample size), observed heterozygosity (H_o), expected heterozygosity (H_e) according to Nei (1987), and the inbreeding coefficient (F_{IS}), using the FSTAT software package (V2.9.3.2, Goudet 2001). Deviation from Hardy-Weinberg equilibrium was assessed per population and per locus using exact tests (Guo and Thompson 1992) based on Markov chain Monte Carlo as implemented in Genepop version 4.0.10 (Raymond and Rousset 1995). These parameters were also estimated for each geographical group of populations as defined in Table 1. The term 'Geographical group' is used here to describe the geographical origin of the trees and also its taxonomic species class. Pairwise differentiation coefficients (F_{ST}) (Weir and Cockerham 1984) among geographical groups were also estimated. R_{ST} (Slatkin 1995) was also used as it takes into account the length difference between alleles (and allele frequency) and uses a stepwise mutation model that is more appropriate for microsatellite markers. F_{ST} and R_{ST} were calculated with ARLEQUIN version 3.5 (Excoffier et al. 2005). The significance of the population differentiation parameters F_{ST} and R_{ST} were tested with 10,000 permutations using the same software. Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was also performed using ARLEQUIN to investigate intra- and inter-population differentiation among geographical regions as defined in Table 1 (and above). AMOVA was also performed to investigate the differentiation among species (*F. excelsior, F. angustifolia* and the plantation trees). The variance components and inbreeding indices were tested by non-parametric randomization tests using 10,000 permutations.

3.2.5.b Morphological analysis

All variables were tested for normality using the Shapiro-Wilk W-test (Shapiro and Wilk 1965) and the Bartlett test for variance homogeneity (Bartlett 1937). One-way analysis of variance was followed by Tukey HSD post-hoc tests (at 5% probability) to make comparisons among the different geographical groups. Characters with significant variation at $P<0.001$ were then used in multivariate analysis. Quantitative data were standardized prior to multivariate analysis, in order to remove biased character weighting, using the formula 'Std. score = (raw score - mean) / std. deviation'. Data analysis for basic statistics and data transformation were performed in Statistica version 6.0 (Statsoft, 2004).

3.2.5.c Assignment and individual classification

- *Multivariate analysis*

Diploid multilocus genotypes were transformed into a binary data matrix. For each individual, data were coded as a row vector presenting as many columns as alleles in the whole dataset. A homozygote was coded as 2 in the corresponding allele and a heterozygote as 1 at each allele. After coding all the individuals, a random column was removed to avoid inner linearity in the data that will produce non-singular matrices in the multivariate analysis. The random drop of an allele per locus has little effect on the estimated genetic structure when several alleles are present (R. Dyer, personal communication). To depict the genetic clustering of each geographical group, we performed a discriminant analysis from the transformed multivariate genotypes using the DISCRIM procedure in SAS ® software (SAS version 8).

In order to compare the morphology of the introduced plantation trees with the two parental species, we ran a multivariate stepwise Canonical Discriminant Analysis (CDA) (Legendre and Legendre 1998) using the procedure DISCRIM in Statistica version 6.0 (Statsoft, 2004) with geographical groups defined in Table 1 as class variables. By using a stepwise method, the variables were added one at the time on the basis of a Wilks' lambda test.

We combined the transformed molecular data set and the standardized morphological data and ran a CDA using PROC DSICRIM in SAS with geographical group and the two plantations as the classification variables to detect hybrids and the genetic structure in the introduced populations. We also recorded the INTO variable provided by the output of the discriminant analysis that classifies individuals as a function of the posterior probabilities of the canonical scores in one of the predefined populations.

- *Characterization of individuals by Bayesian methods*

We used the Bayesian genetic approach implemented in the software STRUCTURE (Pritchard *et al.* 2000) to infer population structure, to assign introduced individuals to reference populations, and to identify admixed individuals. This approach uses multilocus genotype data and runs over a cluster of K groups. Iteratively, the program searches for the cluster of K groups that minimizes the within-group linkage disequilibrium and that optimizes consistency of allele frequencies assuming Hardy-Weinberg equilibrium. The software generates, for each individual, a posterior probability of belonging to one of the groups. For each analysis, twenty independent runs were performed with 100,000 Markov chain Monte Carlo simulations and with the burn-in period length set to 10,000. To avoid over-estimating the number of groups, we estimated the most likely number of clusters detectable by STRUCUTRE with the ΔK method according to Evanno *et al.* (2005) using the software Structure Harvester (Earl 2009).

Firstly, to determine if the model can differentiate pure individuals of each species and separate clusters, we ran an analysis with all the 11 control populations of *F. excelsior* and *F. angustifolia* using an admixture model without using population or species groups (USEPOPINFO=0) from K=1 (no structure) to K=11 (11 clusters). Once the most likely number of groups was identified, we thus continued our analyses to explore population structure and group assignment of the plantation trees. This time we ran the model using prior population information (USEPOPINFO=1) but only on the reference populations (USEPOPFLAG) for K=2. During the assignment procedure it is important to set a threshold value for q (proportion of membership of each individual genotype to the different genetic

groups). Setting thresholds is crucial because it can greatly affect the proportion of individuals assigned to each species or to the hybrid groups. Two approaches exist: a) to predefine arbitrarily a given threshold (ie, 0.8 or 0.9) (Pritchard *et al.*, 2000; Vähä and Primmer, 2006); b) to use mean assignment values from the control group. Both approaches present advantages and disadvantages. If thresholds are set too high, either by decision or because control groups are very homogeneus, the number of hybrids will be over-estimated (few individuals will fit a stringent species definition). Moreover basing membership on the two reference populations is actually too stringent because it does not encompass the full variation found in the species, it only includes that found in those populations. So in fact, it is looking at population rather than taxon membership. Hence we used values applied to other species as a compromise. Following the recommendation of Vähä and Primmer (2006) and Pritchard *et al.* (2000), we used a threshold value *Tq* of 0.9. Considering that both species are very closely related, we also used a threshold value of 0.8 that has been useful in previous analyses (Fernández-Manjarrés, unpublished data). For a value of *q* higher or equal to the threshold, individuals were assigned to a 'purebred' cluster; a lower value of *q* indicated introgressed genotypes.

To further validate the identification of admixed individuals we complemented our analysis with the Bayesian approach in NEWHYBRIDS beta 1.1 (Anderson and Thompson 2002). In contrast to STRUCTURE, NEWHYBRIDS assumes a simpler population structure of two parental species A and B only, but with the advantage of classifying putative hybrid individuals in more precise classes. Individuals are assigned to one of the following six genotypic classes: parental species, F_1, F_2, and backcrosses with each parental species. The analysis was run using the z and s options to incorporate knowledge about our reference sets. Z as defined by Anderson and Thompson (2002) is used for individual which have known genotype frequency category and s indicates individual that is not considered part of the mixture.

To verify our ability to identify putative hybrid individuals, we simulated hybrid populations. From the first analysis performed with STRUCTURE (described above), we randomly selected 100 *F. excelsior* and 100 *F. angustifolia* that had a

posterior probability superior to 0.9 of pertaining to their prior assigned species. We then used these individuals to simulate the genotype of 100 F_1, 100 F_2 hybrids and 100 backcrosses of first generation with *F. angustifolia* and *F. excelsior,* respectively, using the software HYBRIDLAB (Nielsen *et al.* 2006). An analysis was carried out with STRUCTURE using an admixture model and no prior population information. In this way, we calibrated the proportion of individuals correctly identified as hybrids in the simulated data set with *Tq* set at 0.8 and 0.9 (Vähä and Primmer 2006).

Next, we tested the simulated data using NEWHYBRIDS (Anderson and Thompson 2002). The simulated data set was run using the default parameters for the six genotype class frequencies, with uniform priors, a burn-in phase of 100,000 steps and 1,000,000 sweeps. We decided to apply the most relaxed criterion, so *Tq* was only applied to the purebred category. We assigned an individual to one of the parental categories when its inferred probability to belong to this category was at least 80% or more. All other individuals were classified as hybrids.

We finally assigned individuals using the Bayesian methods outlined above. In the assignment method, trees from the plantations were grouped as hybrid type, based on the results from the STRUCTURE analysis, as those not receiving a probability greater than 0.8 of membership to one of the parental clusters (*F. excelsior* or *F. angustifolia*).

- *Characterization of hybrid types*

The STRUCTURE classification was used to evaluate how the morphological characters differentiated these genotypes and to detect cryptic hybrids. We performed a discriminant analysis based on the morphological data with individuals from the plantation identified as *F. angustifolia* or hybrid type and the control geographical group as category. Hence our classes for the CDA were: hybrid type or *F. angustifolia* from the plantation, local ash, control ashes from France (*F. excelsior* and *F. angustifolia*).

To determine the degree of consistency between molecular and morphological data, Mantel tests were performed (999 permutations) using the software GenAlEx

version 6.3 (Peakall and Smouse, 2006). The canonical score of the first two axes of the CDA, based only on morphological traits, were used as input geographical variables. We performed three different Mantel tests for three different data sets: one with all the individuals, one where all individuals from the plantation were discarded and one test with only plantation individuals categorized earlier as hybrids or *F. angustifolia*.

Table 3.2 Within-population diversity at all loci for all populations and plantations.

Populations and genetic parameters	Microsatellite Locus							
	Fem12	Fem4	M2-30	Fem16	Fem19	Fem11	Mean	SE
Ang-Bu (32)								
A	11	14	21	3	10	15	12.3	2.4
Rs	9.904	12.284	17.097	2.581	7.14	13.005	10.3	1.8
H_o	0.682	0.667	0.815	0.226	0.323	0.786	0.583	0.101
H_e	0.812	0.877	0.922	0.318	0.476	0.878	0.714	0.103
F_{IS}	0.183*	0.258***	0.135*	0.305*	0.337***	0.123*	0.202***	0.037
Ang-Cr (32)								
A	14	9	19	5	13	21	13.5	2.4
Rs	12.216	6.62	15.705	4	9.466	17.196	10.9	2
H_o	0.781	0.656	0.903	0.156	0.594	0.938	0.671	0.117
H_e	0.884	0.569	0.906	0.204	0.608	0.922	0.682	0.115
F_{IS}	0.132*	-0.138	0.019	0.248*	0.039	-0.001	0.032	0.053
Ang-Ni (24)								
A	15	9	19	4	10	18	12.7	2.5
Rs	13.811	8.839	17.501	3.714	9.235	15.919	11.5	1.6
H_o	0.818	0.632	0.762	0.333	0.545	0.958	0.681	0.09
H_e	0.872	0.774	0.916	0.454	0.773	0.901	0.784	0.07
F_{IS}	0.085	0.21*	0.192**	0.288*	0.315**	-0.042	0.16***	0.054
Ang-Or (24)								
A	17	11	20	5	10	12	12.5	2.2
Rs	14.939	10.674	17.902	4.739	9.122	10.686	7.3	1.3
H_o	0.739	0.619	0.818	0.652	0.826	0.913	0.761	0.046
H_e	0.879	0.858	0.92	0.671	0.843	0.853	0.837	0.035
F_{IS}	0.181*	0.301**	0.134*	0.05	0.042	-0.049	0.114**	0.05
Ang-StJ (24)								
A	10	8	18	4	11	15	11	2
Rs	9.408	7.535	18	3.987	9.499	13.148	10.3	1.5
H_o	0.81	0.571	0.833	0.667	0.833	0.917	0.772	0.052
H_e	0.794	0.698	0.923	0.65	0.808	0.857	0.788	0.041
F_{IS}	0.004	0.205*	0.125*	-0.004	-0.01	-0.049	0.045	0.039
Ang-M (24)								
A	12	10	19	3	13	11	11.3	2.1
Rs	11.374	9.13	17.017	3	10.999	9.666	10.2	1.7
H_o	0.739	0.792	0.875	0.5	0.609	0.875	0.732	0.062
H_e	0.848	0.836	0.93	0.603	0.691	0.792	0.783	0.048
F_{IS}	0.15*	0.074	0.08	0.192	0.141	-0.084	0.087**	0.04
Exc-LF (24)								
A	10	12	20	4	11	12	11.8	2.1
Rs	8.883	10.178	17	3.926	10.684	10.693	10.2	1.2
H_o	0.75	0.75	0.875	0.333	0.8	0.773	0.722	0.081
H_e	0.792	0.766	0.904	0.327	0.845	0.791	0.74	0.085
F_{IS}	0.074	0.043	0.053	0.003	0.079	0.047	0.055	0.011
Exc-LP (24)								
A	9	10	18	6	12	10	10.8	1.6
Rs	8.416	9.141	16.926	5.826	11.839	9.286	10.2	1.7

H_o	0.682	0.625	0.952	0.522	0.632	0.762	0.696	0.061
H_e	0.762	0.767	0.926	0.515	0.892	0.776	0.773	0.059
F_{IS}	0.129	0.206*	-0.004	0.009	0.316**	0.042	0.124**	0.052
Exc-Pr (24)								
A	13	12	22	5	12	11	12.7	2.2
Rs	12.235	11.092	22	4.485	11.669	10.148	11.9	2.2
H_o	0.833	0.625	1	0.375	0.65	0.5	0.672	0.092
H_e	0.876	0.849	0.943	0.328	0.874	0.747	0.771	0.092
F_{IS}	0.07	0.283**	-0.032	-0.122	0.28**	0.349***	0.16***	0.08
Exc-Cl (96)								
A	19	18	34	7	21	26	20.8	3.7
Rs	11.399	11.739	17.452	4.514	11.358	12.86	11.6	1.8
H_o	0.787	0.747	0.768	0.521	0.842	0.875	0.757	0.051
H_e	0.755	0.827	0.933	0.526	0.874	0.87	0.798	0.059
F_{IS}	-0.037	0.101**	0.182***	0.015	0.042	0	0.056***	0.032
Exc-Kild (96)								
A	21	19	40	7	19	20	21	4.3
Rs	11.542	12.736	19.049	4.708	11.449	12.167	11.9	2.2
H_o	0.72	0.7	0.95	0.48	0.89	0.89	0.772	0.071
H_e	0.788	0.87	0.943	0.504	0.884	0.875	0.811	0.065
F_{IS}	0.091*	0.2***	-0.002	0.053	-0.001	-0.012	0.053**	0.033
Plantation-Cl (192)								
A	23	20	41	9	28	26	24.5	4.3
Rs	12.202	11.011	21.096	4.923	14.792	12.484	12.8	3.1
H_o	0.696	0.594	0.898	0.324	0.861	0.821	0.699	0.088
H_e	0.826	0.843	0.957	0.471	0.922	0.888	0.818	0.072
F_{IS}	0.161***	0.297***	0.064***	0.313***	0.068**	0.079**	0.148***	0.047
Plantation-Kild (192)								
A	23	19	38	6	22	22	21.7	4.2
Rs	12.237	10.389	17.151	3.91	13.661	11.461	11.5	2
H_o	0.795	0.471	0.716	0.375	0.906	0.917	0.696	0.092
H_e	0.799	0.717	0.931	0.363	0.914	0.875	0.767	0.087
F_{IS}	0.008	0.345***	0.234***	-0.029	0.012	-0.045	0.094**	0.066
All pop								
A	15.154	13.154	25.308	5.231	14.769	16.846	15.077	0.92
Rs	13.299	11.832	20.704	4.975	14.688	13.969	13.245	0.503
H_o	0.756	0.65	0.859	0.42	0.716	0.84	0.707	0.021
H_e	0.822	0.789	0.927	0.456	0.8	0.848	0.774	0.02
F_{IS}	0.104**	0.254***	0.127**	0.148**	0.118**	0.053*	0.131***	0.088

N, number of individuals per group; *A*, mean number of alleles; *Rs*, allelic richness per locus and per population; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, Wright's inbreeding coefficient. Exact tests were performed with 1,000 random permutations (*P<0.05, **P<0.01,***P<0.001)

3.3 Results

3.3.1 Genetic diversity and molecular differentiation

A total number of 193 alleles was recorded across all 13 populations for the six screened loci. The number of alleles per locus ranged from 14 for FEMSATL 16 to 52 for M2-30. All loci were highly polymorphic in all populations (Table 3.2) with the mean number of alleles varying between 24.5 for the population with the largest sample size (Clonee, n=192) to 10.8 for the smallest population size (Exc-LP; n=24) (Table 3.2). Within the 13 populations, the levels of expected heterozygosity (averaged across loci) ranged from 0.682 for the Ang-Cr population to 0.818 for Cl-plantation (Table 3.2). Significant heterozygote (F_{IS}) deficiency was detected for almost all the populations and all loci.

Table 3.3 Summary of the genetic diversity statistics for the 6 different geographical/species groups.

Geographic regions	*A*	*Rs*	No. Private Alleles	H_o	H_e	F_{IS}
ANG-Balkan	18	17.532	0.667	0.63	0.723	0.137***
ANG-France	20.167	17.628	1.667	0.737	0.834	0.122***
EXC-France	17.333	16.723	0.167	0.689	0.786	0.130***
EXC-Ireland	24.5	18.236	0.5	0.764	0.808	0.056***
Plantation-Clonee	24.5	18.965	1.333	0.699	0.818	0.148***
Plantation-Kildalkey	21.667	16.865	0.5	0.696	0.767	0.094***

A, mean number of alleles per locus; *Rs*, allelic richness per locus and per population; No. private alleles, Number of alleles unique to a single population per locus; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, Wright's inbreeding coefficient. Exact tests were performed with 1,000 random permutations (*P<0.05,**P<0.01,***P<0.001)

When the 13 populations of ash were grouped into six geographical regions according to species and source (Table 3.1); the genetic diversity results were variable (Table 3.3). The number of alleles differed very little among the geographical groups ranging from 17.33 for *F. excelsior* from France (Exc-France) to 24.5 for native Irish *F. excelsior* (Exc-Ireland) and also for Plantation-Clonee trees (Table 3.3). High values of H_o (0.630 to 0.764) and H_e (0.723 to 0.834) were observed. A significant heterozygote deficiency was detected for all the geographical groups. The deficiency was lower for native Irish trees (Exc-

Ireland=0.056) than for the Clonee (0.148) and Kildalkey (0.094) plantations. *Fraxinus angustifolia* from France (Ang-France) and from the Balkan area (Ang-Balkan), and *F. excelsior* from France (Exc-France) had similar F_{IS} values ranging from 0.122 to 0.137. Private alleles were rare (Table 3.3). We detected 29 unique alleles with very low frequency ranging from 0.003 to 0.016 (data not shown). The geographical groups, which displayed the highest number of private alleles, were Ang-France with 11 and the Clonee plantation with 8 alleles.

3.3.2 Molecular differentiation among populations and geographical groups

Pairwise F_{ST} and R_{ST} values among geographical groups (Table 3.4) were generally moderate but all were significant ($P<0.05$). The highest among geographical group differentiations were found between Ang-Balkan and Exc-France ($F_{ST}=0.086$ and $R_{ST}=0.495$) and between Ang-Balkan and Exc-Ireland ($F_{ST}=0.086$ and $R_{ST}=0.410$). The least differentiation was found between the two Irish plantations ($F_{ST}=0.009$, $R_{ST}=0.017$) and between the Irish plantations and Exc-Ireland (Plantation-Clonee vs. Exc-Ireland, $F_{ST}=0.010$, $R_{ST}=0.010$; Plantation-Kildalkey and Ex-Ireland, $F_{ST}=0.013$, $R_{ST}=0.014$).

Table 3.4 Population differentiation calculated from nuclear microsatellite data. Above diagonal, pairwise population estimates of R_{ST}; below diagonal, pairwise population F_{ST} values; all statistics were significant at the $P<0.05$.

Pop	ANG-Balkan	ANG-France	EXC-France	EXC-Ireland	Plantation-Clonee	Plantation-Kildalkey
ANG-Balkan	**0**	0.068	0.495	0.41	0.38	0.305
ANG-France	0.055	**0**	0.389	0.327	0.288	0.232
EXC-France	0.086	0.052	**0**	0.029	0.025	0.036
EXC-Ireland	0.086	0.055	0.034	**0**	0.01	0.014
Plantation-Clonee	0.064	0.042	0.025	0.01	**0**	0.017
Plantation-Kildalkey	0.061	0.051	0.034	0.013	0.009	**0**

The three-level AMOVA revealed that 96.09% of the genetic variation is contained within populations, whereas 2.11% was due to differences among geographical regions (Table 3.5) and 1.79% among populations within regions/species. AMOVA showed that there was significant ($P<0.001$) differentiation among geographical regions ($\Phi_{CT}=0.02115$), among populations within region/species groups

(Φ_{SC}=0.0183) and within populations (Φ_{ST}=0.03906) (Table 3.5). When the data were partitioned into species groups instead of geographical groups, the among species differentiation was also significant (Φ_{CT}=0.01159; P<0.001). In this case, the highest proportion of the genetic variation was again found within populations (96.44%) (Supplementary material, Table S3.1).

Table 3.5 Analysis of molecular variance (AMOVA) within and among the populations grouped according to geographic region.

Source of variation	df	Sum of squares	Variance Components	Percentage of variation	Fixation indices
Among regions	5	111.394	0.0493	2.11	Φ_{CT}=0.02115***
Among populations within regions	7	36.481	0.0417	1.79	Φ_{SC}=0.0183***
Within populations	1601	3583.675	2.2384	96.09	Φ_{ST}=0.03906***
Total	1613	3731.55	2.3294	100	

*** P<0.001

3.3.3 Morphological differentiation

All morphological variables exhibited significant effects in the ANOVA analysis with respect to geographical groups. The four most significant variables were Leaflet Max ($F_{4,\ 738}$=197.29, P<0.001) LeafletN ($F_{4,\ 738}$=166.65, P<0.001) LeafL ($F_{4,\ 738}$=135.66, P<0.001) and TeethN ($F_{4,\ 738}$=108.525, P<0.001).

The Tukey HSD test showed that *F. angustifolia* and *F. excelsior* were significantly different (P<0.001) for all morphological characters. The two parental species exhibited the most differentiation. *Fraxinus angustifolia* had smaller leaflets (LeafletA, LeafletL, LeafletW), a smaller number of leaflets (LeafletN, Max, Min) and a reduced number of teeth compared to *F. excelsior* (Table 3.6).

The two geographical groups of *F. excelsior* were not significantly different for all the morphological variables. The two Irish plantations of *F. excelsior* were significantly different from *F. angustifolia* for all variables. However, not all variables were significantly different in comparisons between Irish *F. excelsior* and French *F. excelsior*. Individuals from both plantations exhibited a high number of

leaflets (Table 3.6); 11.46 and 10.50 for Clonee and Kildalkey respectively. Leaf area (LA) values were also high with a mean of 129 for Clonee and 139.26 for Kildalkey. The Clonee plantation exhibited the highest value for Max leaflet number (13.27).

3.3.4 Multivariate analysis

The results of the CDA based on the microsatellite data are summarized in Figure 1A for the two principal axes (Can 1 and Can 2) of the analysis. The first axis explained 42% of the variation and the second 19%. The two parental species, *F. excelsior* and *F. angustifolia*, are genetically distinct with almost no overlap in the CDA, and are mainly separated by the first axis. The two geographical groups of *F. angustifolia* (France and the Balkans) were separated by the second axis. The distribution of individuals in the two introduced Irish plantations overlapped with the distribution of *F. excelsior* (especially native *F. excelsior*). However, a few individuals were grouped in an intermediate position between the two species, or closer to *F. angustifolia* than *F. excelsior*.

We performed a second CDA based only on morphological data. The stepwise discriminant analysis was used to select morphological variables which best discriminated among the groups. Each variable was kept as they all contributed in a significant way in the model ($P<0.05$). The CDA based on the morphological characters yielded four out of five significant canonical values that explained 99.54% of the variation. The first axis (Wilks'λ=0.132; df=45; $P<0.001$) and the second axis (Wilks'λ=0.4259; df=32; $P<0.001$) explained 68.72% and 16.72% of the variance respectively. The scatter-plot (Figure 1B) of Can 1 and Can 2 showed that *F. angustifolia* was separated from the other groups along the first axis. Nevertheless, an overlap at the extreme of the scores distribution of *F. excelsior* and *F. angustifolia* in the multivariate space was observed. The two groups of *F. excelsior* (Irish and France) showed a highly overlapped distribution. However, individuals of *F. excelsior* from France were differentiated from *F. excelsior* from Ireland. Individuals from the Irish plantations (Clonee and Kildalkey) were mainly positioned within the "*F. excelsior*" distribution (Figure 1B). However, some

individuals were positioned with either of the two species. The multivariate classification of the individuals according to their canonical scores indicated that 93.5% of the *F. angustifolia* were placed within their *a priori* defined group. For the French *F. excelsior*, 68% were classified within their predicted group and 25% were classified within the *F. excelsior* group from Ireland. For native Irish *F. excelsior*, 70.4% were classified within the predicted group, 13.02% within *F. excelsior* from France, 9.37% within the Clonee group and 6.2% within the Kildalkey group. Only 57% and 62.5% of the trees were correctly assigned for the Clonee and Kildalkey sites respectively. When plantation trees were misclassified they were mainly placed into the other plantation or into the *F. excelsior* group from Ireland. Misclassified plantation trees were rarely classified into the *F. angustifolia* or *F. excelsior* groups from France (between 1 and 2%).

A third discriminant analysis was performed combining the two data sets (morphology and microsatellite markers), and the results are shown in Figure 1C. The first axis (Wilks'λ=0.0101; df=1015; P<0.001) and the second axis (Wilks'λ=0.0526; df=808; P<0.001) explained 46.21% and 29.4% of the variance respectively. The two parental species were clearly differentiated with little overlap at the extreme of their distribution. As with the previous discriminant analyses using morphology and molecular markers separately, individuals from the Clonee plantation were grouped very close to *F. excelsior*. However, in contrast to the previous analysis for the Kildalkey site, several individuals were completely differentiated from the two parental species and formed a group on their own. Combining morphology and molecular data allowed 97.9% of *F. angustifolia* and 93.6% for all the *F. excelsior* to be classified in to their presumed species group. The Clonee plantation had 86.1% individuals correctly assigned and the Kildalkey plantation had 94.8% of individuals correctly classified into their *a priori* defined group.

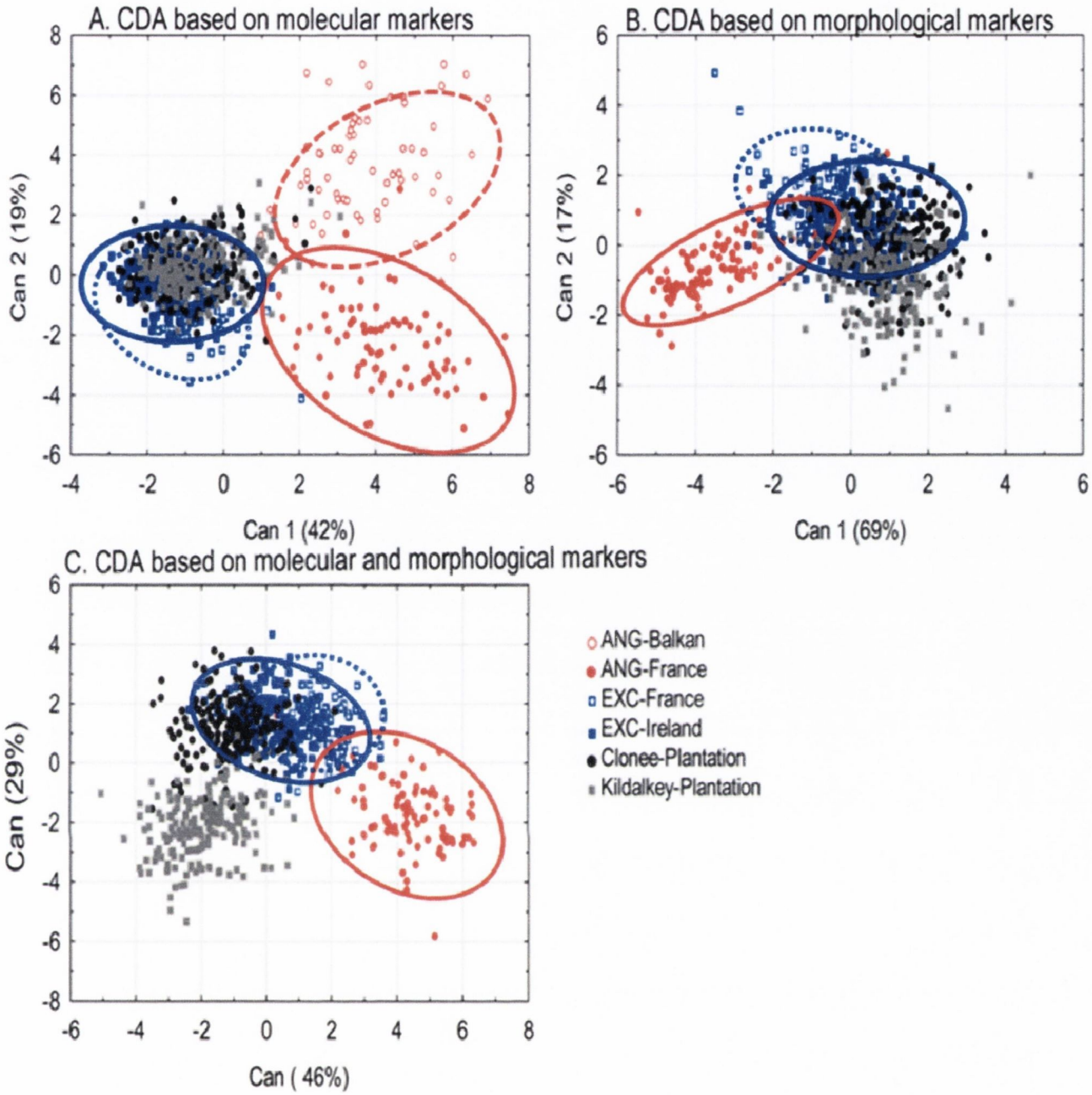

Figure 3.1A. Canonical discriminant analysis of six microsatellites markers and all groups of 808 samples. **B.** Canonical discriminant analysis of 10 phenotypic characters and all groups of 744 samples. **C.** CDA based on morphological variables and molecular data for the Irish, French and plantation populations only. Scatter plot of individual scores for the first two axes of CDA based on morphological variables and molecular data. *Fraxinus angustifolia* from the Balkan area (empty red circles), *F. angustifolia* from France (closed red circles), *F. excelsior* from France (open blue squares), *F. excelsior* form Ireland (closed blue squares) and the two plantations (closed grey squares for Kildalkey and closed black circles for Clonee). 95%confidence intervals around the mean canonical score for *F. excelsior* and F. *angustifolia*.

Table 3.6 Summary of morphological variation for leaf characters within geographical groups.

Geographical groups	LeafletN		LA		LeafletA		LeafL		LeafW		LeafletL		LeafletW		TeethN		Min		Max	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ANG-France	7.42	1.94	49.30	22.44	6.77	3.21	16.58	3.49	11.10	2.15	5.74	1.21	1.45	0.39	10.14	2.13	6.40	1.93	8.44	1.96
EXC-France	10.89[a, b]	0.99	135.8[a, b, d]	51.16	12.61[b]	5.11	25.40[a, b]	4.37	15.72[a]	3.18	7.56[a]	1.33	2.06[a]	0.52	20.04[a]	4.14	9.86[a]	1.05	11.92[a]	1.06
EXC-Ireland	10.62[a, b]	0.94	99.80[d]	44.91	9.13[b]	4.58	24.84[a]	4.40	13.54[a]	2.86	6.94[a]	1.60	1.99[a]	0.58	23.14[a]	5.45	9.19[a]	1.00	12.05[a]	1.30
Plantation-Clonee	11.46	1.26	129.1[a, c]	42.42	10.50[a]	4.17	28.63	4.32	13.86[a]	2.06	6.91[a, b]	1.23	2.29[b]	0.56	19.84[a, b]	5.84	9.66[a]	1.64	13.27	1.37
Plantation-Kildalkey	10.53[b]	1.29	139.3[b, c]	53.78	10.83[a]	4.21	26.75[b]	4.32	14.52[a]	2.49	7.10[a, b]	1.48	2.36[b]	0.58	21.56[a, b]	5.69	8.25	1.74	12.8	1.47

According to the results of the Tukey HSD post-hoc tests, the means followed by the same letters (a, b, c or d) were not statistically different ($P>0.001$).

3.3.5 Bayesian clustering of reference populations

Results with the software NEWHYBRIDS and STRUCTURE for the simulated data were highly correlated (R^2=0.955). Therefore, only results from the STRUCTURE analyses are presented in this paper. STRUCTURE was used to infer the ancestry of native Irish and French *F. excelsior*, Bulgarian, Croatian and French *F. angustifolia*. The highest value of ΔK was found at K=2 (ln$K$=-11730 and ΔK=191.43) with the clusters corresponding to each parental *Fraxinus* (*F. excelsior* and *F. angustifolia*) species. Cluster 1 grouped all the populations of *F. angustifolia* (with a mean proportion of membership q_1=0.973, SD=0.033) and cluster 2 grouped all the French and native Irish populations of *F. excelsior* (q_2=0.951, SD=0.096). However, 31 individuals of *F. excelsior* and 8 individuals of *F. angustifolia* showed less than 0.90 probability of belonging to their *a priori* defined species cluster. If *Tq* was taken as 0.8, all *F. angustifolia* individuals were assigned into their presumed species cluster. However still 12 *F. excelsior* individuals were not assigned to their presumed species cluster even if a *Tq* of 0.8 was used.

3.3.6 Performance of the hybrid assignment based on Bayesian methods assignment

Results of the simulation showed the importance of the threshold selection (Table 3.7). When *Tq* was decreased to 0.8, the percentage of hybrid detected decreased in a significant way for both the NEWHYBRIDS and STRUCTURE software. Similar results were obtained for the F_1 and F_2 hybrids with both NEWHYBRIDS and STRUCTURE, as both correctly assigned almost 100% of the F_1 and between 81% to 97% of the F_2, even with a relaxed threshold of 0.8. However in advanced generations of hybrids (backcrosses with *F. angustifolia*, BCA and backcrosses with *F. excelsior*, BCE) with *Tq*=0.9, STRUCUTRE detected a high percentage of true hybrids (90%, Table 3.7), while the percentage of hybrids correctly assigned by NEWHYBRIDS dropped to 70%.

Table 3.7 Results of STRUCTURE and NEWHYBRIDS analyses with simulated samples

		Tq=0.8			*Tq*=0.9		
	Simulated data	Estimated Exc (%)	Estimated Ang (%)	Estimated Hybrid (%)	Estimated Exc (%)	Estimated Ang (%)	Estimated Hybrid (%)
STRUCTURE	F_1	2	4	94	0	0	100
	F_2	9	10	81	1	2	97
	BCA	0	55	45	0	10	90
	BCE	45	1	54	12	0	88
NEWHYBRIDS	F_1	0	0	100	0	0	100
	F_2	10	11	79	5	6	89
	BCA	0	41	59	7	26	67
	BCE	45	0	55	27	0	73

Tq threshold (q-value); Exc=*F.excelsior*; Ang=*F.angustifolia*; BCA=backcrosses with *F. angustifolia*; BCE=backcrosses with *F. excelsior*.

3.3.7 Genetic admixture analysis and assignment testing

STRUCTURE was used to infer the ancestry of the plantation trees using the prior population information model (Figure 3.2). Several individuals had a likelihood of *F. angustifolia* membership clearly superior to 0.8, signalling the likely presence of hybrid individuals in the stands. With *Tq*=0.9, we detected 113 out of 187, and 109 out of 192, admixed individuals for Clonee and Kildalkey respectively. Furthermore, 13 individuals (i.e. 6.9%) from the Clonee plantation and 8 individuals (4.1%) of the Kildalkey plantation were assigned to *F. angustifolia* (Figure 3.2). For a threshold set at 0.8, the number of individuals classified as hybrids decreased to 53 out of 187 for Clonee plantation (28.3%) and 65 out of 192 for Kildalkey (33.8%). Moreover, the number of individuals classified as *F. angustifolia* increased to 24 individuals (12.8%) for Clonee and 17 individuals (8.8%) for Kildalkey. Given the results of the simulations and the fact that with a threshold to high *F. excelsior* will be misclassified as hybrid, we decide to set up the threshold at 0.8 for further analysis.

Using the canonical scores of the first axis of the CDA based only on morphological traits and the genetic data we conducted three correlations. First, we estimated a low but significant correlation between the genetic distance and morphological distance with the entire data set (R^2=0.177, P<0.001). Second, when only pure *F. excelsior* and *F. angustifolia* were included, the correlation was

higher (R^2=0.267, P<0.001). However when individuals from the plantations, that had been identified as either hybrid or *F. angustifolia* type were analysed alone there was no correlation (R^2=0.04; P=0.467).

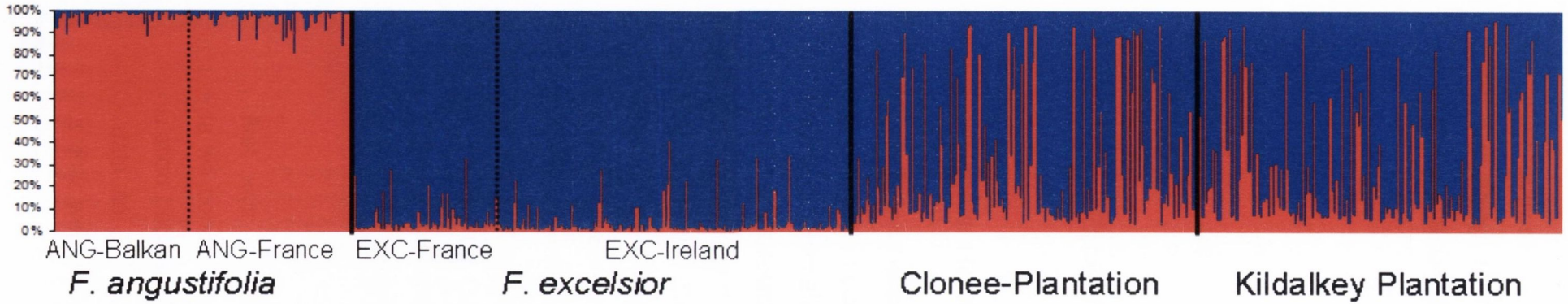

Figure 3.2 Histogram of estimated membership proportions for each parental species. Each vertical bar represented a single individual. Red corresponds to the *F. angustifolia* gene pool and blue corresponds to the *F. excelsior* gene pool.

3.3.7 Characterization of hybrids types

In the multivariate analysis based on the molecular markers only (Figure 3.1A), nine individuals clustered with *F. angustifolia*, all the nine individuals had a likelihood of *F. angutisolfia* membership based on the STRUCTURE superior to 0.9.

We examined the morphology of the plantation individuals in more detail, by limiting the analysis to material identified as hybrids or *F. angustifolia* (Figure 3.3). We thus performed a new discriminant analysis based on morphological markers as before (same control populations) but this time only including individuals, which were identified as hybrids or *F.* angustifolia in the analysis. The first axis (Wilks'λ=0.108; df=54; P<0.001) and the second axis (Wilks'λ=0.382; df=40; P<0.001) explained 68.7% and 18.0% of the variance respectively. At Least three individuals identified by the software STRUCTURE as *F. angustifolia* in Kildalkey had intermediate morphology between the two parental species and several showed unusual morphology. Only one individual at Clonee, indentified as *F. angustifolia,* showed intermediate morphology (Figure 3.3) and instead several individuals were more closely related to *F. excelsior.* The morphology of individuals identified mainly as hybrids appeared to overlap with the distribution of *F. excelsior* (as the first discriminant analysis based on morphology), however a few of them showed unusual and unpredictable morphology specially at kildlakey site.

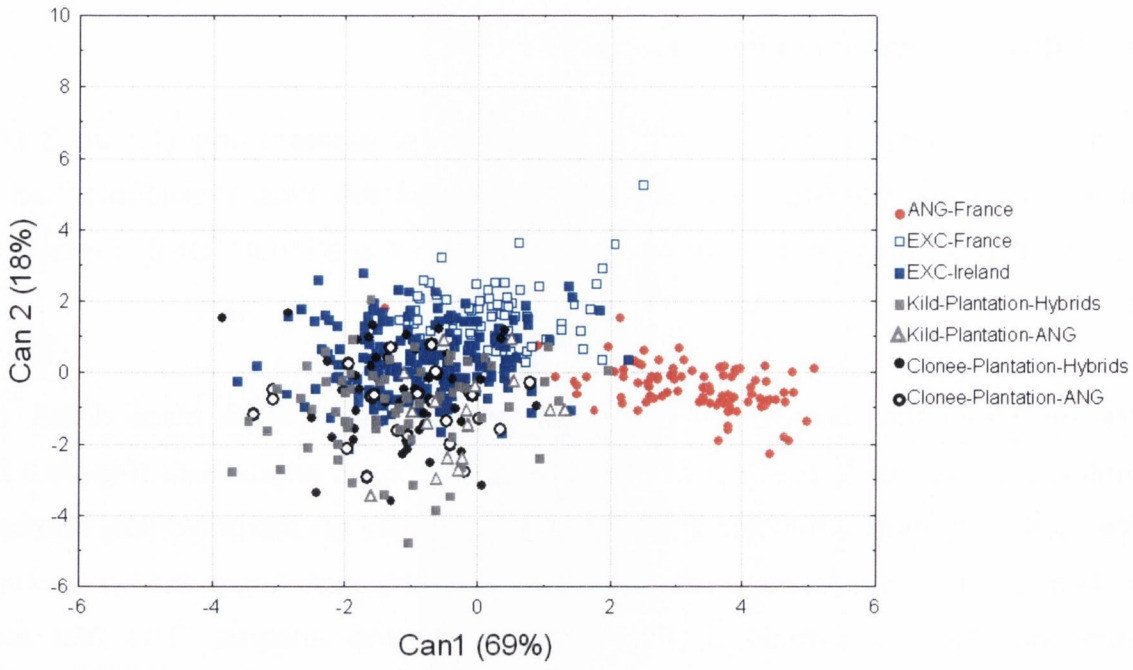

Figure 3.3 CDA based on morphological variables.

The category variable was based on the geographical groups for the control populations (closed red circles for *F. angustifolia* from France and open and closed blue squares for *F. excelsior* from France and from Ireland) and on the previous results of STRUCTURE for the introduced trees (closed grey squares if they were assigned as hybrids, open grey triangles as *F. angustifolia* for Kildalkey; closed dark circles if classified as hybrid individuals or open dark circles if classified as *F. angustifolia* for Clonee).

3.4 Discussion

We have presented molecular and morphological evidence to show that the Irish ash plantations contain numerous *F. angustifolia* and interspecific hybrid individuals. Analysis of the microsatellite data, using Bayesian model-based approaches or discriminant analysis, clearly differentiated individuals belonging to *F. angustifolia* or *F. excelsior* from each other (Figure 3.2 and Figure 3.3). Furthermore, we observed that the introduced hybrids were likely to be advanced beyond the first generation. These represent cryptic hybrid individuals that are hard to detect with morphological markers and are therefore difficult to identify without molecular markers.

3.4.1 Power of the admixture analysis and molecular detection of the hybrids

Our results with simulated data showed that the detection of advanced generation hybrids is highly sensitive to the admixture threshold set under the Bayesian models (Table 3.7). STRUCTURE and NEWHYBRIDS software were both able to accurately detect first generation hybrids. However, performance of STRUCTURE is variable when working with backcrossed material (Table 3.7). Hybrid detection in these programs dropped from 89% with a threshold value of 0.9 to less than 50% with a threshold of 0.8. Therefore, the proportion of hybrids will be potentially underestimated using these methods if threshold values are not calibrated in advance. Such results are common outcomes of hybrid identification assessments, especially those using a moderate or low number of markers (Boecklen and Howard, 1997; Vähä and Primmer, 2006).

At both plantation sites, hybrid and *F. angustifolia* trees were clearly detected (Figure 3.2), even when using the more stringent threshold of 0.9. The influence of the threshold value was less extreme with the empirical data than the simulated data. The percentage of hybrids detected using the empirical data was relatively high at both sites, ranging from 28.3%-58% for Clonee and 33.8%-56.8% for Kildalkey depending on the chosen threshold value (Tq=0.9 or 0.8, respectively). Therefore, our conservative estimates of hybrid occurrence at Clonee and Kildalkey were around 30% but it is likely that some hybrids had been misclassified into *F. excelsior* or *F. angustifolia* groups.

CDA based on molecular data (Figure 3.1A) was less efficient at recognizing hybrids than the Bayesian approach performed with the STRUCTURE software, at least when only the first two axis are used. In the CDA, plantation trees did not form an intermediate group and merged mainly into the distribution occupied by *F. excelsior*. Pairwise F_{ST} values were congruent with these results (Table 3.4) and indicated that the hybrid trees were more closely related to *F. excelsior* than to *F. angustifolia*. With the F_{ST} statistics, the introduced plantation trees were, however, significantly differentiated from *F. excelsior* (F_{ST}=0.010, P<0.05 and 0.013, P<0.05 for Clonee and Kildalkey respectively).

Moreover, on average individuals of *F. excelsior* contained 5% of the *F. angustifolia* gene pool with a maximum of 42% (Figure 3.2). *Fraxinus excelsior* was the only species present in Ireland in the past. This result suggests ancient geneflow between the two species and may reflect directional introgression. Gerard *et al.* (2006c) showed that *F. angustifolia* tends to pollinate *F. excelsior* more readily than the reverse parental combination. Moreover directional geneflow has been observed in other tree species, for example between *Quercus petraea* and *Q. robur* (Petit et al., 2004a).

3.4.2 Morphological variation

Significant differences were observed between *F. excelsior* and *F. angustifolia* for the mean values of all the morphological characters included in the study (Table 3.6, Figure 3.1B). The most significant differences between the two parental species were found for leaflet number and the number of leaflet teeth that were reduced in *F. angustifolia* compared to *F. excelsior*. This pattern was also found in previous studies (Fernández-Manjarrés *et al.*, 2006; Thomasset *et al.* 2011a). Environmental factors have important impact in leaf morphology (Van Splunder *et al.*, 1996; Niinemets *et al.*, 1999; Fitter and Hay, 2002). However, Thomasset *et al.* (2011a) undertook a common garden study using *F. angustifolia* and *F. excelsior*, (and artificially generated F_1 hybrids) and showed, using univariate and multivariate analyses, that the observed differences in morphology had a genetic basis. Nevertheless, our results showed a difference between *F. excelsior* from France and *F. excelsi*or from Ireland. It is not known what proportion of this difference was due to the environment. Some of it could be explained by environmental heterogeneity between France and Ireland (e.g. in light, soil moisture, soil type, or competition) and/or by the age of the trees. However, we might also expect to see intraspecific variation, due to genetic adaptation, across such a large geographical distance with contrasting environment.

3.4.3 Advanced generation hybrids and cryptic hybrids

There are no general rules regarding hybrid morphology compared to their parental species; thus hybrid individuals can exhibit a mosaic of phenotypes

(Rieseberg and Ellstrand, 1993) including intermediate morphology (Tovar-Sanchez and Oyama, 2004; Fernández-Manjarrés *et al.*, 2006), parental morphology (Craft *et al.*, 2002; Usher *et al.*, 2010), novel and/or extreme morphological traits (Rieseberg and Ellstrand, 1993; Johnston *et al.*, 2004). Our results from morphological and DNA markers indicated that hybridization also has unpredictable consequences in ash. Many of the hybrid individuals are cryptic. Several individuals exhibited novel traits, in particular individuals from the Kildalkey plantation. Some others seemed to have intermediate morphology but several others did not (Figure 3.3). Previous work (Fernández-Manjarrés *et al.*, 2006) on ash trees suggested only the presence of hybrids with intermediate morphologies especially in the Loire valley of France where ecological conditions appear to have allowed for both molecular and morphological introgression. On the other hand, hybrid zones in Eastern France could only be detected with genetic markers, suggesting that hybridization in continental areas may have different dynamics. In fact, if provenance certification is correct for Kildalkey, and many cryptic ash individuals are indeed from Alsace, we could hypothesise that hybrid trees may originate in temperate regions from continental Europe but this would need to be verified with sequence data for example. It is clear, however, that we would have underestimated the number of hybrids considerably if we had relied entirely on morphology. Similar results have been found with other tree species. For example, hybrid detection based on molecular markers in oak (*Quercus* spp.) indicated a higher estimation of hybridization rates than studies based solely on morphological characters (Craft *et al.*, 2002; Valbuena-Carabanã *et al.*, 2005).

Artificially generated F_1 hybrids between *F. angustifolia* and *F. excelsior* show intermediate morphology (Thomasset *et al.*, 2011a, Chapter II), therefore the present results indicated that introduced trees were mainly not first generation hybrids but were more likely to be advanced backcrosses between the two species. A significant correlation was found between the morphological and genetic distance of the parental species, however there was no correlation between morphological and genetic distance when the dataset was reduced to include only individuals classified as hybrids. Moreover, since hybrid individuals

mainly cluster with *F. excelsior*, the results suggest that they are highly introgressed to *F. excelsior*.

The power of the molecular and morphological markers to detect hybrids in the discriminant analysis was increased when the markers were combined (Figure 3.1C). All the individuals of the Kildalkey plantation were differentiated from the other clusters. However, the majority of trees at the Clonee plantation still clustered with *F. excelsior* individuals. The two plantations analyzed in the present study showed different morphology (Figure 3.1B) and low but significant genetic differentiation (F_{ST}=0.009, P<0.05). Thus, it was highly likely that the introduced ash at Clonee and Kildalkey came from different sources.

3.4.4 High genetic diversity of natural populations and introduced plants

The genetic diversity for *F. excelsior* and *F. angustifolia* in our samples was high (overall H_e=0.774) and comparable with values obtained in previous studies of natural populations (Heuertz *et al.*, 2001; Morand *et al.*, 2002; Fernández-Manjarrés *et al.*, 2006; Ferrazzini *et al.*, 2007). Even though the genetic differentiation between the two parental species was low (Table 3.4 and Table 3.5 and supplementary material table S3.1), they were significantly distinct (Table 3.4 and supplementary material S3.1; Figure 3.1A). This differentiation was mainly due to differences in allele frequencies rather than allele type, such as species-specific alleles.

A significant excess of homozygotes was observed in all populations except two of *F. angustifolia* (AngCr and Ang-StJ) and one of *F. excelsior* (Exc-LF) (Table 3.2). A deficiency of heterozygotes in *F. excelsior* was also reported for different populations in Europe (Heuertz *et al.*, 2001; Morand *et al.*, 2002; Fernández-Manjarrés *et al.*, 2006; Ferrazzini *et al.*, 2007; Sutherland *et al.*, 2010). Morand *et al.* (2002) observed a significant homozygote excess and concluded that it was due to a persistent Wahlund effect in *F. excelsior* and a preferential mating system. A Wahlund effect indicates that population does not interbreed randomly so that it is substructured. In *F. excelsior*, Heuertz *et al.* (2003) estimated that

restricted seed movement compared to pollen dispersal might explain the genetic structure within populations and increase the relatedness among neighbouring trees. Moreover, previous studies including hybrid zones and different populations of *F. excelsior* and *F. angustifolia* (Gérard *et al.*, 2006b; Gérard *et al.*, 2006c) showed an assortative mating system due to differences in flowering time. The lowest heterozygote deficiencies were observed for the two native Irish populations (Exc-Cl and Exc-Kild; Table 3.2). These were F_{IS}=0.056 (P<0.001) and 0.053 (P<0.001) for Clonee and Kildalkey respectively. The two sites are fragmented agricultural landscapes, open to strong wind. Bâcles *et al.* (2006) highlighted that landscape fragmentation can increase pollen dispersal in ash and tends to reduce population isolation. Whether these plantations are sources or sinks of pollen compared to native ash populations remains to be explored.

It is also noteworthy that genetic diversity was high for the two introduced populations suggesting that they contain a mixture of provenances (Table 3.2 and Table 3.3). In many cases, the limited diversity of introduced individuals (founder effect) and extension of range following introduction can cause population genetic bottlenecks and will lead to increased loss of genetic diversity in introduced populations (Amsellem *et al.*, 2000). However, some studies showed that introduced populations were able to maintain a high level of diversity (Squirrell *et al.*, 2001; Genton *et al.*, 2005). In general, the success of colonization and rapid adaptation to a new environment is facilitated by high genetic variation. In our case, there has not yet been time for the expansion or colonization so we cannot predict the effect of a bottleneck. However, the genetic diversity of introduced plantation populations was similar to the genetic diversity of native Irish control populations (H_e=0.808 for Exc-Ireland and respectively 0.818 and 0.767 for Clonee and Kildalkey plantations). The plantations therefore contain considerable amounts of variation on which natural selection might act.

3.4.5 Conclusions and implications for conservation

In this study, we have clearly demonstrated the hybrid origin of some alien ash in Ireland and also discovered introduced *F. angustifolia*, a non-native ash species.

To summarize, introduced trees in each plantation were a mix of *F. excelsior*, *F. angustifolia* and hybrids trees. Some of the hybrid trees had intermediate morphology but some were cryptic and when hybrids were detected, the majority was found to be backcrossed hybrids with *F. excelsior*.

Globally, reforestation projects often use exotic species and there are several reports of introduced species hybridizing with native species because of gene flow between planted stands and natural populations (Barbour *et al.*, 2002; Potts *et al.*, 2003a; Vanden Broeck *et al.*, 2003b; Ziegenhagen *et al.*, 2008). Invasions by introduced species are also well documented (Rhymer and Simberloff, 1996; Richardson, 1998; Ellstrand *et al.*, 1999; Rouget *et al.*, 2002; Titus and Tsuyuzaki, 2002). The lag time between the introduction of new species and colonization is highly variable and can take over 200 years (Kowarik, 1995). So far, in Ireland the introduction of alien ash as plantations, whether *F. angustifolia* or hybrid origin, is a recent phenomenon. The trees are just reaching maturity and starting to flower. Even though it still too early to talk about invasion, there is risk of introgressive gene flow with natural populations. We can take some comfort from the observation that introduced horticultural *F. angustifolia* has not become naturalized in Ireland. However, it is harder to predict what will happen with the introduced hybrid material. In particular, some trees are highly introgressed with the better-adapted *F. excelsior* at least for neutral markers.

It could be argued that introduced plantation ash may provide a source of novel genes for native *F. excelsior* to adapt to changing environmental pressures. Nevertheless, it is unknown if *F. angustifolia* or hybrids from continental Europe would have beneficial adaptive genes. The climatic conditions of known hybrid populations has little resemblance to that predicted for Ireland in the future (Thomasset *et al.*, 2011b, Appendix 1) and there may actually be counter selection. Future conservation, breeding programmes, and gene bank collection of ash trees in Ireland should ensure that alien ash material is not sampled. Moreover, authorities need to establish whether plantations of cryptic hybrid ash should be removed. By using different approaches combining molecular and morphological markers, it seems possible to recognize hybrid trees of recent generation. Moreover, as plantation trees in Ireland are reaching reproductive age,

it seems necessary to advise foresters on the management of the introduced plantation. As trees at Clonee and Kildalkey sites had different sources, each of the suspected sites should be assessed individually.

Much needs to be discovered before we can adequately assess the extent of gene flow possible between these aliens and native ash. We have addressed elsewhere the phenology of the alien ash, the potential of gene flow and seed viability for these plantations, but much more research effort is needed to determine if all plantation sites contain the same type of individuals as those analysed here.

Acknowledgements

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Supplementary material

Table S3.1 Analysis of molecular variance (AMOVA) within and among the geographic region grouped according to the species level (*F. excelsior, F. angustifolia* and plantation trees).

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices
Among species	2	62.941	0.02705	1.16	Φ_{CT}=0.01159***
Among geographical group within species	3	48.453	0.05614	2.4	Φ_{SC}=0.02433***
Within geographical group	1608	3620.156	2.25134	96.44	Φ_{ST}=0.03564***
Total	1613	3731.55	2.33454	100	

*** P<0.001

Chapter IV

Flowering and bud flushing patterns in native and introduced ash trees (*Fraxinus* spp.) highlights potential for hybridization

To be submitted as Muriel Thomasset, Trevor R. Hodkinson, Juan F. Fernández-Manjarrés, Atchara Teerawatananon and Gerry C. Douglas

Abstract

Phenological patterns of flowering are determined by the interaction of complex genetic mechanisms and environmental signals. As such, gene exchange between populations of the same species can be highly variable in space and time. Consequently, introduced plant populations can have different impacts on local populations depending on the resulting phenological patterns in the novel environment. *Fraxinus excelsior* is the only indigenous ash in Ireland and has economic value as a hardwood. Alien ash saplings, including: a) hybrids of *F. excelsior* × *F. angustifolia*, b) pure *F. angustifolia*, and c) provenances of *F. excelsior*, have been imported from continental Europe to Ireland for use in the forestry industry. These were generally poorly adapted to the Irish environment and after a few years began to exhibit poor stem formation. The presence of introduced plantation trees (hybrid or not) is considered to be a potential threat to the status of native *F. excelsior*. We examined the hybridization potential of the introduced trees by comparing flowering and vegetative phenology of ten variables in two populations of alien (plantations) and wild Irish ash. We collected data over two years (2009 and 2010) with contrasting climatic conditions. The results indicated that flowering time did not constitute a breeding barrier between native and introduced trees, as a significant overlap (over one month) in flowering period was observed in both years. Although trees in one of the plantations showed a significantly earlier start to flowering than native trees in 2009 and 2010, an overlap period was still observed with the native trees. Moreover, results from a multivariate analysis (PCA) showed that introduced trees, which had similar flowering phenology to the native trees, could be either hybrids or *F. angustifolia* or *F. excelsior*. Variation in climatic conditions between study years did not affect the phenology of the plants as much as genotypic differences due to contrasting source populations. This may suggest a genetic basis to the phenology. Vegetative bud burst was earlier for the introduced trees than native trees, which could explain the poor stem form observed in some of the alien cultivars. Future studies will need to focus on phenology of stigma receptivity as pollen based observations may overestimate the degree of overlap between alien and introduced trees.

4.1 Introduction

Around the world, many exotic plant species have been accidentally or deliberately introduced into the native flora, mostly for agricultural, forestry (1999; Mack and Lonsdale, 2001), horticultural or amenity purposes (Zobel *et al.*, 1987; Richardson, 1998). Most of the time, pre-mating barriers prevent hybridization and hence preserve the integrity of native species (Levin, 1978; Rathcke and Lacey, 1985; Arnold, 1997). Sometimes pre-mating barriers are removed or breakdown and this leads to potential or actual interbreeding between introduced species and their native relatives (Ellstrand *et al.*, 1999; Barbour *et al.*, 2002; Burke *et al.*, 2002; Vanden Broeck *et al.*, 2003b; Burczyk *et al.*, 2004a; Vanden Broeck *et al.*, 2005). One of the pre-zygotic barriers, which can prevent interspecific crossing in plants, is temporal isolation by flowering asynchrony (Levin, 1978).

Natural hybridization has been recognized as a strong evolutionary process (Stace, 1975; Rieseberg, 1995; Arnold, 1997; Ellstrand and Schierenbeck, 2000; Rieseberg *et al.*, 2003; Arnold, 2004b; Rieseberg and Willis, 2007) so hybridization between exotic and native species may have favourable consequences, such as increasing genetic diversity (Abbott, 1992). However, negative consequences of hybridization and introgression with wild relatives have also been documented (Ellstrand, 1992; Levin *et al.*, 1996; Rhymer and Simberloff, 1996; Vila *et al.*, 2000). Hybridization might play a role in enhancing invasion and colonization (Potts and Reid, 1988; Petit *et al.*, 2004b) and can also be a source of genetic pollution that may result, in some cases, to extinction by hybridization (Ellstrand and Elam, 1993; Rhymer and Simberloff, 1996).

Phenology is defined as the study of timing of life cycles (Lechowicz, 2001). The regulation of these events is determined by the complex interaction of genetic mechanisms (Boss *et al.*, 2004; Jack, 2004) and environmental signals (Rathcke and Lacey, 1985). Studies of phenology of different plant communities over the last decade have shown an advance in bud burst dates, flowering dates and dates of pollen release all of which have been correlated with rising temperatures (Emberlin *et al.*, 2002; Fitter and Fitter, 2002; Parmesan and Yohe, 2003; Miller-

Rushing *et al.*, 2007). One of the responses of plants to climatic change could be that sympatric species with non-synchronous flowering could become synchronous. Another concern is that vegetative phenology such as leaf bud burst, might also change and this has already been found to be strongly linked to climatic conditions in some species (Fenner, 1998; Partanen *et al.*, 1998; Wielgolaski, 1999; Linkosalo *et al.*, 2006; Hänninen and Kramer, 2007; Doi and Katano, 2008). The timing of dormancy break and leafing is important in temperate tree species, as they have direct consequences on annual growth (Heide, 1985). Furthermore, a significant advance in the timing of vegetative bud burst has been linked to rising spring temperatures in many European countries including Ireland (Chmielewski and Rötzer, 2001; Kunkel *et al.*, 2004; Donnelly *et al.*, 2006; Menzel *et al.*, 2006a; Menzel *et al.*, 2006b; Parmesan, 2006). The earlier timing of bud burst makes tree species more susceptible to damage from late spring frost (Kramer, 1996; Inouye, 2008).

In Ireland, in order to meet the needs of farmers and foresters who in the 1990s received grants from the Irish government to plant native broadleaf trees, seeds of *Fraxinus* were imported from continental Europe. The plantations have been characterised using molecular markers (Chapter III) and contained *F. angustifolia* Vahl and *F. excelsior* L., and hybrids between them (between 28% and 58% of the plantation). More than 100 plantations were found to be affected (G. Douglas, personal communication) with trees developing poorly (deformation and excessive branching). *Fraxinus excelsior* is the only native ash in Ireland but in continental Europe two other species occur, *F. angustifolia* and *F. ornus* in addition to *F. excelsior*. Moreover, *F. angustifolia* and *F. excelsior* are known not only to be cross compatible (Raquin *et al.*, 2002a), but also to produce natural hybrids where they have been found to grow in sympatry (Fernández-Manjarrés *et al.*, 2006), indicating that an overlap in the flowering time occurs at those latitudes (Gérard *et al.*, 2006b; Gérard *et al.*, 2006c).

The establishment of exotic mixed plantations of *F. excelsior*, *F. angustifolia,* and hybrids throughout Ireland has created the potential for gene flow from exotic trees into native ash populations. However, little is known about the phenology of ash species or their hybrids. Furthermore, it is not known how Irish climatic conditions

might alter the flowering time of the introduced plantation trees in comparison to that expected in their native range.

The aim of this study was to assess the phenology of alien ash in Ireland and to help determine the potential for gene flow between alien ash and native ash. To estimate the phenological contribution to reproductive isolation, we recorded the flowering time during two seasons of reproduction for both native and non-native trees (2009 and 2010). Moreover, we investigated vegetative bud burst in the ash populations to determine whether this could be an explanation for the poor growth of plantation trees under current Irish climatic conditions. We undertook the study over two growing seasons to check for consistency in the timing of the phenophases in question and to investigate how different climate conditions may influence phenology in these taxa.

4.2 Materials and methods

4.2.1 Study species

Fraxinus excelsior and *F. angustifolia* generally differ in their distribution (Thomasset *et al.*, 2011b), their climate and soil requirements (Marigo *et al.*, 2000; Oddo *et al.*, 2002; Jaeger *et al.*, 2009), and their flowering phenology (Raquin *et al.*, 2002a; Jato *et al.*, 2004; Gérard *et al.*, 2006b). However they grow in sympatry at the northerly distribution limits of *F. angustifolia* (Fernández-Manjarrés *et al.*, 2006), and natural hybrids in these regions indicate flowering time overlap (Gérard *et al.*, 2006b; Gérard *et al.*, 2006c). Both species are wind pollinated with complex polygamous mating systems (Wallander, 2001; Morand, 2003) and wind dispersed fruits (winged samaras).

Climatic preferences of the two species differ. Indeed Tal (2006) and Peeters (2000) revealed that cold weather, especially low temperature in February and March, and strong rainfall might be responsible for delayed onset of flowering and for the start of pollen release in *F. excelsior*. For *F. angustifolia*, Jato *et al.* (2004) suggested that the accumulation of chilling period in November determined the

heat requirement and the start of the pollen season in *Fraxinus*. They highlighted differences in chill requirement between *F. angustifolia* and *F. excelsior* and found that flowering in *F. angustifolia* will be delayed if temperature drops below 0°C in December and January. Moreover, Van Vliet *et al.* (2002) showed, in a study lasting over 30 years, that the starting date of flowering *of F. excelsior* did not differ much. In contrast, Penuelas *et al.* (2002) showed that flowering of *F. angustifolia* was delayed for more than 37 days between 1952 and 2000.

4.2.2 Sampling site

Two sites, Kildalkey (53° 34' 17.8"N, 6° 54' 54.21"W) and Clonee (53° 28' 03.09" N, 6° 28' 58.9"W), were selected for this study. Both sites contained a plantation with imported trees exhibiting poor stem form. The plantation trees have started to reach maturity age (approximately 12 years) and have begun to produce flowers and seeds. Not all the plantation trees had reached the flowering maturity stage. The plantations were shown, using morphological and molecular markers, to be mixed stands of *F. excelsior*, *F. angustifolia* and their hybrids (Chapter III). Moreover, the same study had shown that the two plantations were stocked from different source populations. In each plantation, we randomly selected 70 imported ash trees from a sample of 192 trees analysed for a paternity and gene flow study (Chapter V). During successive years of the study, observations were made on the same trees. Tree diameter at breast height (DBH) was also recorded for the plantation trees. The plantations were established adjacent to populations of native Irish ash trees, generally present in the hedgerows of fields. 60 native trees were randomly selected from these mature populations. The closest native trees were located on the hedgerow of the planations (less than 5m), the furthest was slected to be at less than 500 m of the plantations. GPS coordinates of all trees were taken using MobileMapper™ Pro from Thales so that they could be monitored over the study period. The system is a mobile mapping system combining full GIS data collection and navigation software in a handheld GPS receiver with an accuracy in Real-Time Autonomous between 7-10 m

Phenological observations were made every 10 days between the end of January and June in 2009 and 2010 and climatic data during these years were obtained from the Dublin Airport Meteorological Station, the nearest station to both plantations (approximately 40 to 60 km from the two study sites) and at similar elevation (http://www.met.ie/climate/monthly-data.asp). A summary of average monthly temperatures and rainfall for this station is given in Table 4.1.

Table 4.1 Mean temperature and rainfall values for Dublin airport during the two years of the study (2009 and 2010), and the monthly averages between 1961 and 1990.

	Year	Jan	Feb	Mar	Apr	May	Jun	Annual
Rainfall in millimetres for Dublin Airport	2009	62.1	56.1	26.2	71.1	75.5	64.3	978.9
	2010	45.2	36.7	54.8	26.7	38	50.3	628
	1960-1991	69.5	50.4	53.5	51.1	54.8	55.8	732.8
Mean temperature in degrees C. for Dublin Airport	2009	4.3	5.1	6.7	8.3	10.9	13.2	9.6
	2010	1.7	2.3	4.9	7.7	9.6	14.1	8.8
	1960-1991	5	5	6.3	7.9	10.5	13.4	9.6

4.2.3 Phenological survey

Previous studies on ash have established a classification of the phenological flowering phases (Gérard *et al.*, 2006b) and the classification of different inflorescence and tree gender types (Wallander, 2001). Categories for the gender were simplified for our study and classified as follows:

1. Male (large stamens with no visible pistils- or a rare rudimentary pistil)
2. Balanced hermaphrodite (large pistils and medium sized stamens)
3. Female-biased hermaphrodite (a large pistil, small (fertile) stamens)
4. Female (large pistils, no or rudimentary (sterile) stamens).

Phenological phases for the flower were scored according to the scale in Figure 4.1. Phenophases are defined from the initial flushing date (Stage 1) to the wilting of the flower (Stage 5); Stage 2.5 is a key stage (pollen starting to be released and stigma completely extended and formed). For the leaf bud phenology we used a five point scale as reported in Jouve et al. (2007) from dormant buds (Stage 0) to fully expanded leaves (Stage 4) with stage 1 being the start of the first swelling of apical vegetative buds.

Trees were monitored in 2009 and 2010 every 10 days by visual observation using binoculars when necessary. Given the predicted flowering periods of *F. angustifolia* and *F. excelsior* (Raquin *et al.*, 2002a; Jato *et al.*, 2004; Gérard *et al.*, 2006b; Gérard *et al.*, 2006c), our first field observations were made from the beginning of January and continued until the beginning of June, using January 1st as Julian day 1. On each census day, we recorded the number of trees in flower and their phenological stage. A given tree was considered to be in flower or to be leafing if more than 10% of the buds start to flush, and continuing to flower if there were still anthers releasing pollen. We also noted the timing of peak flowering for each year (date when a maximum number of trees were between Stage 2.5 to 4).

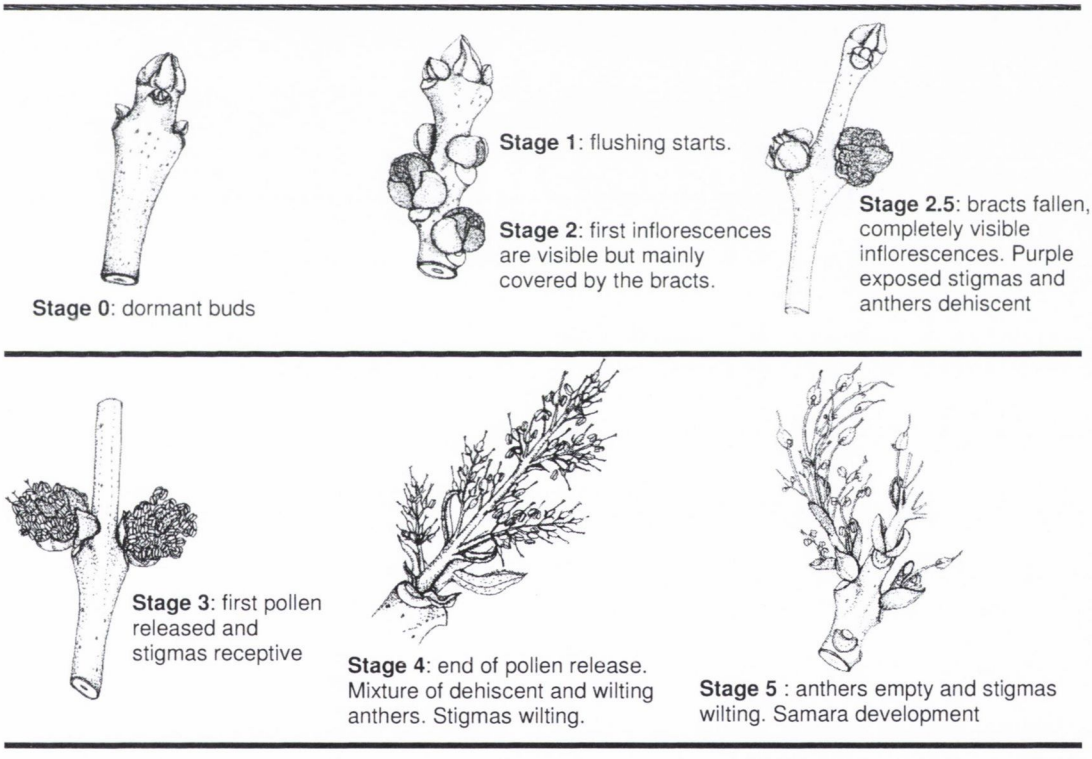

Figure 4.1 Description of the flowering stages and score notation.
Source: drawings Atchara Teerawatananon

In order to assess the risks for hybridization between introduced and native trees, individual phenologies were described at the individual crown level and not at a branch level, and were summarized using three variables for flowering phenology and two for the vegetative phenology:

1. The onset of the flowering period (Onset) (i.e. the first flowering date when trees are at Stage 1);
2. The first date when trees started to release pollen or had receptive stigmas (Stage 2.5);
3. The duration of the flowering period (DFP): number of days between the first date that trees reached Stage 2.5 and the end of the flowering period when trees reached stage 5;
4. The first date when apical vegetative buds started to swell (Apical);
5. The duration of leaf expansion (DLE), taken as the number of days between the swelling date of the apical buds to fully expanded leaves.

4.2.4 Statistical analysis

The proportion of trees flowering each year followed a binomial distribution hence we explored the relationship between flowering (i.e. proportion of trees flowering each year: response variable) and the values of the predictors (populations: explanatory variable) using a generalized linear model (GLM) with binomial error and logit link function (McCullagh and Nelder, 1989). Pairwise comparison of the means were realised using Fisher's protected least significant difference, significance was declared if $P<0.05$.

Normality of all phenological variables, except for the proportion of trees, was examined by the Kolmogorov-Smirnov test and homogeneity of the variance by Levene's test. Although plots of variables looked approximately normal, statistical tests revealed that the residuals deviated from normality for each variable ($P<0.001$), data transformation did little to improve the situation. Therefore, data were not transformed and non-parametric tests were carried out (as described below). All analyses were carried out using the software Statistica v.6.0 (Statsoft, 2004).

We computed the mean and the standard deviation for each of the five variables for each group of trees (i.e. native or introduced plantation trees at each site) and each year. Only trees that flowered during the observation period (i.e. 2009 or

2010) were used to calculate the mean of the reproductive characters. We compared the variable means between each of the different pairs of population groups for each year (Plantation-Clonee versus Native-Clonee; Plantation-Kildalkey versus Native-Kildalkey, Plantation-Clonee versus Plantation-Kildalkey and Native-Clonee versus Native-Kildalkey) using the Mann–Whitney *U* test (Underwood, 1997). Spearman rank correlations were used to assess the correlation between the different variables and to examine the consistency of flowering and vegetative phenology over time. To further analyse the difference between years by comparing the difference between each pair of populations, a matched pairs Wilcoxon test was carried out. For the reproductive variables, only trees that produced flowers in the two consecutive years were analyzed.

Given the different flowering times of *F. angustifolia* and *F. excelsior* (Raquin et al., 2002a; Jato et al., 2004, Gerard et al., 2006b), it is known that some trees flower much earlier than others. We decided to divide our data in three flowering time categories- early, intermediate, or late. To determine the best arrangement of our dataset, the first day of budburst for all the trees over the two years were pooled together. We used the Jenks Natural Breaks Classification method to categorize our data into classes. The data were analyzed using the software package ClassInt, implemented in R (R Foundation for statistical software 2007). To obtain three categories, the results suggested partitioning our data as follows: category one from Julian day 26 to Julian day 69; category two from Julian day 69 to Julian day 89; and category three from Julian day 89 to Julian day 110. Hence, given the different times of our field observations, individuals that started to flower before 10th March were classified as early; those between the 10th March and 5th April were considered as intermediate; and those after the 5th April were classified as late. The same classification was used for 2009 and 2010.

We used principal component analysis (PCA) to provide overall information about phenological similarities between native and plantation trees. Results of the PCA were compared with the classification of each tree as either: 1) hybrid, 2) *F. excelsior*, or 3) *F. angustifolia* (Chapter III)[1]. Classification of each tree as early,

[1] Threshold values (*Tq* see Chapter III) for the group delimitation was set up at 0.8

intermediate, or late flowering was also compared with the hybrid classification from the molecular data (Chapter III). Spearman correlation between the onset of the flowering or the onset of the leaf flushing and the DBH was also calculated.

4.3 Results

4.3.1 Climatic differences between years

January and February 2010 were much drier than the same two months in 2009 but also much colder (4.3°C average for January 2009 compared to 1.7°C for January 2010). In 2010, the weather remained cooler until June and in general less rainfall was observed except for March (Table 4.1). January 2010 until March 2010 was a colder period compared to the average mean observed for the same over a period of 30 years (Table 4.1).

4.3.2 Proportion of flowering trees

Trees from the two plantations produced less flowers than native trees during each year of the survey (Figure 4.2). For the first year, 2009, only 47% and 56.8% of the plantation trees flowered for Clonee and Kildalkey, respectively. Mean comparisons indicated that the proportion of plantation trees that flowered was not significantly different between the two plantations ($P>0.05$) but was significantly lower ($P<0.05$, results not shown) than the proportion of flowering native trees. The proportion of flowering trees from the plantation increased slightly during the second year (2010), however this increase was not significant between years ($P>0.05$). However, the native trees produced significantly ($P<0.05$) more flowers the second year (from 87.6% in 2009 to 100% in 2010, and from 67.5% in 2009 to 98.3% in 2010 for the Clonee and Kildalkey plantations respectively).

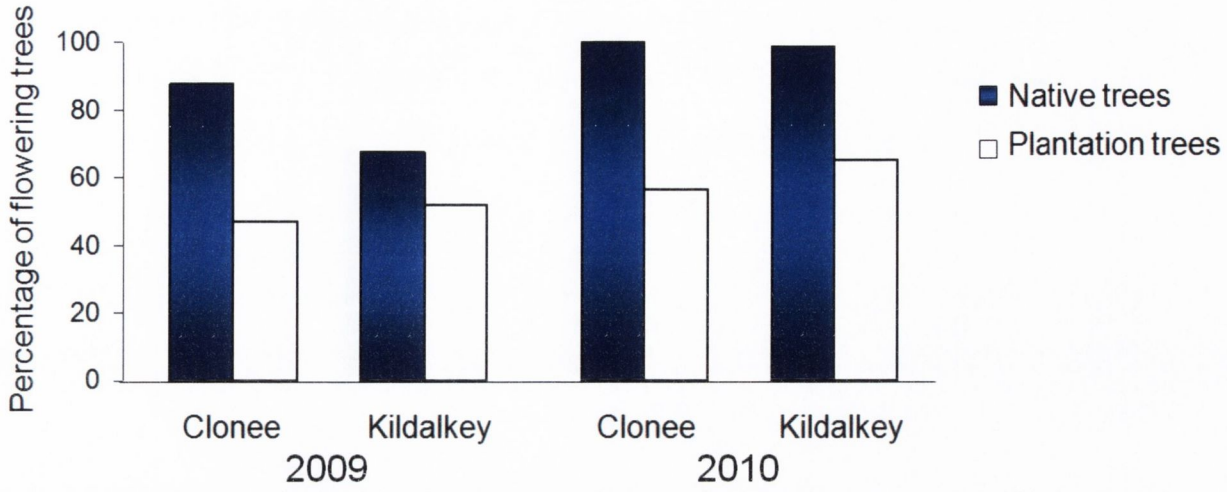

Figure 4.2 Distribution of flowering intensity measured as number of flowering trees in 2009 and 2010 for native and introduced trees at Clonee and Kildalkey sites.

4.3.3 Gender of the trees

No pure female or female biased hermaphrodite flowers were observed. Trees, whether native or introduced, were either pure male or hermaphrodites with functional anthers. Similarly, the plantation trees, which confirmed as hybrids by molecular and morphological study (Chapter III) were also either male or hermaphrodite: equal proportion.

4.3.4 Flowering phenology

4.3.4.a Clonee site

- Within years

In 2009, no significant difference between native and plantation trees was observed for the mean flushing date of flowering buds (Onset) (Table 4.2, and Figure 4.3). However, even though the majority of the plantation trees flowered at the same time as the native trees, few displayed a very early date of flushing (minimum Onset of 36 days for plantation individuals and 55 for the native trees, data not shown). A strong correlation (Table 4.3) was found between Onset values and Stage 2.5 values. Indeed, in 2009, a similar pattern for Onset and Stage 2.5 was observed (Figure 4.3). In addition, at this site, there was no difference between the first pollen release of the introduced and the native trees (Figure 4.3).

In 2010, a low but significant difference was observed between the plantation and the native trees for the start of flowering period (Onset) and the start of the pollen release (Stage 2.5) (Table 4.2).

The duration of the flowering period (DFP) was not correlated with any of the other variables (Table 4.3), and was significantly shorter for the plantation trees in 2009 ($P<0.01$; Table 4.2). However, in 2010, no difference was observed for the DFP between the plantation and the native trees.

Table 4.2 Pairwise comparisons between populations for flowering phenology. Significance of the *P*-values for Mann-Witney U test.

	Kildalkey (Native vs. Plantation)		**Clonee (Native vs. Plantation)**		**Native-Clonee vs. Native-Kild**		**Plantation-Clonee vs. Plantation-Kild**	
	2009	2010	2009	2010	2009	2010	2009	2010
ONSET	***	***	0.475	*	0.457	0.345	***	0.058
Stage 2.5	***	***	0.330	*	0.861	0.623	***	0.814
DFP	***	**	**	0.444	0.567	0.729	***	0.503
Apical	***	***	***	***	0.014	***	0.391	0.163
DLE	***	0.159	***	0.920	0.029	0.353	0.069	***

If $P>0.05$ its value was indicated by the real value, * indicates $P<0.05$; ** $P<0.01$, *** $P<0.001$
ONSET: onset of the flowering; Stage 2.5:first pollen release; DFP: duration of the flowering period; Apical: first swelling in Apical vegetative buds; DLE: duration of leaf expansion.

- Between years

For both years, the distribution of pollen release was unimodal for the native and the plantation trees. Native and plantation trees had the same flowering peak (15th of April; Figure 4.4) in 2010 as they had in 2009. Thus, flowering in native and plantation trees appeared synchronized. The overall duration of the flowering period for the native trees and the plantation trees (Figure 4.4) was over 70 days in 2009 and 2010. Moreover for the tree flowering variables, Onset, Stage 2.5 and DFP, there were not significantly different between the two years (Wilcoxon matched pairs test $P>0.05$; data not shown). The cold weather observed in 2010 (Table 4.1) for January and February did not affect the flowering period in Clonee.

Trees were categorized each year regarding their flowering date as early, intermediate and late as defined in material and methods section. At Clonee, 69.1% and 44.3% of the flowering native trees had intermediate flowering time in 2009 and 2010 respectively. Over the two years, 2009 and 2010 respectively, 12.6% and 29.5% of the native trees showed late phenology and 18.3% and 26.2% showed early phenology start (Table 4.4). Few differences were observed by comparing the individual phenological classification of the native trees over the two years (comparison between trees, which had produced flowers over the two years; data not shown). Six individuals, which had intermediate flowering in 2009, flowered late in 2010 and three trees with intermediate flowering in 2009 flowered early in 2010. However, for native trees, no trees that flowered late in 2009 showed early flowering in 2010.

For the introduced plantation trees, only a few differences in flowering group (intermediate, late or early) were observed between 2009 and 2010. At Clonee, one tree with early flowering in 2009 was classified as intermediate the second year, two trees classified as intermediate were identified as early in 2010 and four classified as intermediate became late in the second year (Table 4.4). Therefore, plantation trees, which flowered early in 2009 still tended to flower early in 2010.

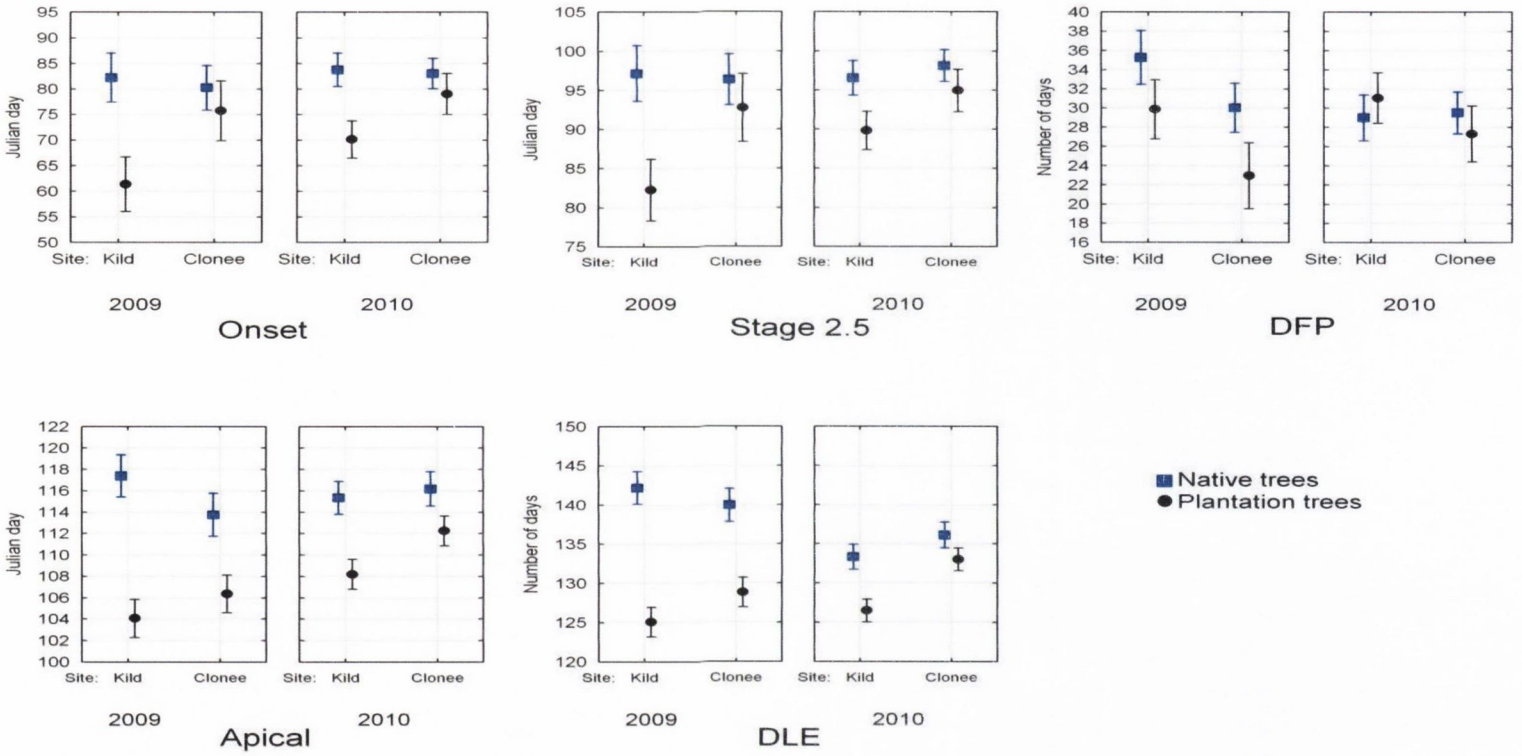

Figure 4.3 Means and standard deviations of the different phenological variables among populations (native or introduced), site (Clonee or Kildalkey), and year (2009 or 2010). Onset: onset of the flowering; Stage 2.5:first pollen release; DFP: duration of the flowering period; Apical: first swelling in Apical vegetative buds; DLE: duration of leaf expansion.

Table 4.3 Spearman rank correlations between the different variables in 2009 and 2010. Correlations significant at $P<0.05$ are above the diagonal.

	Onset 2009	Stage 2.5 2009	DFP 2009	Apical 2009	DLE 2009	Onset 2010	Stage 2.5 2010	DFP 2010	Apical 2010	DLE 2010
Onset 2009	-	0.894	0.113	0.445	0.097	0.765	0.621	0.422	0.200	0.216
Stage 2.5 2009	***	-	0.209	0.478	0.202	0.739	0.58	0.373	0.244	0.247
DFP 2009	ns	ns	-	0.188	0.124	0.019	0.038	0.193	0.151	0.017
Apical 2009	***	***	ns	-	0.241	0.422	0.387	0.042	0.45	0.28
DLE 2009	ns	ns	ns	***	-	0.217	0.11	0.009	0.253	0.125
Onset 2010	***	***	ns	***	ns	-	0.82	0.536	0.242	0.178
Stage 2.5 2010	***	***	ns	***	ns	***	-	0.709	0.245	0.058
DFP 2010	***	***	ns	ns	ns	***	***	-	-0.014	0.065
Apical 2010	ns	ns	ns	***	ns	ns	ns	ns	-	0.17
DLE 2010	ns	ns	ns	***	ns	ns	ns	ns	ns	-

4.3.4.b Kildalkey site

- Within years

In 2009 at Kildalkey, the mean flushing date for flowering buds (Onset) of the introduced plantation trees was significantly ($P<0.001$) earlier than the native trees (Table 4.2, and Figure 4.3). Onset and stage 2.5 showed similar pattern (Figure 4.3). A significant difference was observed between the date of the pollen release between the native and the plantation trees. In 2009, plantation trees had a significantly shorter duration of flowering (DFP) than native trees (Table 4.2 and Figure 4.3).

The male function phenology (pollen release) of the plantations was found to be heterogeneous. Introduced plantation trees started to release pollen at the beginning of February in 2009 (Figure 4.4). In this plantation, pollen was released

over a period of 85 days in 2009 and the flowering peak occurred during the beginning of April. In contrast, for the native trees, the flowering peak was around the 20th of April in 2009 (Figure 4.4), indicating a difference of more than 15 days for peak flowering at between the native population and the plantation trees. However, a substantial overlap (more than 50 days) between the flowering periods was observed.

In 2010, plantation trees flowered significantly ($P<0.001$) earlier than the native trees (Table 4.2 and Figure 4.3). However, the DFP was not significantly different between the native and the plantation trees (Table 4.2). In 2010 the overall duration of the flowering period (Figure 4.4) for the introduced trees was around 60 days and similar to the native trees. The flowering peak occurred around the same time, the 10th of April (Figure 4.4). Therefore even if the plantation trees flowered earlier (on average) than the native trees, there was a large overlap of the flowering periods.

Table 4.4 Percentage of individuals classified as early, intermediate, and late flowering according their origin and the year. The classification of individual trees was made according their flowering bud burst date.

	Clonee				Kildalkey			
Flowering	**Native**		**Plantation**		**Native**		**Plantation**	
group	2009	2010	2009	2010	2009	2010	2009	2010
Early	18.3	26.2	32.9	36.6	25.9	23.2	65.9	64.3
Intermediate	69.1	44.3	47.1	51.4	50	60	25.5	26.1
Late	12.6	29.5	20	12	24.1	16.8	8.6	9.6

- Between years

At the two sites, a strong correlation (Spearman coefficient r=0.765) between Onset values of the two years was observed. There was also a correlation between Stage 2.5 values over the two years (r=0.580) (Table 4.3).

For the native trees, there were no significant differences between the two years (Wilcoxon matched pair test $P>0.05$; data not shown). The cold weather observed in 2010 (Table 4.1) for January and February did not affect the start of the flowering period (Onset) or the time when the flowers of the native trees reached maturity (Stage 2.5). The majority of individuals showed intermediate flowering

dates. 50.0% and 60.0% of the flowering trees had intermediate flowering phenology for 2009 and 2010, respectively. For Kildalkey, changes among flowering categories were only observed for the trees showing intermediate and early phenology (two trees with intermediate flowering the first year became early the second year and *vice versa*); trees with late flowering remained late. We never observed a tree with late flowering the first year and early flowering the second year (or *vice versa*). The category appeared to be maintained over the time.

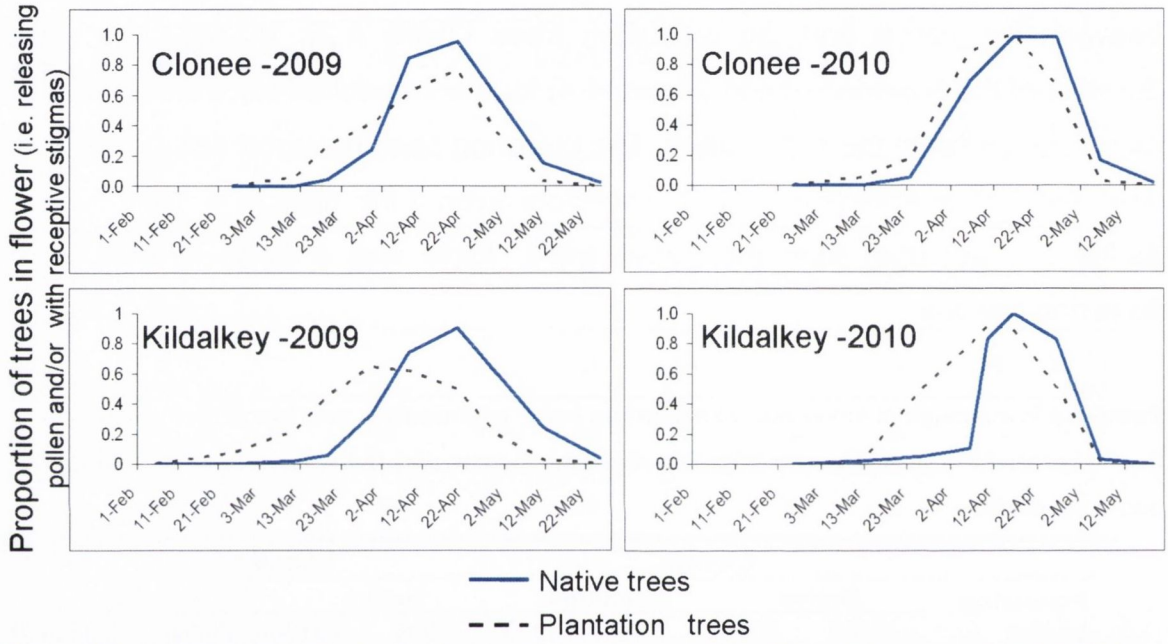

Figure 4.4 Frequency distribution for flowering phenology of the different sites monitored in 2009 and 2010.

For plantation trees in Kildalkey, the only significant difference was observed for the Onset values (data not shown) between the two years; they flowered later on average in 2010 than in 2009, N=34, Z=3.015, *P*=0.002). As mentioned previously, even if the overall duration of the pollen release of the introduced trees was 20 days shorter in 2010 than in 2009 (Figure 4.4), no significant difference was observed for the individual DFP (Figure 4.3). The duration of the pollen release (DFP) was the same between the two years, even though DFP was slightly longer for the introduced trees in 2010 (Figure 4.3). For the plantation trees, a few changes were observed regarding their classification status: one tree classified as late in 2009 was classified as intermediate in 2010 and one tree classified as early

became intermediate (and *vice versa*). The group classification appears to be consistent over the two years of the study.

4.3.4.c General flowering pattern

There were no significant differences between the two sites for Onset, Stage 2.5 and DFP values in 2009 and 2010 (Table 4.2) for native trees. However, plantation trees at Kildalkey flowered significantly earlier in 2009 than plantation trees in Clonee (Table 4.2, Figure 4.3). There was a large difference between the plantation trees at Clonee and Kildalkey sites for their first flushing date (early, intermediate or late; Table 4.4). Averaged over the two years, 65% of the flowering plantation trees had an early start at Kildalkey, compared to 35% at Clonee. We have observed that during hard frost, the inflorescences of plantations trees, which flowered very early (End of January, beginning of February) might be killed. The majority of the plantation trees at Clonee had an intermediate flowering period (52%), compared to approximately 25% at Kildalkey. At both sites, for both years, some plantation trees with very late flowering phenology were also observed (Table 4.4). Using a Spearman correlation, no correlation for the two plantations and over the two years of observations was found between DBH and the onset of flowering (Data not shown).

4.3.5 Leaf flushing phenology

A strong positive correlation (r=0.445) was observed between the flowering Onset and values for the initiation of vegetative bud swelling (Apical) in 2009. However, there was no such correlation in 2010. At the two sites, leaves of the plantation trees started to expand significantly earlier than native trees in both years (Table 4.2 and Figure 4.3). The native trees of the two sites showed significantly earlier leaf bud flushing in 2010 than 2009 (Wilcoxon matched pair test, $P<0.05$; data not shown). However, opposite results were found for plantation trees. Leaves of the plantation trees burst significantly later in 2010 than 2009 (Wilcoxon matched pair test, $P<0.05$; data not shown). No significant difference in flushing time between the plantation trees of the two sites was observed during the two years; likewise

similar results were observed for the flushing time of native trees at the two sites except for the year 2010 (Table 4.2).

The period of leaf expansion in vegetative buds (DLE) was significantly reduced for the plantation trees in 2009 (Table 4.2) but not in 2010. No difference between the native trees, at each site over the two years, was observed for DLE. However, in 2010, a significant difference was observed between the DLE of plantation trees, Kildalkey having a shorter expansion period than Clonee. DLE was not correlated with any other variable (Table 4.3) in 2009 or 2010, apart for DLE in 2010 that was correlated with Apical in 2009. No correlation between the DLE values over the two years was observed for the plantation trees. Whereas DLE increased in a significant way for the plantation trees at the two sites in 2010 compared to 2009 (Figure 4.3, $P<0.05$; data not shown), no differences between the native trees at each site were observed.

For the two plantations, no correlation between DBH and the onset of the leaf flushing was found (Data not shown), indicating that smaller trees were no more likely to flush earlier.

4.3.6 Phenology and hybrid recognition

Results of the PCA based on the ten phenological variables showed that a few individuals did not cluster with the native trees (Figure 4.5). The first two axes of the PCA explained 37.6% and 16.6% of the variation respectively for the Clonee site. The first two axes of the PCA for the Kildalkey site accounted for 42.9% and 15.5% of the variation, respectively. For Kildalkey, of the 33 trees that flowered in both years, 13 (36.33%) did not cluster with the native trees. Of the 27 trees that flowered in both years for the Clonee site, 16 (59.25%) did not cluster with the group of native trees.

For Clonee, out of the 16 trees, which did not cluster with the native trees, 10 were classified as hybrids, and five as *F. angustifolia* (Chapter III). For Kildalkey, for the 13 trees which did not cluster with the native trees, only one was previously

identified as *F. excelsior*; nine were identified as hybrid and two were identified as *F. angustifolia* (Chapter III). Among the trees that could not be distinguished from the natives based on the phenology, few individuals were identified as hybrids. However, among the trees, which clustered with the natives (Figure 4.5), 55% and 45% were previously classified as hybrids or *F. angustifolia* type for the Clonee and Kildalkey sites, respectively.

For Clonee, 40.7% of the trees, which flowered over the two years, were classified as early flowering trees (data not shown). The early trees were all identified as hybrids or as *F. angu*stifolia, except for one individual. Trees with intermediate phenology were classified as *F. excelsior*.For Kildalkey, out of the 33 trees that flowered in 2009 and 2010, 25 were classified as early flowering (75.57%); among these 23 (92%) have been identified as hybrids or *F. angustifolia*. None of the trees, which were classified as late flowering, showed *F. angustifolia* characters (Chapter III).

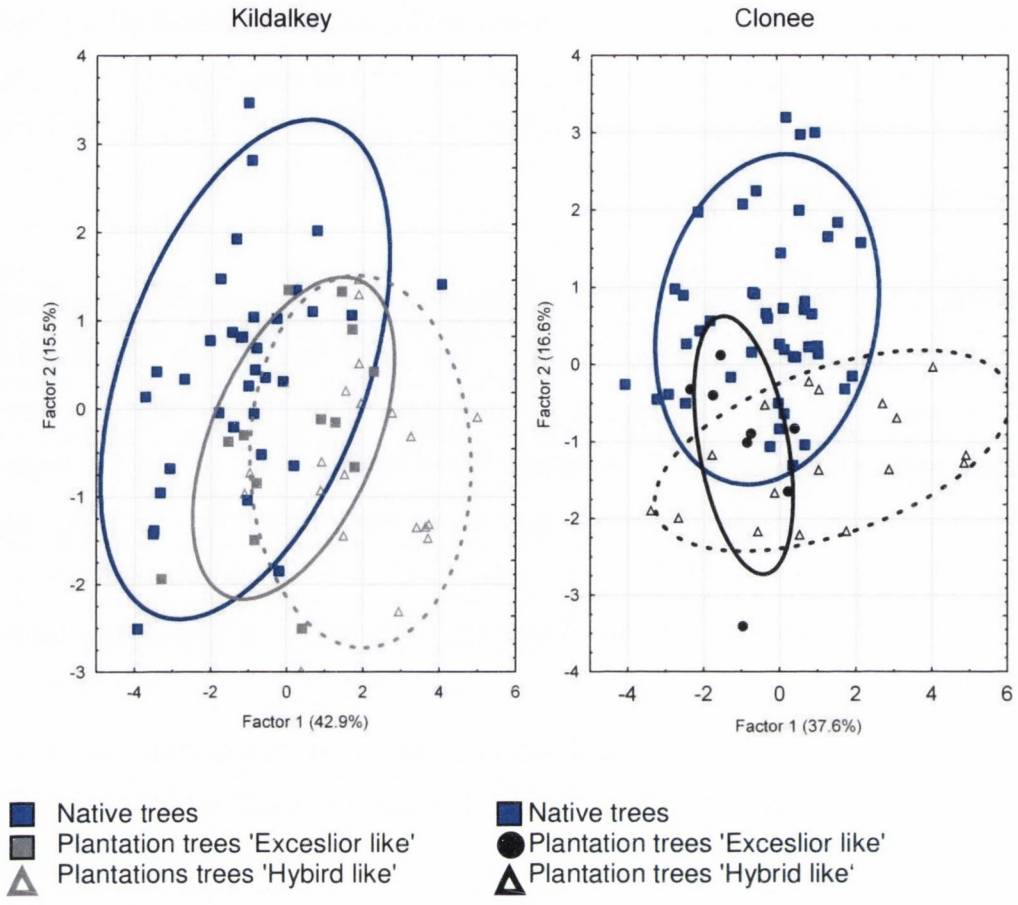

Figure 4.5 Representation of the individual trees on the first two axes of the principal component analysis (PCA) based on the ten phenological variables collected over the two years of the study. 95% confidence intervals around the mean score for native trees and plantation trees are indicated with ellipsoids.

4.4 Discussion

Our results show that the flowering period of the introduced and native ash trees overlapped for a long period. Flowering phenology is not a strong barrier to gene flow. Moreover, we show that 1) the genetic identity of the plantation trees and 2) the variation in weather might have an important impact on the potential for hybridization and introgression.

4.4.1 Overlapping flowering time and introgression

Individuals trees with synchronized flowering time are more likely to mate with each other (Fox, 2003; Weis and Kossler, 2004; Hendry and Day, 2005), but flower opening can be deceptive because stigma receptivity may not always coincide with flower opening or pollen release. For example, in *Fraxinus,* it has been reported (Wallander, 2001) that stigmas in hermaphrodite inflorescences can be receptive up to a few weeks before pollen release. However, in the present study, we mainly measured pollen release since we did not observe any pure female flowers, and stigma receptivity was not practical to determine on site. In consequence, our measures of flowering time overlap may be overestimated.

Despite the fact that plantation trees showed an early start to the flowering period, especially for Kildalkey (suggesting the possibility of bi-modal peaks of flowering), it is clear that the flowering of plantation and native trees in our study was overlapped for several weeks. The overall flowering period of imported plantation trees and native trees was found to overlap for over 50 days during the two years of observation if we consider the male function only.

There is a potential for pollen transfer and interbreeding between native trees and plantation trees identified as hybrids or *F. angustifolia*. Several of the plantation trees are known to be *F. angustifolia* or *F. angustifolia x F. excelsior* hybrids (Chapter III). It was evident that many of these trees showed early flowering compared to native *F. excelsior* (consistently over the two year study period) which would limit their chance of cross-pollination with the native trees. However, we also observed trees, identified as introgressed hybrids and/or *F. angustifolia* that had an intermediate flowering period that overlapped with the native trees. Moreover, in the multivariate analysis based on all the phenological variables over the two years (PCA, Figure 4.5), we observed that if some plantation trees showed unusual phenological traits compared to native *F. excelsior*, several of the plantation trees showed similar patterns to the native ones.

In a study of natural hybrid zones of *F. excelsior* and *F. angustifolia*, Gérard *et al.* (2006c) showed that trees with early flowering participate more as pollen donors

and have a higher mating success than later flowering individuals. In our case, this might indicate that early flowering plantation trees (that are often hybrids or *F. angustifolia*) have the potential to contribute more as pollen donors than later flowering trees, if the latter do not flower enough to compensate for this early pollen release. This could have important consequences on the probability of introgression between native and alien plantation trees.

4.4.2 Intensity of flowering

More trees flowered in 2010 than 2009 for both plantation and native populations (Figure 4.2). This finding supports the results of some previous studies on ash that have shown that flowering intensity can vary from year to year (Wallander, 2001; FRAXIGEN, 2005). Flowering intensity and linked pollen release will influence the levels of potential gene flow within and among populations. In *Eucalyptus* plantations, it has been shown that flowering intensity increased with the age of the plantation (Barbour *et al.*, 2008b) and the spacing of trees (Williams *et al.*, 2006). We can not ascertain whether the increase in flowering intensity we observed from 2009 to 2010 was due to the age of the plantations (that were only just reaching reproductive maturity at c. 12 years) or due to the different climatic conditions experienced by the plants in the two years. It is interesting to note that some trees that flowered the first year did not flower the second year and *vice versa*, trees which did not flower the first year did flower the second year (data not shown). This pattern of alternation is widely known for many species including *F. excelsior* (Tapper, 1992; Tapper, 1996). However, Tapper *et al.* (1996) found no evidence that fruiting in one year will decrease the fruiting probability in the next year. Both studies showed that the alternation of fruiting was not due to resource being limited and was not correlated to climatically favourable years.

4.4.3 Possible sources of variation

Our previous study (Chapter III) has shown that plantations are a mixture of *F. excelsior*, *F. angustifolia*, and hybrids of different types. Therefore, the high variability in phenology of the plantation trees (Table 4.4) may reflect the diversity of tree types that were plantation at those sites. In fact, results from previous

studies suggest that the phenological variation we observed in and between plantations may be explained by genetic differences in the source material. Provenance trials in a project called RAP (Improving Ash Productivity for European needs by testing, propagation and promotion of improved genetic resources; Report 4), showed highly significant provenance effects for the bud flushing dates. Moreover, Cundall *et al.* (2003) showed that provenances from continental Europe (France, Germany, Czech Republic and Romania) performed differently in different sites in Britain for height growth.

Additional lines of evidence suggest that the differences observed in the phenological patterns have a genetic basis and are less dependant on the environmental conditions of the plantation sites per se. We only observed a significant difference for the beginning of the flowering between plantation trees from the two different plantations but not between the native trees in either site. In addition, the two sites have similar soil type[2], and they receive a similar amount of precipitation[3]. They have the same degree of insolation, and only 0.5 °C difference in the mean daily temperature for January[4] . They are both uniform, flat and surrounded by farm land, which make them equally sensitive to wind. The two plantations have the same age. This supports the hypothesis that the differences observed for the plantation trees between sites were more likely due to differences in source trees at the two sites and not due to differences in the local environment. Nevertheless, influence of local microclimate variation cannot be ruled out but we do not have access to these data.

Previous studies (Gérard *et al.*, 2006b; Tal, 2006) revealed consistency for the onset of the flowering despite environmental variation, suggesting the existence of a strong genetic basis for flowering phenology. In our case, a strong correlation for Onset, and Stage 2.5, was observed in individual trees studied over the two years (Table 4.3). Moreover, most trees that had an early start to flowering in 2009, still flowered earlier in 2010. Only a few changes in terms of flowering class between the two years were observed. Therefore flowering phenology was very consistent

[2] http://erc.epa.ie
[3] precipitations map on http://www.wesleyjohnston.com /users/ireland/geography/climate.html
[4] http://www.wesleyjohnston.com/users/ireland/geography/climate.html

between the years even though the weather conditions of the two years varied considerably. Indeed, on average, the temperature for January and February 2010 was 2.5°C lower than the temperatures observed for the same months in 2009. Precipitation was less important in 2010 than in 2009 (Table 4.1). Therefore, 2010 was a colder and dryer year.

Despite the inter-year phenological consistency, observed in our and in several previous studies, we cannot neglect completely the effects of weather on phenological patterns. A significant delay in the initiation of flowering for the plantation trees in Kildalkey in 2010 compared to 2009 could be attributed to the colder year. Several studies (Fitter and Fitter, 2002; Linkosalo *et al.*, 2006; Hänninen and Kramer, 2007) recognized environmental conditions (mainly winter chilling and spring temperatures) as a trigger for the timing of bud burst. However to draw defebtive conclusion on the influence of the climatic conditions on phenology, observations should ideally be made over a longer period of time.

Cold weather could have influenced the length of the flowering period during the two years of observations. The mean flowering duration period (DFP) did not differ between years for the native trees ($P>0.05$, Wilcoxon matched pair test), however we observed a slight DFP increase for the Kildalkey and Clonee plantation trees. In ash, the flowering period duration has been previously demonstrated to lengthen when flowering is interrupted by cold weather periods (Wallander, 2001; Tal, 2006). Individual plantation trees, which flowered earlier in 2010 for Clonee and Kildalkey, could have undergone slower flowering bud development due to cold weather.

4.4.4 Vegetative bud flushing and leaf unfolding

Premature vegetative flushing may explain the unusual morphology of the plantation trees including the poor stem form with forking which may be the result of frost killing the terminal buds. However, our analysis cannot rule out other genetic effects. Leaf flushing phenology showed similar trends to flowering phenology (Figure 4.3). The leaves generally started to develop earlier in the

plantations than the native trees (Figure 4.3, Table 4.2). Onset of the vegetative growth and leaf unfolding in temperate forest are key processes influencing tree fitness and productivity (Rathcke and Lacey, 1985; Kramer *et al.*, 2000). Kerr and Boswell (2004) suggested that late spring frost was mainly responsible for forking in *F. excelsior*. In addition, Cundall et al. (2003) indicated that provenances of ash, which flushed earlier, had poorer growth than later flushing trees in provenance trials in England and Wales. It is well known that frost might damage plant tissue (for review see Kratsch and Wise, 2000) by killing cells, damaging or stopping cell elongation and by influencing the apical meristem activity. However, in our study no correlation between the DBH and the leaf flushing onset was observed indicating that trees which flowered earlier had the same diameter than trees flowering later. Whether or not mal-adaptations of alien trees to Irish conditions, other than leaf flushing times, are responsible for tree form, the mechanism remains to be explored in further studies.

Finally, our results of vegetative phenology show that plantation and native trees did not react in the same way to cold weather. The onset of leaf flushing was earlier for the native trees in the colder 2010 compared to 2009 but the reverse was observed for the plantation trees, especially for Clonee. No change for the duration of leaf unfolding (DLE) was observed for the native trees over the two years, whereas a longer DLE was observed for the introduced plantation trees in 2010. The duration of leaf development lengthened in the coldest year for trees with earlier leaf flushing date. Even if the exact pattern of the interaction remained difficult to precisely establish, we clearly observed the presence of an interaction between weather and genotypes. Indeed the different genotype groups (i.e. *F. excelsior* or plantation trees) reacted in a different way for leaf phenology in response to cold temperature.

4.4.5 Conclusions and perspectives

To conclude, pollen transfer and hence cross pollination between native and plantation trees is possible at the two plantation sites. The climate conditions of each year will, in combination with genotypic effects, have an impact on the hybridization rate by influencing flowering period overlap. Exceptionally hard

winters which can delay bud burst in *F. angustifolia* (Jato *et al.*, 2004), might increase the flowering overlap period and hence the level of interbreeding between the hybrid and the native ashes, provided that flowers are not lost to frost (Gerard et al. 2006).

Temporal overlap in pollination is a necessary factor for potential introgression, but it is not sufficient to imply successful fertilization and geneflow. Other mechanisms such as pollen competition (Boavida *et al.*, 1999) could prevent introgression between introduced plantation and native trees. Also post-zygotic barriers might be important such as seed development, germination, dispersal and survival (Coyne and Orr, 2004; Lowe *et al.*, 2004; Costa *et al.*, 2007; Varela *et al.*, 2008). Hence, we are currently undertaking further analyses on gene flow in these populations and are assessing seed survival (Chapter V and Chapter VI).

For future management, as almost all the plantation trees which did not cluster with native *F. excelsior* in the PCA analysis (Figure 4.5) were *F. angustifolia* or hybrids (Chapter III), phenology should be recommended as one of the criteria to recognize hybrids. However, many plantation trees of hybrid or *F. angustifolia* type showed reproductive and vegetative phenology similar to the native Irish trees and therefore phenology should not be used on its own to determine the genetic identity of any specific introduced tree.

Acknowledgements

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Chapter V

Paternity analysis and pollen mediated gene flow between native and no- native populations of *Fraxinus* (Oleaceae) in Ireland

To be submitted as Muriel Thomasset, Juan F. Fernández-Manjarrés Gwendal Restoux, Natalie Frascaria-Lacoste, Gerry C. Douglas, and Trevor R. Hodkinson

Abstract

Patterns of pollen-mediated gene flow and mating system were investigated between populations of native ash (*F. excelsior*) and mixed plantations comprising non-native ash, (*F. excelsior* and *F. angustifolia*) and putative hybrid ash (*F. excelsior* x *F. angustifolia*) at two different sites in Ireland. Analyses were performed on progeny arrays and potential parents using six microsatellite loci and phenological data. High levels of multilocus outcrossing rates (t_m > 0.99) where observed using mating system analysis. In addition, maximum likelihood methods to identify pollen donor suggested that long-distance pollen dispersal was frequent. More than 50% of the seeds were sired with pollen coming from over 200 m away. For the fraction of trees for which a pollen donor was detected within each site, paternity assignment indicated that the plantation trees were pollinated by native trees but also that native trees pollinated plantation trees. We did not find a significant spatial genetic structure using a KinDist analysis between introduced and native ashes. However, we observed a significant genetic structure related to different phenological groups. Our results suggest that gene flow between non-native plantation trees and native ash occurs, but that not all introduced populations present the same risk of contamination via gene flow because of partial phenological isolation.

5.1 Introduction

The introduction of non-native plant populations from different regions of provenance, species, intraspecific taxa, or new commercial varieties into natural landscapes for ornamental, agricultural and forestry purposes can lead to the spread of novel genes (Anderson and Hubricht, 1938; Thompson *et al.*, 2010). Introduction of novel genes into small or inbred, populations may be beneficial to the future success of that population (Byrne and Macdonald, 2000). However, intra or inter specific gene flow is often seen as a source of genetic contamination (Levin *et al.*, 1996; Rhymer and Simberloff, 1996; Schierenbeck *et al.*, 2005). Interspecific gene flow might be a real threat for many native plant species around the world especially if hybrids are more competitive and reproductively successful (Vila *et al.*, 2000). For conservation, it is important to distinguish between natural hybrids that might need to be protected and hybrids that have occurred because of human intervention (Allendorf *et al.*, 2001). Hybridization might lead to the introgression of deleterious genes and therefore decrease the mean population fitness (Waser and Price, 1994; Storfer, 1999; Keller *et al.*, 2000).

Many reforestation and aforestation programmes introduce imported provenances, as local seeds are often scarce, creating the possibility of hybridization between native and alien populations with unknown consequences for local tree populations. It is therefore important to evaluate the risks of gene flow between native and introduced populations such as those of the forest tree genus *Fraxinus* wich is the topic of this study. Indeed, the Irish government introduced substantial planting grants in 1992 for farmers to plant trees with an extra premium for planting native hardwood species. This led to a shortage of native trees from home-collected seeds. Consequently, plants and seeds of supposedly *Fraxinus excelsior* L. (common ash) were imported to fill the forester's needs. After a few years, some of the plantation trees where found to have unusual growth, and in addition, the terminal buds were often pale in colour, more brown than black and hence were more characteristic of another ash species, *Fraxinus angustifolia* Vahl (narrow-leaved ash). *Fraxinus excelsio*r is the only native ash species in Ireland but on continental Europe, where the plantation seeds were collected, *F. ornus* L. (flowering ash) and *F. angustifolia* are also found (Wallander, 2008). Whilst no

hybrids between *F. ornus* and either ash species are known, there is a large contact zones in Europe where *F. excelsior* and *F. angustifolia* hybridize (Fernández-Manjarrés *et al.*, 2006; Gérard *et al.*, 2006a; Thomasset *et al.*, 2011b). These contact zones run from Turkey to northern Spain and to the Mediterranean basin (Thomasset *et al.*, 2011b and Figure 1.2 in Chapter I). Artificial control crosses (Raquin *et al.*, 2002a) and studies on hybrid zone populations in France (Fernández-Manjarrés *et al.*, 2006) have clearly demonstrated the extensive potential and occurrence of hybridization between these two species. Introduced ash plants in Ireland that show unusual morphology were identified (Chapter III) to be a) hybrid trees between *F. excelsior* and *F. angustifolia*, b) non-hybrid *F. angustifolia* or c) provenances of *F. excelsior*. Today, there are at least 100 afforested sites in Ireland with suspected alien ash dating from the plantings made between 1992 and 2000. The introduced plantation trees have started to produce flowers and are often established very close to native ash populations raising concerns of genetic pollution because of potential gene flow

Gene flow, along with genetic drift, mutation and selection, is one of the most important factors that determine the genetic structure of populations (Ellstrand, 1992; Burczyk *et al.*, 2004a). In seed plants, gene movement among and within populations is mediated by pollen movement and seed dispersal. Trees are often characterized by long-distance pollen movement (Streiff *et al.*, 1999; Dick *et al.*, 2003; Bâcles *et al.*, 2005; Valbuena-Carabanã *et al.*, 2005; Goto *et al.*, 2006; Byrne *et al.*, 2008). Thus, pollen dispersal is of major importance for the connectivity among populations and consequently for adaptation or hybridization (Oddou-Muratorio *et al.*, 2001; Lopez *et al.*, 2008). Many factors may influence pollen dispersal, such as population size and density (Treuren *et al.*, 1993; Richards *et al.*, 1999; Willenborg *et al.*, 2009), breeding system (Lowe and Abbott, 2004), flowering phenology (Robledo-Arnuncio *et al.*, 2004; O'Connell *et al.*, 2006; Sampson and Byrne, 2008) or fecundity variations among individuals (Oddou-Muratorio *et al.*, 2005; Oddou-Muratorio *et al.*, 2006; Klein *et al.*, 2008). Moreover, pollen dispersal can drive the mating system of trees when the selfing rate is not genetically controlled and only depends on the relative amount of selfed and outcrossed pollen on stigmas (the mass action law *sensu* Holsinger, 1991).

Mating systems, including the proportion of selfed and outcross events and the distribution of parentage in offsprings, have not only an important impact on the structure and evolution of plants in hybrid zones (Valbuena-Carabanã *et al.*, 2005; Gérard *et al.*, 2006c) but also impact on newly created hybrids when alien populations reproduce with local ones. Populations with large selfing rates do not exhibit a high level of introgression even in a hybrid zone. Moreover, in wind pollinated species the level of outcrossing is related to the quantity of external pollen available, driven by the mass-action law, (Holsinger, 1991). Mating systems of plants are thus largely variable and dependent on the density of populations (Lu, 2000; Restoux *et al.*, 2008) and their phenological synchrony (Fukue *et al.*, 2007; de-Lucas *et al.*, 2008). This is of particular importance in hybrid zones where different provenances might not share their pollen emission and female receptivity periods. In such cases, this leads to inbred populations despite the apparent high density of potential parents for hybridization. Thus, real-time estimates of mating systems are needed to better understand the dynamics of progeny populations compared to the parent generation. Such estimates are possible using progeny array methods (Ritland and Jain, 1981; Ritland, 2002) and rely on samples of progenies like seeds in the case of plants.

Evaluating the impact of gene invasion in plant populations depends largely on our ability to track the location of pollen donors and to identity those individuals siring the seeds in the native populations. The development of highly polymorphic molecular marker systems has facilitated the study of gene flow in forest stands using parentage assignment techniques (Streiff *et al.*, 1998; Streiff *et al.*, 1999; Craft and Ashley, 2007). Pollen-mediated gene flow analysis based on molecular markers can be undertaken using direct or indirect approaches that provide complementary information. Direct methods use paternity exclusion (Marshall *et al.*, 1998; Burczyk and Chybicki, 2004) or paternity assignment (Marshall *et al.*, 1998; Neff *et al.*, 2000; Nielsen *et al.*, 2001) to infer the identity of putative parents. Indirect approaches use synthetic parameters (e.g. F-stats) and isolation by distance models. For example, the TwoGener method (Smouse *et al.*, 2001), allows the estimation of the pollen cloud differentiation across the landscape using a parameter Φ_{ft} obtained from AMOVA (Excoffier *et al.*, 1992) on the male gametes. The Φ_{ft} can be translated into the correlation of paternity (i.e. the

probability of two randomly drawn seeds having been pollinated by the same father), and when associated with an isolation by a distance model allows for real-time (i.e. for a unique reproduction event) pollen dispersal estimation (Austerlitz and Smouse, 2001; Austerlitz *et al.*, 2004). Unlike parentage analysis, pollen structure based methods present the additional advantage to not require an exhaustive genotyping of the potential fathers.

Fraxinus excelsior and *F. angustifolia* are wind pollinated species with a complex polygamous mating system (from strictly male individual to strictly female individuals with hermaphrodite intermediates occurring on the same or different individuals within a population) (Wallander, 2001; Morand, 2003). In both species, fruits are winged samaras that are wind dispersed. In continental Europe, *F. angustifolia* usually flowers a few months earlier than *F. excelsior* (Raquin *et al.*, 2002b; Jato *et al.*, 2004). However, in the Loire hybrid zone (France), flowering overlap has been recorded in the two species (Gérard *et al.*, 2006b). Several studies using microsatellite markers have described the genetic structure of *F. excelsior* populations and gene flow among them (Heuertz *et al.*, 2001; Morand *et al.*, 2002; Heuertz *et al.*, 2003; Morand, 2003). These studies found heterozygosity deficiency, very low selfing rates and high pollen dispersal with relatively moderate patterns of isolation by distance. However, Gérard *et al.* (2006c) showed that assortative mating might strongly influence genetic isolation, and Bâcles *et al.* (2005) also highlighted the essential contribution of long distance pollen dispersal on the genetic structure of remnant populations of *F. excelsior*.

In this study, we examined progeny arrays, collected from native and introduced plantation ash trees within two different sites in Ireland, characterised using nuclear microsatellite markers (Chapter III). We used a combined approach including both phenological observations and genetic analyses, to determine the mating patterns and the temporal and spatial genetic patterns of pollen dispersal. Our main aims were to describe the patterns of pollen flow between native *F. excelsior* and non-native ash trees and to quantify the magnitude of contemporary gene flow. Finally, we explored the relative importance of evolutionary forces acting on the genetic structure in the studied populations.

5.2 Materials and methods

5.2.1 Study material and sites

Two Irish plantations consisting of imported trees, showing poor stem form, were studied. The first one in Kildalkey (53° 34' 17.8" N, 6° 54' 54.21" W) (Figure 5.1A), is a site of 20 ha with provenances mainly from North-eastern France (Alsace). Planted in 1999, the trees had initially grown vigorously but showed crooked growth after 4-5 years. The second plantation, located at Clonee (53° 28' 03.09" N, 6° 28' 58.9"W) (Figure 1B), is over 3.5 ha and was also planted in 1999. At Clonee, some of the poor growth trees were cut back and stumps treated with herbicide. They had been replaced approximately 3-4 years after planting with native ash. Some original trees are still present and are clearly distinguishable from the recently planted native ash. Trees at these sites are now old enough to flower and to produce seeds. Both stands are surrounded by native trees growing in hedgerows (Figure 5.1).

5.2.2 Sampling

We sampled 192 adult trees from each plantation and 96 native ash trees around each site (Sampling design as described in Chapter III). We recorded the GPS coordinates of each tree and collected two leaf discs of 12.5 mm diameter for DNA extraction (stored in 1:1 ethanol and acetone solution), and 48h-72h later the solution was decanted and discs were air dried for 48h in a lamina-flow cabined. Dried discs were stored in tubes at -18°C until DNA extraction (from 8 weeks up to 6 months)..

We studied 20 mother-trees from each plantation. For each mother-tree, we sampled 20 seeds resulting from open-pollination and thus a total of 400 seeds per plantation (Table 5.1). We were only able to include a limited number of mother-trees because very few trees at each site were setting seed due to their young age. At the Clonee site, some trees produced very few seeds and some of them only produced seeds that were either infested or empty. Thus after DNA extraction and PCR amplification, a total number of 357 seeds were successfully

included in the study for the Clonee site from the initial collection of 400 seeds. More than half of the native trees produced seeds, so the design was set up to sample several trees with less seeds per tree rather than a few large progenies, as recommended for landscape level studies (Austerlitz and Smouse, 2002; Miyamoto *et al.*, 2008). Thus, eight seeds per tree were sampled for 45 and 47 maternal native trees at Clonee and Kildalkey sites, respectively (Figure 5.1; Table 5.1).

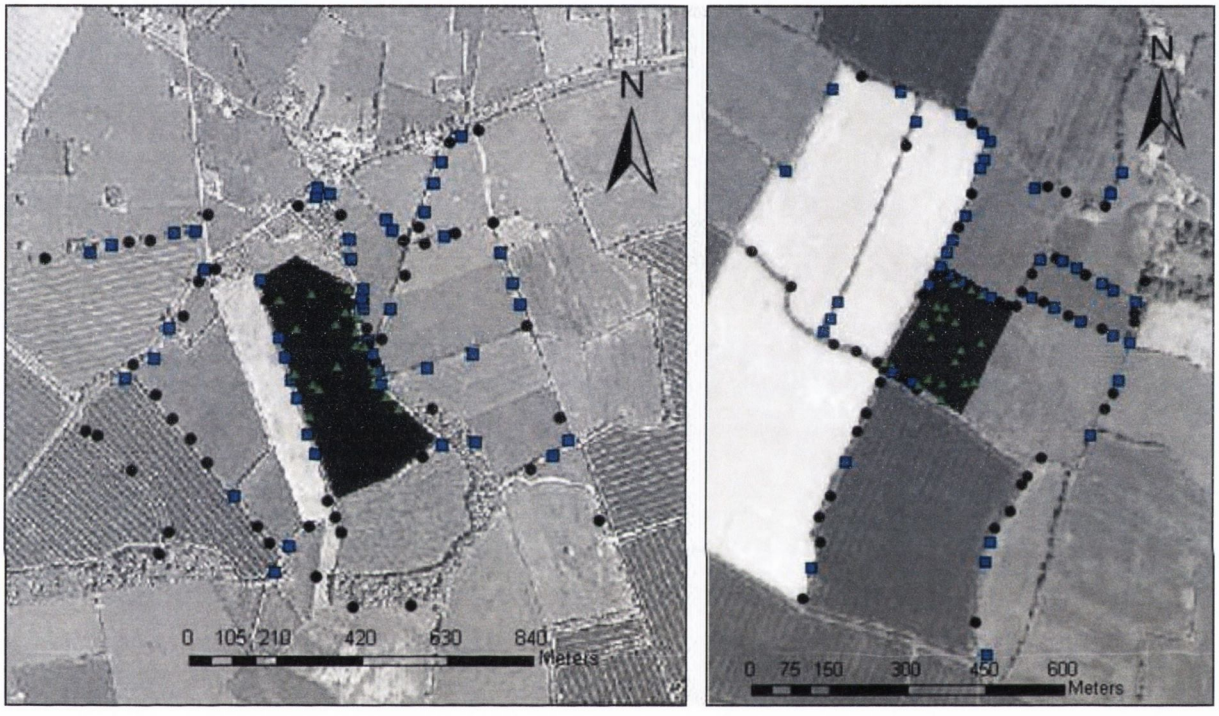

Figure 5.1 Map of the two study sites.

The Kildalkey plantation site is shown on the left and Clonee on the right (areas in dark grey). For the plantation trees, only the maternal trees are represented (green triangles). All sampled native trees are represented: maternal native trees (blue squares) and the other native adult trees (black circles).

5.2.3 Phenological data

Floral phenology of maternal trees was recorded every 10 days during the flowering season from mid-December to mid-April as described in chapter IV. Flower phenology was scored using a scale from 0 to 5 (0 for the dormant flower bud stage to 5 for the seed stage); stage 2.5 was estimated to represent the point of anther dehiscence/pollen release using visual inspection (for details see Figure

4.1). Trees were categorized into three classes depending on the flowering date, just before pollen release (Chapter IV): 1) early flowering: before the 10th of March; 2) intermediate flowering between the 10th of March and 5th of April; and 3) late flowering after the 5th April.

5.2.4 DNA extraction and microsatellite marker genotyping

Seeds were dried for 2 weeks and then slightly rehydrated for one night in water at 4℃, then submersed in 0.3 M NaOH at room temperatu re for 20 min, and sterilized for two hours in calcium hypochlorite, 70% active chlorine, at 4℃ (Raquin *et al.*, 2002b). Seed wings were removed and then seeds were checked using a microscope. Darkened embryos were discarded as they were found, by experience, to be destroyed by fungi or insects. To prevent grinding issues because of their elasticity, seeds were sliced into sections. In order to maintain uniformity in the quantity of tissue used, two thirds of large seeds were retained and whole small seeds were used. The DNA from 1,493 seeds and 576 leaves (from adult trees), for the two stands, was extracted using the DNeasy Plant Mini Kit ® (QIAGEN) following the manufacturer's instructions. Quality and quantity of DNA (from 10 to 50 $ng.\mu l^{-1}$) were checked on 10% of the samples using NanoDrop® ND-1000.

Six microsatellites markers (FEMSATL 4, FEMSATL 11, FEMSATL 12, FEMSATL 16, FEMSATL 19 (Lefort *et al.*, 1999) and M2–30 (Brachet *et al.*, 1999; Lefort *et al.*, 1999), which have previously been used successfully to characterize common ash populations (Heuertz *et al.*, 2001; Morand *et al.*, 2002), were used to genotype all the samples. PCR reaction methodology followed Morand *et al.* (2002). Allele sizes were determined using an ABI Prism™ 3130xl Genetic Analyser (Applied Biosystems). Raw data were analyzed using GeneMapper version 4.0 (Applied Biosystems). Individuals with doubtful and /or missing peaks were genotyped a second time. If a mismatch between mother and offspring genotypes was observed we repeated the PCR reaction and genotyping. After repeating these, we obtained a Mendelian segregation in all cases for all loci (i.e. congruent maternal and offspring genotypes).

5.2.5 Data analysis

5.2.5.a Genetic diversity

Genetic diversity of adult and seeds was quantified by standard measures of genetic variation, including average number of alleles per locus (A), effective number of alleles (A_e), observed heterozygosity (H_o) and Hardy-Weinberg expected heterozygosity (H_e) (according Nei, 1978), using the software GenAlEx 6.3 (Peakall and Smouse, 2006). Departures from Hardy-Weinberg equilibrium were determined using Genepop web Version 3.4 (Raymond and Rousset, 1995) using the exact probability test. Inbreeding coefficients (F_{IS}) (Weir and Cockerham, 1984) and their significance (with 1000 permutations) were computed using the program FSTAT version 2.9.3.2 (Goudet, 2001). An estimation of the inbreeding coefficient was also calculated using a Markov chain of Monte Carlo developed by Ayres and Balding (1998). This method represents visually the inbreeding coefficient in terms of posterior density curves and allows a graphic interpretation of the observed values. Null allele frequencies and the paternity exclusion probability for the second parent, across all loci, were estimated using the program CERVUS version 3.03 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007). Genetic differentiation among adult populations was evaluated by calculating overall and pairwise F_{ST}. The significance of pairwise F_{ST} values was tested using 1000 permutations with the FSTAT software.

5.2.5.b Mating system

Mating system parameters were estimated using a maximum likelihood procedure as implemented in the MLTR3.2 software (Ritland, 2002). The model is based on a mixed mating reproduction system where offspring could be derived from both selfed and outcrossed fertilization (s and t). Maternal genotypes were known because leaves from maternal trees were genotyped. We estimated multilocus outcrossing rates (t_m) rates and the average single-locus estimate (t_s) according to the sampling site (i.e. Clonee or Kildalkey), the considered groups (i.e. plantation

or native) and the flowering score (i.e. early, intermediate or late). The biparental inbreeding was calculated using the difference between the multilocus and the single locus estimates of the outcrossing rate (t_m - t_s). Using the same experimental design, we also estimated the correlation of paternity within the outcrossing pollen pools (i.e. the probability that two randomly drawn offspring from the same mother share the same father) (r_p). This correlation was translated into an estimate of the effective number of pollen donors ($N_{ep}=1/r_p$). Standard errors were computed using 1000 bootstrap iterations.

5.2.5.c Paternity assignment

Paternity analysis was performed using CERVUS Version 3.03 (Kalinowski *et al.*, 2007). Seed paternity was assigned with a threshold of 80% (a relaxed confidence level; Marshall *et al.*, 1998) based on the maximum likelihood method given the genotypes of the offspring and the adult trees. Assigned paternity was assessed if the difference (Δ) between the log of odds (LOD) score of the two most likely candidate fathers exceeded a certain threshold of Δ, computed by simulation in order to correctly assign the father in 80% of the cases (Meagher, 1986). Input simulation parameters were used with 10000 cycles including all adult trees per population as candidate fathers. Data were double checked for accuracy and error rate. The scoring error was set at 1%. The CERVUS software also estimated the error rate of incompatibility between the offspring and the known mother to be inferior to 1%. The minimum number of loci required for assignment was set at four. Given the high density of introduced trees and the potential for long distance gene flow in *Fraxinus* (Bâcles *et al.*, 2005), we assumed that 10% or less of the potential fathers were sampled. Pollination distances were calculated for seeds as the distance between mother trees and the most likely candidate pollen donor parents. The taxon identity of the trees in the plantation, either *F. excelsior*, *F. angustifolia* or hybrid group, was based on a prior multivariate morphological and molecular assignment[5] classification (Chapter III). To compare the numbers of seeds sired, either by plantation trees classified as *F. excelsior,* or as hybrid type, we applied a non parametric Chi square test.

[5] With a threshold value *Tq* set up at 0.8 for the molecular analysis (see result Chapter III)

5.2.5.d Genetic structure of pollen pools

Pollen pool genetic structure in the two study sites was analyzed using TwoGener (Smouse *et al.*, 2001) and KinDist (Robledo-Arnuncio *et al.*, 2006) methods, both implemented in the software package POLDISP (Robledo-Arnuncio *et al.*, 2007). We used this software to: i) estimate the genetic structure of the fertilizing pollen pool and ii) estimate pollen dispersal assuming an isolation by distance model based on the previously estimated genetic structure. TwoGener relies on Φ_{ft}, a differentiation index, derived from an AMOVA computation (Excoffier *et al.*, 1992) on the male gametes. Φ_{ft} can be computed at the population level (i.e. global Φ_{ft}; Smouse *et al.*, 2001) or for each pair of maternal sibships (i.e. pairwise Φ_{ft}; Austerlitz and Smouse, 2002). KinDist computes relative measures of correlated paternity between maternal sibship pairs, Ψ (z), separated by a distance *z*. The methods figure out the genetic structure of the male gametic pool using differentiation or similarity indices, respectively. We used TwoGener to estimate i) the global Φ_{ft}, ii) a Φ_{ft} per phenological group and iii) a pairwise Φ_{ft} for each pair of maternal sibship for each site (Clonee and Kildalkey) and for each population (native and plantation). The results of the pairwise Φ_{ft} were compared with the Ψ (z) resulting from KinDist.

The second step of the analysis consisted in estimating the pollen dispersal function and relies on the isolation by distance model. To estimate pollen dispersal using such an indirect method, the genetic diversity must be significantly spatially structured and therefore relate the pairwise genetic indices (either Φ_{ft} or Ψ) to the pairwise geographic distances. The TwoGener method jointly computes two related parameters, the dispersal function parameters and the effective density of pollen donors (d_e), whereas the KinDist method computes the dispersal function parameters independently of the effective density of pollen contributors. Thus Robledo *et al.* (2007) suggested the use of a sequential method, with a first estimation of the pollen dispersal function using KinDist and then an estimation of the effective density in TwoGener using the previously estimated pollen dispersal function. We applied this latter approach to our dataset.

5.2.5.e Gametic assay

To estimate the genetic composition of the fertilizing pollen pool of each mother trees, we directly determined for each seed which alleles were paternally inherited (i.e. via pollen). The male gametic contribution was inferred by subtracting the female gametic contribution (known using leaf genotype from Chapter III) from the offspring genotype. However when mother-offspring pairs share the same heterozygote genotype for a particular locus, it leads to an ambiguous determination of the paternal contribution. Thus, in these cases, we used a fractional probability for each of the potential alleles to be paternally inherited. Given the ambiguous offspring genotype for a particular locus (A, B) and the similar genotype for the mother-tree (A, B), the frequencies of each allele, $f(A)$ and $f(B)$, at the considered locus were computed using the unambiguous paternity determinations. Thus the probability that the paternal contribution was the A allele is $\frac{f(A)}{f(A)+f(B)}$, whereas the probability for the father to have transmitted the B allele is $\frac{f(B)}{f(A)+f(B)}$. This method is very conservative and is the same as the one used in TwoGener (Smouse *et al.*, 2001). When there was no ambiguity, the probability that, at a given locus, the allele was inherited from the father was one. A Perl script, for those calculations, is available upon request from M. Thomasset. To compute the genotype frequencies we considered all loci to be non-linked and thus we computed the frequencies of each genotype by multiplying the frequencies of each allele for all the combinations of alleles over all the loci. We then used these genotype frequencies of each mother-tree fertilizing pollen cloud to discriminate the pollen pools according to their groups (plantation vs. native) and their flowering scores, using a discriminant analysis (CDA) carried out with the R software (2009, R Foundation for Statistical Software).

5.3 Results

5.3.1 Genetic diversity

The total paternity exclusion probability over the six loci was 0.997 and 0.996 for Clonee and Kildalkey, respectively. All the loci were highly polymorphic and a total of 190 alleles were observed. All the computed genetic diversity indices are summarized in Table 5.1. The average allele number per locus was 22.7 ranging from seven (FEMSATI16) to 47 (M2-30) alleles per locus. Fifteen and eight unique alleles and a mean of 2.5 and 1.3 alleles per locus were observed for the adult trees in Clonee plantation and Kildalkey plantation respectively. The frequency of unique alleles was always really low and ranged from 0.003 to 0.008. Each of the four populations of adult trees showed high and similar levels of genetic diversity (H_e=0.767-0.818; Table 5.1). Adult populations exhibited a positive and significant inbreeding coefficient indicating an excess of homozygotes (F_{IS} from 0.053 to 0.147; Table 5.1). F_{IS} was lower within the native tree populations than within the plantations (Table 5.1). Deviation from Hardy-Weinberg proportions was variable among loci and among populations (data not shown). F_{ST} values computed for the adult native and introduced adult trees at the two sites ranged from 0.0033 to 0.0144. Low but significant differentiation was observed among adult populations for five of the six pairwise comparisons (Table 5.2). The only undifferentiated comparison (at the P<0.01 level) was observed between the two native Irish populations.

The mean number of alleles observed within the progenies (22.7-24.7; Table 5.1) was similar to the values observed within the adult trees (20.8-24.7; Table1). For the progeny population the expected heterozygosity ranged from 0.777 to 0.805. The inbreeding coefficient also showed a significant and positive deviation from zero (F_{IS} from 0.104 to 0.131; Table 5.1). The inbreeding coefficient was higher within the progeny individuals than within adult trees for native trees (Supplementary.material, Figure S5.1)

Table 5.1 Parameters of genetic diversity for the adult *F. excelsior* trees and their progeny.

		Adult trees						Progeny					
Site	**Locality**	*N*	*A*	A_e	H_O	H_e	F_{IS}	*N*	*A*	A_e	H_O	H_e	F_{IS}
Clonee	Plantation	192	24.7	9.87	0.700	0.818	0.147***	357	22.7	7.82	0.705	0.793	0.117***
	Native	96	20.8	7.12	0.757	0.798	0.057***	360	23.2	7.29	0.713	0.805	0.122***
Kildalkey	Plantation	192	21.7	7.38	0.696	0.767	0.094***	400	24.7	7.35	0.697	0.777	0.104***
	Native	96	21.0	8.11	0.772	0.811	0.053**	376	23.0	7.25	0.692	0.796	0.131***

N , number of individuals per group; *A*, mean number of alleles; A_e, effective number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, Wright's inbreeding coefficient. Exact tests were performed with 1000 random permutations (**P<0.01, ***P<0.001)

No obvious presence of null alleles was observed. However, the software CERVUS estimated a positive null allele frequency averaging at 0.093. Low variation of the null allele frequency between native and plantation populations and between sites was observed. The locus FEMSATL4 always showed the highest null allele frequency (ranging from 0.1342 to 0.1505) and FEMSATL19 the lowest (ranging from 0.0125 to 0.0182). No clear relationship between the proportion of null alleles and inbreeding was observed.

Table 5.2 Genetic differentiation measured by pairwise F_{ST} (Weir and Cockerham, 1984) (below diagonal) and significance (above diagonal) among the four adult populations.

	Plantation-Clonee	Native-Clonee	Plantation-Kildalkey	Native-Kildalkey
Plantation-Clonee	0	*	*	*
Native-Clonee	0.0127	0	*	ns
Plantation-Kildalkey	0.0086	0.0144	0	*
Native-Kildalkey	0.0077	0.0033	0.0143	0

P-values determined using 1000 bootstrap replicates
*: $P < 0.01$, ns: not significant ($P>0.05$).

5.3.2 Mating system

Trees were largely outcrossing, with estimated multilocus population outcrossing rates ranging from 0.980 to 0.997 (Table 5.3). Outcrossing rates (t_m) were similar between plantation and native Irish trees. Trees showing self-fertilization had an individual outcrossing rate ranging from 0.625 to 0.95 and were not found preferentially in one of the populations. For Clonee, only two trees from the plantation experienced self-fertilization, both were classified as intermediate flowering individuals. Three native trees (two with intermediate flowering time and one with late flowering time) showed an outcrossing rate ranging from 0.625 to 0.875. At Kildalkey, three plantation trees showed self-fertilization; two of them had an early flowering period (t_m=0.685 and 0.95 respectively) whereas the latter one had an intermediate flowering date (t_m=0.95). Comparisons of outcrossing rates among phenological groups revealed that flowering time did not affect the mating system (Table 5.4).

For all the analyzed populations, a small proportion of siblings shared the same father (r_{pm} in Table 5.3 ranging from 0.015 to 0.115). This correlated paternity coefficient was always smaller for the plantation trees than for the native trees. Indeed the estimated effective number of pollen donors was between four to six times higher for the plantation than for the native trees (Table 5.3). When trees were grouped according to their phenological class, offspring from early flowering trees had a higher coefficient of correlated paternity. In contrast, late flowering trees during showed a lower correlated paternity and a higher number of effective fathers (Table 5.4).

Table 5.3 Estimated mating system parameters for the progeny from Clonee and Kildalkey.

Site	Locality	t_m	t_s	t_m-t_s	$r_{p(m)}$	N_{ep}
Clonee	Native	0.983 (0.053)	0.842 (0.017)	0.141 (0.048)	0.095 (0.022)	10.5
	Plantation	0.994 (0.079)	0.864 (0.010)	0.131 (0.078)	0.015 (0.016)	66.7
Kildalkey	Native	0.997 (0.093)	0.823 (0.013)	0.175 (0.092)	0.155 (0.026)	6.5
	Plantation	0.980 (0.007)	0.855 (0.013)	0.125 (0.013)	0.043 (0.017)	23.3

t_m multilocus outcrossing rate; t_s single-locus outcrossing rate; t_m-t_s biparental inbreeding; $r_{p(m)}$ paternity correlation, N_{ep} the effective number of pollen donors per mother tree. Standard errors based upon 1000 bootstrap replicates between parentheses.

Differences between single-locus and multi-locus estimates of outcrossing rates indicated biparental inbreeding in all populations. The biparental inbreeding was higher for the native trees than for the plantation trees (Table 5.3).

Table 5.4 Estimated mating system parameters for the progeny of the different phenological groups (Early, Intermediate or Late) from the Clonee and Kildalkey sites.

Site	Locality	Phenological group	Number of mother trees	t_m	t_s	t_m-t_s	$r_{p(m)}$	N_{ep} ($1/r_{p(m)}$)
Clonee	Native	Early	11	0.998 (0.000)	0.850 (0.001)	0.148 (0.016)	0.119 (0.084)	8.4
		Intermediate	26	0.986 (0.011)	0.852 (0.021)	0.133 (0.016)	0.092 (0.035)	10.9
		Late	8	0.950 (0.043)	0.850 (0.026)	0.099 (0.022)	0.051 (0.104)	19.6
	Plantation	Early	3	0.994 (0.001)	0.870 (0.015)	0.124 (0.015)	0.041 (0.041)	24.4
		Intermediate	17	0.993 (0.005)	0.866 (0.009)	0.127 (0.008)	0.021 (0.023)	47.6
Kildalkey	Native	Early	3	0.947 (0.021)	0.842 (0.032)	0.105 (0.035)	0.110 (0.021)	9.1
		Intermediate	29	1.000 (0.000)	0.834 (0.021)	0.166 (0.021)	0.096 (0.024)	10.4
		Late	15	1.000 (0.000)	0.817 (0.023)	0.183 (0.023)	0.126 (0.035)	7.9
	Plantation	Early	9	0.961 (0.035)	0.814 (0.055)	0.147 (0.026)	0.057 (0.027)	17.5
		Intermediate	7	0.995 (0.006)	0.900 (0.017)	0.095 (0.017)	0.023 (0.050)	43.5
		Late	4	1.000 (0.000)	0.875 (0.023)	0.125 (0.023)	0.018 (0.008)	55.6

t_m multi-locus outcrossing rate; t_s single-locus outcrossing rate; t_m-t_m biparental inbreeding; $r_{p(m)}$ paternity correlation, N_{ep} the effective number of pollen donors per mother tree. Standard errors based upon 1000 bootstrap replicates in parentheses.

5.3.3 Gene flow

5.3.3.a Paternity assignment

At the Clonee site, the paternity assignment using CERVUS resulted in a unique father for 108 of the 717 (15%) analyzed seeds. At Kildalkey, unique paternity was determined for 89/776 (11.5%) of the offspring. This meant that 609 seeds for Clonee and 687 seeds at Kildalkey remained without an assigned father because more than one possible local father was found or we lacked compatible male parents in the samples studied. Offspring from native and plantation trees were sired by both native or plantation trees (Table 5.5).

Within the assigned parents, the total average pollination distance at Clonee and Kildalkey was 206 m and 285 m, respectively (Table 5.5). The minimum pollination distance found between a maternal tree and an assigned pollen donor tree was 12 m and the maximum distance was over 1 km. Less than 35% of mating events occurred at distances less than 100 m (Figure 5.2A and Figure 5.2B). More than 30% of the pollen received by the native mother trees came from trees located further than 400 m away (Figure 5.2A and Figure 5.2B).

Most of the candidate pollen donor trees identified were found to sire one or two sampled seeds (Table 5.5). For Clonee, only one plantation tree and two native trees were found to sire more than two seeds. At Kildalkey, only one native tree was found to sire four seeds.

Table 5.5 Results from paternity assignment (using the LOD score method implemented in CERVUS). Number of seedlings assigned with confidence interval of 80%, number of detected fathers and pollen dispersal distances for seed sired from pollen sources and observed standard deviation (sd) within the two sites, Clonee and Kildalkey.

Site	Known mother from	Candidate male group	Number of sired assigned individuals	Number of different fathers	Average distance between parents	sd
Clonee	Plantation	Native	37/59	23	185.58	148.68
		Plantation	22/59	18	123.47	56.64
	Native	Native	30/49	23	207.41	197.77
		Plantation	19/49	18	334.77	141.66
Kildalkey	Plantation	Native	24/53	21	313.17	168.65
		Plantation	29/53	28	143.1	66.5
	Native	Native	25/36	21	365.64	289.26
		Plantation	11/36	10	419.61	260.21

Trees that were assigned as putative hybrids (Chapter III) were shown to pollinate the native trees in addition to other native trees. Indeed, for Clonee, 9 out of 20 (45.5%) offspring collected from native mother trees and sired by plantation trees were pollinated by individuals identified as hybrid. For Kildalkey, 7 among 11 (63.6%) offspring collected from native mothers had a pollen donor identified as a hybrid. For both sites there were no significant differences between the number of mother trees pollinated by hybrid trees or by trees characterised as *F. excelsior* in

the plantation (χ^2=0.42, df=1, P=0.515 for Clonee and χ^2=3.53, df=1, P=0.060 for Kildalkey)

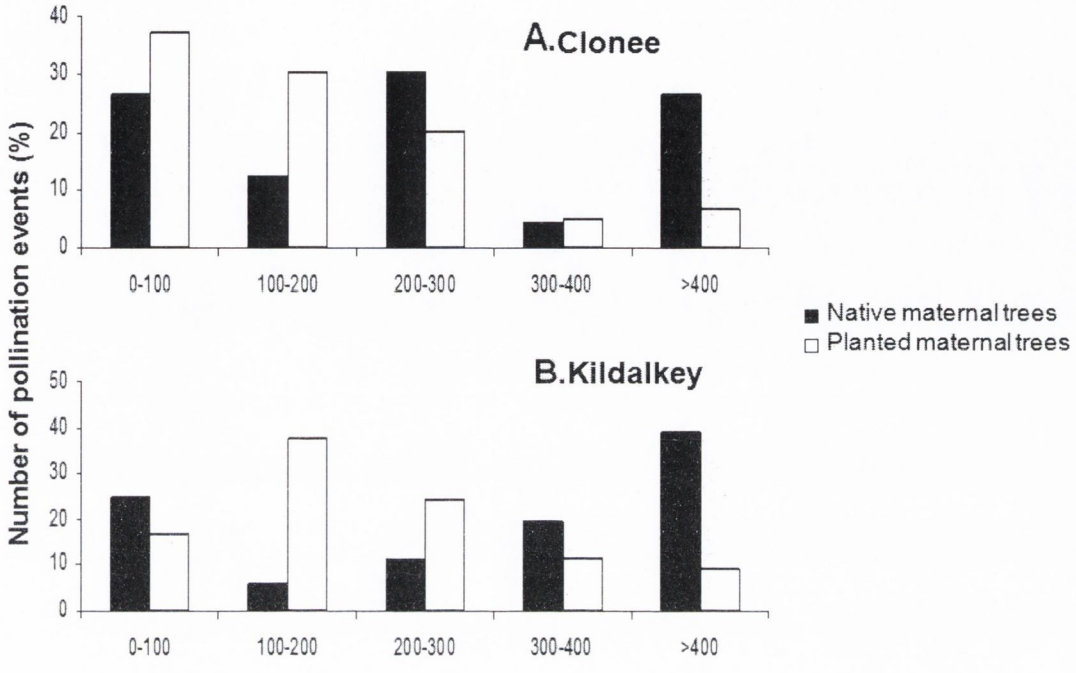

Figure 5.2 Distribution of detected pollen dispersal distance classes when mother trees are either native (in black) or from the plantation (in white) in relation to the position of pollen donor within the sites. **A.** Clonee site and **B.** Kildalkey site.

5.3.3.b Pollen pool genetic structure

The global differentiation among pollen pools sampled by female trees (global Φ_{ft}) was low and similar for both sites (Φ_{ft}=0.055 and Φ_{ft}=0.050 at Kildalkey and Clonee sites, respectively) indicating that over 94% of the genetic variability of fertilizing pollen pool was found within seeds from a given mother. Pairwise Φ_{ft} values were small (Figure 5.3) indicating a low differentiation of the pollen pools of two mother trees. Pairwise Φ_{ft} were slightly higher for the native trees: mean Φ_{ft}=0.062 ± 0.049 and mean Φ_{ft}=0.066 ± 0.045 for the native mother trees at Clonee and Kildalkey, respectively compared to the mean pairwise Φ_{ft}'s, 0.037 ± 0.027 and 0.040 ± 0.035 for plantation mother trees at Clonee and Kildalkey, respectively. Similar results were observed for Ψ, with the KinDist method indicating that the probability was very low for two mother trees to mate with the

same father (results not shown). Moreover, for the two sites (Kildalkey and Clonee), there was no apparent decline in among-sibship correlated paternity with distance. In addition, the Spearman rank correlations of among-sibship correlated paternity ($\Psi(z)$) and the distance z between two mothers trees was negative, -0.05 and -0.04 but not significant. These results suggest that the low but significant genetic structure is not due to a spatial decline in the relatedness of the pollen donors. Therefore, since there was no relationship between the Kinship coefficient Ψ (z) and the distance z between mother trees, it was neither possible to link the pairwise Φ_{ft} between two progenies and the distance between mothers trees. As a consequence pollen dispersal function cannot be computed using the indirect approach at this scale.

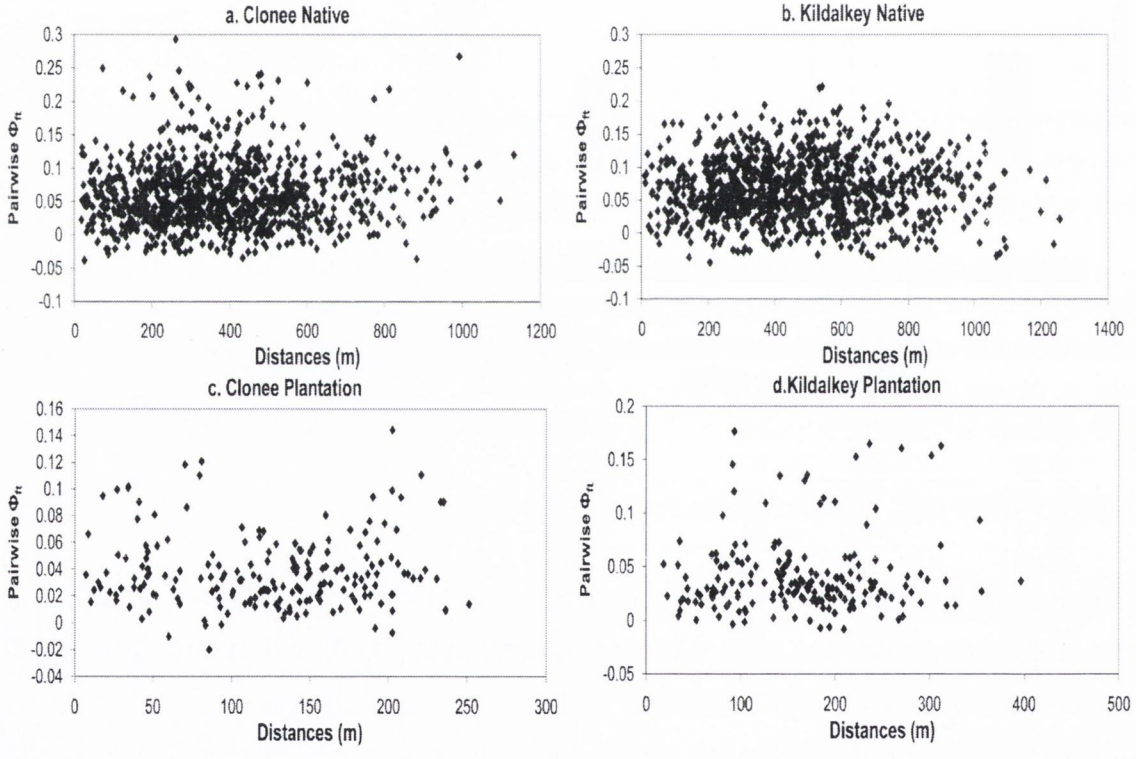

Figure 5.3 Pairwise Φ_{ft} in relation to the distance between mother trees at Native-Clonee (a), Native-Kildalkey (b), Plantation-Clonee (c) and Plantation-Kildalkey (d).

At each site, the KinDist method was applied for the three phenological group datasets: early, intermediate, and late. The Spearman rank coefficient was also inferior to -0.1 and non-significant. This indicated that even within a phenological group, there was no significant spatial genetic structure of the pollen cloud.

However, the discriminant analysis, based on our own estimation of the genetic composition of the fertilizing pollen pool of each mother, revealed that the individual pollen pools could be differentiated according to the three phenological groups (Figure 5.4). At both sites, the first axis of the discriminant analysis separated the composition of the fertilizing pollen pool of each mother tree, which belonged either to the plantation or to the native population. This indicated that native and plantation trees tend to sample different pollen pools overall.

Native and plantation trees with intermediate phenology largely shared their pollen pool (Figure 5.4A and Figure 5.4B). The discriminant analysis showed graphically the same pattern as the Φ_{ft} computed per phenological groups (Table 5.6). Indeed the Φ_{ft} of the groups with intermediate flowering time were inferior for both the plantation site native trees compared to the groups with early phenology (Table 5.6). The results (Figure 5.4A and Figure 5.4B) clearly showed that the native mother trees at both sites sampled different pollen pool for extreme phenological groups (Early and Late). However early flowering plantation trees in Clonee and Kildalkey shared part of their pollen pool with the intermediate group.

Table 5.6 Φ_{ft} per phenological group estimated using TwoGener

Site	Locality	Phenological group	Φ_{ft}
Clonee	Native	Early	0.063
		Intermediate	0.058
		Late	0.051
	Plantation	Early	0.039
		Intermediate	0.031
Kildalkey	Native	Early	0.070
		Intermediate	0.056
		Late	0.083
	Plantation	Early	0.052
		Intermediate	0.031
		Late	0.022

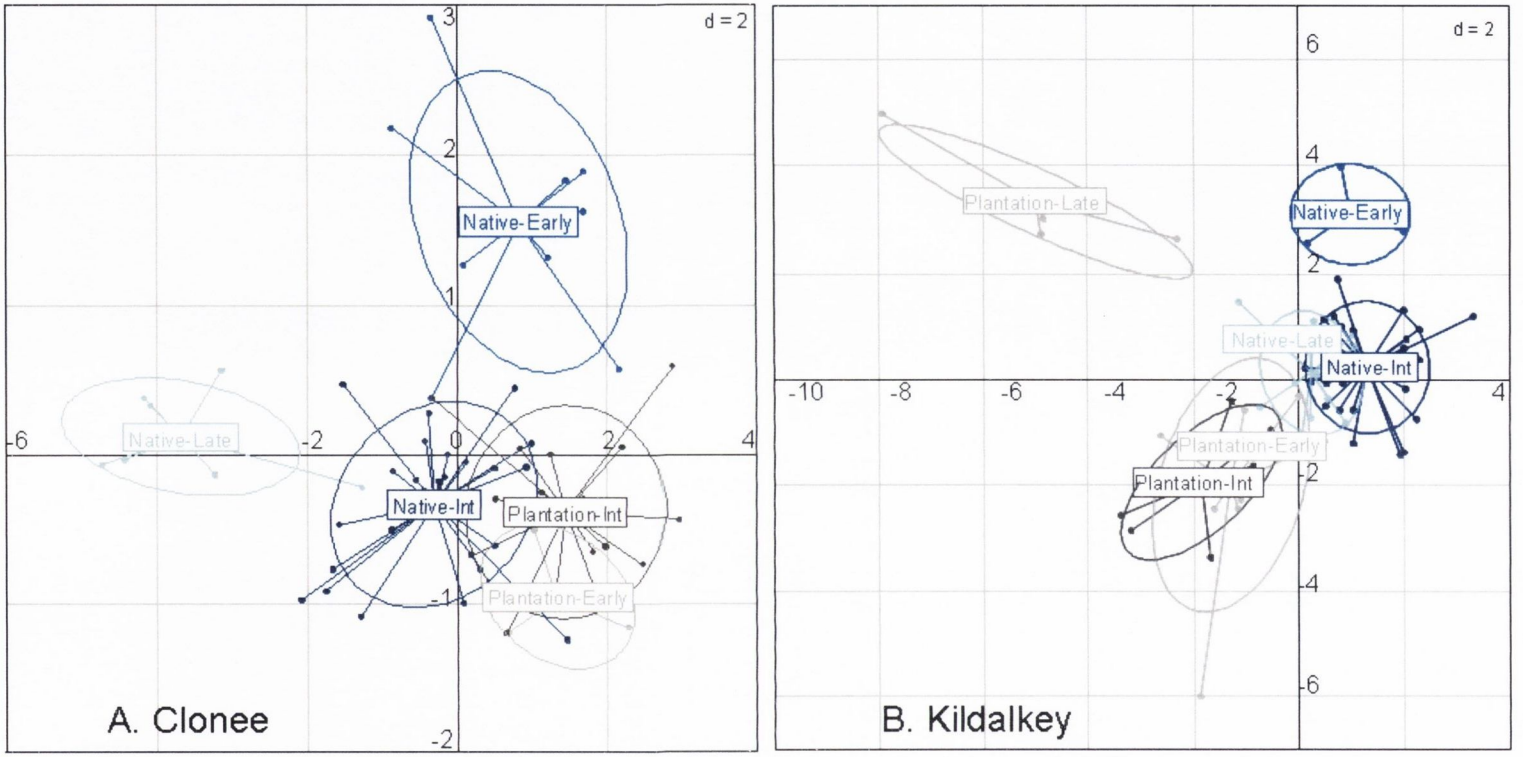

Figure 5.4 Plot of the two first canonical scores of the CDA analysis. The pollen cloud differentiation for each mother tree is shown. **A.** Clonee site and **B.** Kildalkey site. Trees were grouped according to their time of flowering phenology (Int for intermediate dates of flowering period; Early and Late for the two other flowering groups) and their sampling location (plantation trees in different shades of grey and native trees in different shades of blue). 95% confidence intervals around the mean canonical score for the different groups are shown.

5.4 Discussion

Our results show gene flow occurs between native and alien introduced ash. Hence, genetic pollution of native ash population in Ireland is possible. The following discussion examines the implications of our results (parentage and mating system analysis) for gene conservation strategies.

5.4.1 Mating system

Very low population level selfing rates were observed (between 1 and 2%, Table 5.3) which was consistent with results found in previous studies on ash trees (Heuertz *et al.*, 2003; Bâcles *et al.*, 2005; Gérard *et al.*, 2006c). In a European natural hybrid zone, Gérard *et al.* (2006c) found that trees with an earlier flowering period had a higher selfing rate due to a lack of pollen (i.e. mass action law). In contrast, in our study, neither plantation nor native trees with extreme flowering period (Late or Early) exhibited a higher selfing rate (Table 5.4). One possible explanation is that in Ireland, we analysed an artificial mixture of hybrids, *F. excelsior,* and *F. angustifolia* trees. This may result in sufficient pollen donors over all the pollination periods, even if different phenological classes are detected in our sample. In addition, seeds resulting from selfing may be difficult to detect as they may be of sub-optimal quality and thus discarded from the genotyping. Indeed, seeds from many plantation trees had parasites and were non-viable (Chapter VI). This can lead to underestimation of the selfing rates.

We detected a significant level of biparental inbreeding in both plantations and native populations. This suggests that the contributors to the pollen pool of a given seed tree might be closely related. Biparental inbreeding might be the cause for the increased homozygosity relative to random mating that we observed (Table 5.1). However, large F_{IS} values, indicating a heterozygote deficiency, have been well documented in *F. excelsior* populations elsewhere in Europe and appear to be widespread (Morand *et al.*, 2002; Heuertz *et al.*, 2004b; Bâcles *et al.*, 2005; Fernández-Manjarrés *et al.*, 2006; Ferrazzini *et al.*, 2007; Sutherland *et al.*, 2010). Analysis with the software CERVUS suggested they might be present especially

for the locus FEMSATL12 that also generated null alleles in a previous study (Heuertz *et al.*, 2001). Null alleles can also contribute to heterozygote deficiency estimates; Morand *et al.* (2002) and Bâcles *et al.* (2005) also suggested that the presence of null alleles at different loci might explain, to some extent, the deficiency of heterozygotes observed in ash. However after redesigning primers, Morand *et al.* (2002) found that the homozygote rate was unaltered, indicating the possible presence of other effects responsible for heterozygote deficiency in *F. excelsior*. The Wahlund effect has been suggested several times in ash population studies (Morand *et al.*, 2002; Gérard *et al.*, 2006b; Sutherland *et al.*, 2010) and has been observed in other wind pollinated species (Bacilieri *et al.*, 1994). In our case, plantation individuals may come from different provenances, which lead to a bias in our F_{IS} estimations. However, a noteworthy result for the native trees is that F_{IS} was higher for the progenies than for the adult trees (Supplementary material Figure S5.1) suggesting some assortative mating within related individuals and a counter-selection against the inbreeding over the tree life (Table 5.1). This is congruent with previous studies dealing with trees (Farris and Mitton, 1984; Yazdani *et al.*, 1985; Strauss and Libby, 1987; Rossi *et al.*, 1996) suggesting that heterozygosity rate increases with the age of the trees in populations due to selection and inbreeding depression.

The plantation trees showed a lower level of biparental inbreeding than native populations. Seeds of the plantation trees were collected in continental Europe and placed into bags and thus presumably mixed before germinating and planting. Indeed, source material of the plantation trees was suspected to be from mixed provenance origin. Thus, trees could have been collected from different provenances. The higher level of biparental inbreeding estimated for the native trees may indicate a weak but significant isolation by distance effect. Indeed, the degree of genetic relatedness between pairs of individuals within a natural population usually decreases with increasing geographical distance (Wright, 1943). Native trees have more chance to be related because heavy seeds tend to fall close to the maternal tree creating a spatial genetic structure. Under these conditions, it could be expected that a part of the progeny would be the result of mating between related individuals.

5.4.2 Pollen dispersal

We found that most matches between non ambiguously identified pollen donors and offspring occurred between 100 and 299 m for the plantation trees (Figure 5.2A and Figure 5.2B) and over 200 m for the native trees with a peak over 400 m. The furthest observed distance between identified pollen donors and offspring was 1033 m (in Kildalkey, between a plantation and a native tree). Our results indicated that the pairwise correlated paternity among pollen pools did not decline with distance between mothers. In other words, mother trees were not more frequently pollinated by their neighbouring pollen donors than by random trees of the population (Figure 5.3). These results indicate intensive pollen-mediated gene flow and long-distance pollen dispersal. Species with wide-ranging dispersal of pollen are more likely to have spatial distributions of genotypes close to random than those with limited dispersal (Chung *et al.*, 2000). This can be due to the location of our two study sites in a windy open farmland landscapes more likely to be considered as a fragmented landscape than a continuous forest. Indeed, in anemophilous species, pollen movement can be higher in landscapes subject to deforestation and fragmentation in comparison to landscapes with continuous forest due to an airborne pollen movement enhancement (Bâcles *et al.*, 2005; Dick *et al.*, 2003; Nielsen and Kjaer, 2010; Sampson and Byrne, 2008).

Average pollen dispersal distances of 260 m and 382 m (between identified pollen donor and mother trees) were found for the two native populations at Clonee and Kildalkey, respectively. These distances are similar to those found by Bâcles *et al.* (2005) who recorded an effective dispersal distance of 328 m within Scottish remnant ash populations. On a local scale, for *F. excelsior*, the probability of pollen dispersal has been found to decrease rapidly with distance from pollen sources (Gérard *et al.*, 2006c; Bâcles and Ennos, 2008). On the other hand, these two studies also showed high levels of immigrant pollen (i.e. from outside the sampled area), and long distance pollen dispersal following a fat tailed kernel. Bâcles and Ennos (2008) even observed an effective dispersal at 2900 m between two neighbouring populations. Our sampling did not extend to such distances, but some individuals were 1500 m apart. It is not known whether the low paternal assignment values we observed (15 and 11%), and our limited ability to estimate

the parameters of the dispersal curves, were due to the marker system (few markers and low genetic differentiation between native and plantation trees; Table 5.2), or to high pollen immigration rates from trees outside of the study site (see methodological concerns section). This modest success was achieved despite the fact that the genotyped loci were highly polymorphic leading to high cumulative exclusion probabilities (0.996-0.997). Nevertheless, the higher number of effective pollen donors observed in the late flowering trees observed means that they were pollinated by more diverse pollen pools, which may indicate long distance pollen sources. The low level of differentiation between the different populations of native and plantation trees (F_{ST}) could be a factor, which explained the limited paternity assignment success. Also, in our case if trees were not sampled assignment was not possible. Moreover, because of the large size of the plantation, our sampling was not exhaustive inside the sampling area and represented approximately 10% of the adult trees. As such, we consider our estimates as a minimum bound for hybridization levels, but we can not assess what proportion of hybrid pollen is present from the long distance pollen sources. Considering that plantations are separated by several km it is realistic to assume that the proportion of native ash pollen is significant especially in the late flowering class.

5.4.3 Pollen pool genetic structure

The estimated level of differentiation of pollen clouds among mothers was low but significant (Φ_{ft}=0.05) and consistent with previous studies on *F. excelsior* (Φ_{ft}=0.06, Morand, 2003; Φ_{ft}=0.11, Gérard *et al.*, 2006c). These results might indicate a non-random mating system. However, we did not observe a relationship between the genetic structure of pollen clouds and spatial distance (Figure 5.3), indicating an absence of isolation by spatial distance. Indeed, according to our results (Discriminant analysis Figure 5.4 and Φ_{ft}'s computed within groups), the structure of the pollen clouds was not due to spatial proximity between mother trees but was more likely to be due to the different phenological groups or the source of the material (native / plantation) (Figure 5.4).

Some other studies have indicated that temporal variation in individual flowering phenology might greatly affect mate availability. Gérard *et al.*, (2006c) found that male ash trees flowering earlier participated more as pollen donors and had a higher mating success than later flowering individuals. In the present study, we observed that the number of effective fathers was always higher for trees that flowered late (Table 5.4) and that consistently the pollen cloud differentiation was less important for the trees that flowered late (Table 5.6). The observed pattern of increased number of effective fathers as the flowering season advanced can be explained by the increase in mate availability. Surprisingly, the results for Clonee showed that late native *F. excelsior* (Figure 5.4A) that were expected to be able to mate with trees flowering earlier, showed a distinct pollen cloud. The fact that the number of effective fathers was more important in this phenological group than in the trees with earlier flowering period (Table 5.4) could confirm long distance pollen movement. However, for Kildalkey, the native late mother trees, which also showed a higher number of effective fathers, shared their pollen pool with trees with intermediate flowering time (Figure 5.4B). The size of the plantation and in consequence the number of trees was very different: around 3.5 ha for Clonee and 20 ha for Kildalkey. Our results suggest that in Kildalkey where more trees have been planted, the pollen pool was maintained and that there was a reduction in the long distance pollination.. Gérard *et al.* (2006b) found that for mature trees in a hybrid zone between *F. excelsior* and *F. angustifolia,* genetic differences increased with increasing difference in flowering phenology. Other studies in forest tree species (Byrne *et al.*, 2008; Sampson and Byrne, 2008) have highlighted the fact that variation in the pollen source pool can be attributed to flowering phenology rather than geographic distance between two mates.

Moreover, it is remarkable that the pollen cloud of native early flowering mother trees did not cluster with the early flowering plantation trees at both sites (Figure 5.4A and Figure 5.4B) because in general early flowering *F. excelsior* will blossom during the same period of as early flowering plantation trees. We measured our phenological classes from a male function point of view but we did not measure the receptivity of the stigmas. Future studies should focus on observing and quantifying the start of the female function that might be more indicative of phenological isolation. Moreover, gametophitic incompatibility and sporophitic

factors may explain the lake of overlap between the different class (Plantation and native) of early flowering trees.

5.4.4 Consequences of non-native gene flow into native *Fraxinus*

Our study showed that native mother trees around plantation sites were unambiguously pollinated by individuals from the plantations or from other native trees (Table 5.5). Moreover, all types of trees, *F. excelsior*, *F. angustifolia* and ash of hybrid origin in the plantations were able to pollinate the native Irish trees. So far they do not show an unequal mating system. Thus, interbreeding between these taxa is occurring and genes are moving from the plantation to native ash populations. Asynchronous flowering was not found to be a major factor in preventing crossing between introduced plantation and native Irish trees because a considerable overlap in flowering periods was observed between the two populations of plants (Chapter IV). The presence of few selfing trees with intermediate flowering period suggests that phenology is not the only process driving the mating system of Fraxinus.

At the time of our study, the plantation trees were only just reaching the adult flowering stage of their life cycle and were much younger than the native trees (they produced far fewer flowers; personal observation). Furthermore, approximately half of the plantation trees had not yet started to flower (personal observation). In a few years time, all the plantation trees should be able to participate in reproduction at these sites. Thus on the basis of our results, pollen from plantation trees would be expected to compete strongly with pollen from native trees.

In the long term, the fact that individuals from different taxa (*F. excelsior*, *F. angustifolia* and hybrids) and individuals from different phenology groups interbreed, even in low proportions, will lead to the production of more hybrid types. Hence, the overlap of the flowering period between native and plantation might be predicted to increase.

5.4.5 Conclusion

In conclusion, this study has shown that pollen dispersal has occurred over large distances between native and non-native *Fraxinus* trees both into and out of the plantations. Thus, genes from alien ash can potentially spread to Irish ash populations via pollen movement directly from the plantation sites but also via subsequent seed dispersal from plantation sites including hybrid seeds produced by the mating of plantation mother trees and native pollen donors. Our study also showed that the mating system or paternity analyses alone are not enough to describe the pattern of interspecific gene flow. Indeed the ability to detect the pollen cloud structure allowed us to characterize a differentiation of the pollen pool that would have otherwise been overlooked. We observed a structure of the pollen cloud related to different flowering time. This structure due to flowering time might reduce the risk of gene flow between plantation and native trees especially for some years and more likely for small plantations.

The question remains whether the hybrid progeny produced around or inside the plantations will be fit enough to compete in the Irish environment and to invade the landscape. For the time being we have measured a very high rate of predation in the seeds from the plantation trees due to insects (Chapter VI) that could suggest non adaptation. It is not known if zygotes of interspecific crosses (i.e. *F. excelsior* and *F. angustifolia*) will survive in the future but we plan to monitor and assess this in subsequent studies. In the long term, it is also not known whether introgression will have a positive or negative effect on Irish ash populations. Introgression from non-native sources could in fact provide a novel source of genes. However simulation analysis suggest that future Irish climates would not match present day condition that favour hybrid zones of *Fraxinus* in continental Europe (Thomasset *et al.*, 2011b, Appendix 1).

Acknowledgments

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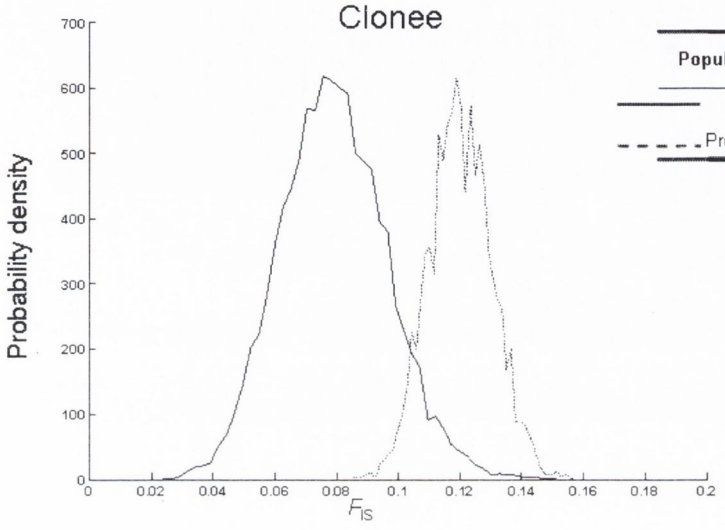
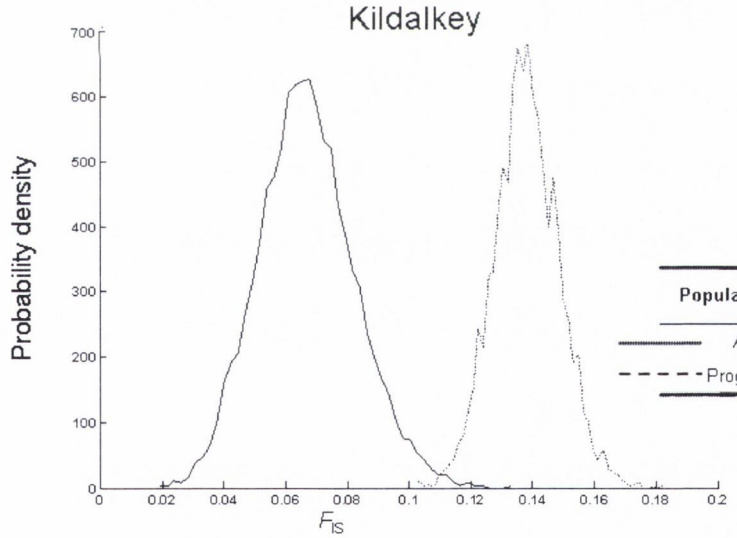
Supplementary material

Figure S5. 2 Posterior density curves for the inbreeding coefficient for the progeny and the adult of the native trees at the two sites. The tables show the mean of the inbreeding coefficient and the confident interval.



Population	Mean	Lower 0.025	Upper 0.975
Adult	0.0790	0.0428	0.0780
Progeny	0.1200	0.0978	0.1195



Population	Mean	Lower 0.025	Upper 0.975
Adult	0.0669	0.0344	0.0660
Progeny	0.1380	0.1147	0.1374

Chapter VI

Fitness and seed characteristics of native ash (*Fraxinus excelsior*) and introduced population of hybrid ash (*F. excelsior* x *F. angustifolia*)

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Abstract

One central question regarding invasive species or populations is whether seeds from alien individuals outperform those from local populations. We determined the germination requirements of native and alien accessions of ash (*Fraxinus excelsior*, *F. angustifolia* and their hybrids) in Ireland and studied seed morphology characteristics that might be linked to fitness. As a control, we compared germination rates and dormancy requirements of seeds from *F. excelsior*, *F. angustifolia* and two hybrid populations from different provenances from France. The results confirmed that *F. excelsior* is not able to germinate without stratification by a warm and cold pre-treatment. Secondly, we compared fitness characteristics of native Irish ash seed (*F. excelsior*) with non-native ash plantation seeds (the two *Fraxinus* species and their hybrids). We analysed dormancy requirements, germination rates, seed quality and seed/embryo size. Seeds from non-native plantation trees expressed dormancy requirements characteristic of *F. excelsior*. However, after a warm and cold stratification treatment, we observed a lower rate of germination in seeds from plantations than native trees especially for seeds from one of the study sites (Plantation-Clonee=22.1% ± 5, compared to Native-Clonee=72.0% ± 4.33). We also observed that seeds from plantation trees were much more damaged by insects (43.8% ± 15.4) than the seeds from native ash (8.8% ± 5.2). Seeds attacked by insects can be difficult to distinguish without removing the pericarp thus insect infestation might have contributed to the lower germination rate observed in seeds from plantation trees. Seed size and embryo size were highly variable and no significant differences were observed among ash groups studied. Therefore, overall, seeds from plantation trees do not show higher fitness than the seeds of native *F. excelsior* and in many cases even show poorer fitness characteristics.

6.1 Introduction

Introduction of exotic species boosted by human activity has become a major concern for the world's biodiversity (Sakai *et al.*, 2001). Introduced populations can displace local ones by competitive exclusion. A second possible consequence of the introduction of exotic organisms is the transfer of non-native genes into native gene pools through introgression (Anderson and Hubricht, 1938; Chapman and Burke, 2006). Recent studies have shown that the invasion of exotic species can be promoted by hybridization (Rhymer and Simberloff, 1996; Ellstrand *et al.*, 1999; Ellstrand and Schierenbeck, 2000; Vila *et al.*, 2000). However, the potential consequences of exotic species' introduction followed by introgression and hybridization will depend, in part, upon the fitness of their hybrids compared with the parental species (Rieseberg and Carney, 1998; Burke and Arnold, 2001). Biological fitness can be defined as the genetic contribution of an individual to the next generation's gene pool relative to the average for the population, i.e. the ability of an organism to produce viable offspring capable of surviving to the next generation. Several studies have shown that hybrids between two closely related species or between geographically distant populations (Rieseberg, 1995; Rieseberg, 1997; Rieseberg and Carney, 1998; Keller *et al.*, 2000; Montalvo and Ellstrand, 2001) have reduced fitness and viability compared to their parents. This fitness reduction is named outbreeding depression (Lynch, 1991). However, several studies (Arnold and Hodges, 1995; Arnold, 1997) concluded that hybrids can also be more fit, or intermediate in fitness, compared to both parents. Hybrid fitness and its variation is affected by both endogenous (genetic) and exogenous (environmental) selection (Barton and Hewitt, 1985; Arnold and Hodges, 1995; Barton, 2001). Hence, understanding the factors that determine newly formed hybrid fitness is essential to prevent unwanted hybrid spread.

Early life history stage characteristics, such as germination, seed mass production, and dormancy may be altered by hybridization (Burke *et al.*, 1998). On the one hand, post mating barriers that prevent hybridization might reduce seed viability (for example by embryo abortion), thus reducing the germination rate and the global seed production (Ellstrand and Elam, 1993). On the other hand,

hybridization between two species will lead to the creation of new genotypes, which will express different phenotypes depending on the relative contribution of nuclear and non-nuclear genes leading to the potential modification of reproductive traits. Nuclear effects include chromosomal rearrangement (Rieseberg and Burke, 2001), interaction between parental genes (Rieseberg *et al.*, 1996; Burke and Arnold, 2001) or interactions between nuclear genotypes of the hybrids and their environment (Arnold, 1997). Non-nuclear effects refer to cytoplasmic effects (Roach and Wulff, 1987; Levin, 2003), and the influence of the parental environment on the offspring traits (Wade, 1998; Lacey and Herr, 2000).

The relationships between germination, seed size, seed mass and seedling establishment or invasiveness have been investigated in some tropical and temperate forest trees (Foster, 1986; Seiwa and Kikuzawa, 1996; Seiwa, 2000; Parolin, 2001). For example, larger seed mass might be advantageous for seedling establishment (Bonfil, 1998; Hewitt, 1998; Seltmann *et al.*, 2007) but the influence of seed size on germination is not always clear (Rejmánek and Richardson, 1996; Jakobsson and Eriksson, 2000; Gomez and Husband, 2004; Larsen and Andreasen, 2004; Willenborg *et al.*, 2005).

Several seed dormancy and germination strategies among species have evolved that are believed to optimise plant establishment (for review see: Baskin and Baskin, 2004; Nikolaeva, 2004; Finch-Savage and Leubner-Metzger, 2006). Seedling survival has been found to be highly connected to seasonal timing of germination (Harper, 1977). Many species have developed mechanisms to prevent germination immediately after seed dispersal so that it will occur when conditions are likely to be more suitable for germination and seedling survival (Baskin and Baskin, 2004; Fenner and Thompson, 2005).

In Ireland during the 1990s, exotic provenances of what was then believed to be *Fraxinus excelsior* L. were introduced to cater for the needs of foresters and farmers. However, these non-native trees showed unusual morphology and bad growth (Douglas *et al.*, 2009). We have shown elsewhere (Chapter III) using morphological and molecular markers that the introduced plantation trees were a mixture of alien provenances of *F. excelsior*, *F. angustifolia* Vahl and introgressed

hybrids between them. *Fraxinus excelsior* and *F. angustifolia* are both wind pollinated species that produce wind dispersed fruits (samaras) capable of invading forest openings. Several reports (Muyt, 2001; Richardson *et al.*, 2006; Gray, 2009) have highlighted *F. angustifolia* as an invader in Australia. *Fraxinus excelsior* is the only native ash in Ireland. However, these two *Fraxinus* species are found on continental Europe and at latitudes from a zone running from Turkey to France (Thomasset *et al.*, 2011b) they are found to grow in sympatry and natural hybrids have been observed in some of these (Fernández-Manjarrés *et al.*, 2006; Gérard *et al.*, 2006b). The introgression and the spread of non-native DNA into natural populations will depend on the frequency of hybrid formation and their fertility. In our case, we found in a previous study that introgressed hybrids (late generation hybrids) made up a large proportion of trees in the plantations, between 28.3%-58% (Chapter III). Moreover, hybrids between the two species have been found to be fertile (Raquin *et al.*, 2002a; Gérard *et al.*, 2006b; Gérard *et al.*, 2006c).

Studies have shown that the two species have different dormancy periods (Piotto, 1994; Raquin *et al.*, 2002b; Gérard *et al.*, 2006a), the dormancy period being particularly long for *F. excelsior* (2 to 6 years). Studies on the effects of the storage, temperature and stratification on germination of *F. excelsior* (Villiers and Wareing, 1964; Tylkowski, 1990), of *F. angustifolia* (Preece *et al.*, 1995; Tilki and Ciçek, 2005) and of both species (Raquin *et al.*, 2002b; Gérard *et al.*, 2006a) have shown that the two species have different pre-treatment requirements. The warm and cold periods need to be shortened in *F. angustifolia* compared to *F. excelsior.*

The aims of this study were firstly, to compare the germination characteristics of *Fraxinus* taxa of known origin from France (*F. excelsior*, *F. angustifolia* and their hybrids) to establish their germination requirements as a control. Secondly, to compare introduced plantation seed from two sites in Ireland (that contain a mixture of the same taxa) with seeds from native Irish *F. excelsior.* We examined if they have: i) different dormancy requirements; ii) different germination rates; iii) different proportions of damage seeds; and iv) different seed and embryo sizes. These results will help determine the survival probability of the seeds in the Irish

landscape and hence the potential for native to non-native competition at early stages of their life.

6.2 Materials and methods

6.2.1 Plant material

To confirm results from previous studies (Piotto, 1994; Raquin *et al.*, 2002b; Gérard *et al.*, 2006a) and to assess the validity of the experimental conditions used in stratification, we performed an initial experiment (Experiment 1 – 'control'). In this case, seeds previously collected from two natural populations of pure *F. angustifolia*, two natural populations of pure *F. excelsior* and two populations where natural hybridization between the two species occurred (Fernández-Manjarrés *et al.*, 2006; Gérard *et al.*, 2006a)were used. All the populations were sampled in France (Table 6.1) and ten trees per population were randomly chosen for seed collection. Seeds were air dried at room temperature (between 15 and 20°C). Thirty seeds per tree were collected from each population, those appearing viable (without visible hole typical of insect attack or obvious empty seeds) were pooled together into a plastic bag. Seeds were stored in the dark at 4°C until required.

Secondly, tests (Experiment 2 – Irish native and non-native material) were carried out on mature seeds collected in September 2008 from two Irish sites: Kildalkey (53° 34' 17.8"N; 6° 54' 54.21"W) and Clonee (53° 28' 03.09" N; 6° 28' 58.9"W) (Table 6.1) that contain introduced hybrids between *F. angustifolia* and *F. excelsior* and also *F. angustifolia* and *F. excelsior* (Chapter III). At each site, seeds were collected from 12 plantation trees. Our sample was limited to 12 trees because very few trees had started to set seed in these young plantations and because of limited resources for experimentation. Mother trees were classified as either *F. excelsior* or hybrid type (hybrid type including hybrids and *F. angustifolia* individuals) (Supplementary material Table S 6.1) on the basis of the molecular results from a previous study (Chapter III). For comparison, control seeds from 12 randomly chosen native mature mother trees were collected around each site.

Seeds were transported to the laboratory in labelled paper bags (one bag per tree), air dried and stored as described above.

Table 6.1 Study populations for Experiments 1 and 2 (sample size, origin, latitude, longitude).

	Population	Country	Latitude	Longitude	N° of mother trees	N° of seeds per treatment & per population
Experiment 1 – 'control'	***F. excelsior***					
	La Preste	France	42°24'	2°41'	10	96
	St Paul de Balers	France	45°08'	2°31'	10	96
	F. angustifolia					
	Cogolin	France	43°12'	6°28'	10	96
	Mas Larrieu	France	42°36'	3°02'	10	96
	Putative hybrids					
	St Dye sur Loire	France	47°39'	1°29'	10	96
	Tavaux	France	47°02'	5°24'	10	96
Experiment 2 – Irish native and non-native material	***F. excelsior***					
	Native-Clonee	Ireland	53° 28'	6° 28'	12	576
	Native-Kildalkey	Ireland	53° 34'	6° 54'	12	576
	Plantation*					
	Plantation-Clonee	Ireland	53° 28'	6° 28'	12	576
	Plantation-Kildalkey	Ireland	53° 34'	6° 54'	12	576

*Plantation trees are a mixture of non-native *F. excelsior*, *F. angustifolia* and hybrids between the two.

6.2.2 Stratification treatment and germination

Damaged seeds or those with unusual morphology were discarded for further analyses. For both experiments (as described above), two treatments were performed to compare germination capacity and dormancy requirement. Seeds were either:

1) germinated in the greenhouse without stratification and hence were regarded as the control treatment;
2) or germinated after applying a stratification pre-treatment.

For the stratification treatment, seeds were placed in bags containing a moistened 1:1 mixture of sand and commercial grade of peat compost. Seeds were stored for 16 weeks at room temperature followed by 16 weeks of cold stratification at 4 °C (C. Raquin, personal communication).

Non stratified seeds were stored in the fridge at 4 °C until February when they were sown. Stratified seeds were stored in the fridge until April, stratified for 32 weeks, and then sown in February of the following year. For the two treatments (with or without stratification) seeds were sown in trays (1/3 perlite, 2/3 commercial peat compost) and germinated in a greenhouse under natural light conditions. 48 seeds per tree were placed in trays to germinate. Therefore, for Experiment 1 ('control'), 48 seeds per population and per treatment were included. For Experiment 2 (Irish native and non-native material), 48 seeds per tree and per treatment were included. The number of seeds that germinated was counted every week until the 75th day after sowing. Seeds were considered to have germinated when they exhibited 1cm long radicles.

6.2.3 Seed characteristics

From the seeds collected in Experiment 2 (Irish native and non-native material) , 60 seeds per mother tree were de-pericarped, rehydrated and sterilized as described in Raquin *et al.* (2002b). When sterilized seeds became translucent, it was easy to visually detect the presence of the embryo and insect infestation. We recorded the number of seeds infested by insect larvae. Moreover, we also recorded when seeds were not infested, and the presence or absence of embryos. These were often detectable with the naked eye, but sometimes seeds needed to be dissected under a binocular microscope (10-x magnification).

To measure seed and embryo size, 20 viable seeds per mother tree were randomly selected after sterilization. Length (SeedL) and width in the widest part of the seeds (SeedW) were measured. Width was measured before cutting the border of the seeds to carefully open the seed so that embryo length (EmbryoL) could be determined. All the measurements were made under an Olympus BH-2

microscope (100-x magnification) and assessed using electronic image analysis equipment (Camera: UI-1545LE and Quick Photo CAMERA 2.3, Software, Promica). The ratios "EmbryoL/SeedL" and "SeedL/SeedW" were also calculated.

6.2.4 Seed production

During seed collection, each mother tree was categorized according to their fruiting level; the categories were as defined by Gardner (1977): 1) Light; 2) Medium; or 3) Heavy.

6.2.5 Data analysis

6.2.5.a Germination

Germination data (proportions) follow a binomial distribution. Hence we explored the relationship between seed germination (i.e. the proportion of germinating seeds per box: response variable) and the values of the predictors (explanatory variables) using a generalized linear model (GLM) with binomial error and a logit link function (McCullagh and Nelder, 1989). For Experiment 1 (control), both direct (species, populations and treatment) and indirect (interactions) effects were considered as potentially explanatory. For Experiment 2, populations (Plantation-Clonee, Plantation-Kildalkey, Native-Clonee and Native-Kildalkey), treatment (stratification or control) and trees within population (nested) were considered as main effects. First order interactions were also included in the model. The best-fit model was selected using a stepwise procedure with Akaike's information criterion corrected (AICC) (i.e. a model minimizing the AICC value). The model was finally selected after checking the deviance statistics and the plots of residuals. F tests were calculated and tested on the basis Chi-square likelihood ratio. Means were then separated using Fisher's protected least significant difference with effects declared significant at $P<0.05$. Statistical analysis were done with the software SPSS, version 16.0 (SPSS, 2007).

6.2.5.b Quality of the seeds

Absence (or presence) of embryo and attack (or non attack) by insects also followed a binomial distribution. Hence, these data were explored using a generalised linear model as described above for the germination experiments. The response variable was defined as the percentage of empty or infected seeds, and populations (Plantation-Clonee, Plantation-Kildalkey, Native-Clonee and Native-Kildalkey within population (nested)) were considered as the main effect. To identify the best-fit model and separate the mean, the same procedure as described above was used.

Based on the results of Bayesian analyses performed with the software STRUCTURE (Pritchard *et al.*, 2000) from a previous Chapter (Chapter III), for taxon identification, the percentage of *F. angustifolia* genotypes of each mother tree from the plantations (which was the basis to recognize non-pure *F. excelsior* genotypes) was compared to the percentage of germination per tree after stratification treatment. The comparison was assessed using a Spearman correlation coefficient. The same approach was used to assess the relationship between the percentage of germinating seeds and the percentage of seeds infected by insects.

6.2.5.c Seed and embryo size

After verifying that the variables (SeedL, SeedW, EmbryoL, SeedL/SeedW, EmbryoL/SeedL) did not deviate from assumptions of normality (using normality plots) and that they showed homocedasticity (using the Levene test) within each population, we compared means between the different populations using a nested ANOVA, with trees nested within. Following ANOVA, post hoc Tukey tests were performed. All statistical analysis were carried out using the software SPSS, version 16.0 (SPSS, 2007).

6.3 Results

6.3.1 Experiment 1: Species comparison and validation of experimental conditions.

The best-fit model (Table 6.2) includes species and treatment effects and the interaction between species and treatment. The best-fit model according to the AICC criteria does not consider the population effect and thus this effect was not included in the model; therefore the two populations of the same species (i.e. *F. excelsior*, *F. angustifolia* and putative hybrid's population) were pooled together. The results of the generalised model (Table 6.2) showed that germination rate was significantly affected by species (taxon) effects, and treatment effects. There was also a significant interaction between species and treatment (thus species responded differently to treatment).

Experiment 1 ('control') used seeds from natural populations of ash with and without stratification treatment. *F. excelsior* did not germinate at all without a stratification treatment (Figure 6.1). In contrast, the germination mean of *F. angustifolia* without stratification was 34.4% (Figure 6.1) and was significantly higher than the germination mean observed for the seeds from putative hybrids (Table 6.3) that was only 13% (Figure 6.1). After the stratification treatment, the mean germination of *F. excelsior* was 23.3% (Figure 6.1).

Pairwise comparisons (Table 6.3) showed that the germination success of the seed from the hybrid population did not change significantly with or without stratification treatments. The germination success of seed from hybrids was also not significantly different from either *F. angustifolia* or *F. excelsior*. In contrast, the germination of *F. angustifolia* was significantly affected by the treatment being reduced after stratification treatment (from 34.4% to 11.9%). After stratification, the germination success was not significantly different between the two parental species.

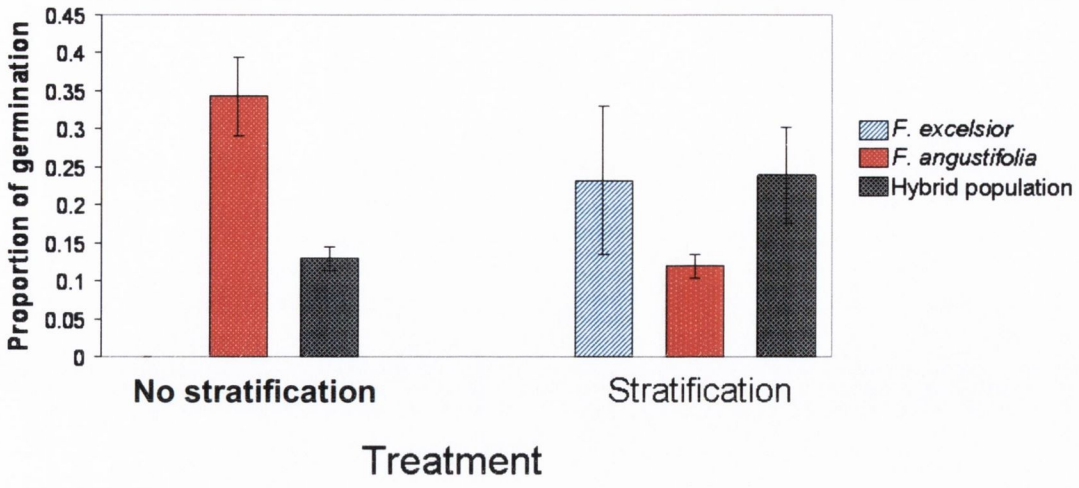

Figure 6.1 Germination rates (means ± SE if >5%) and effects of stratification, for Experiment 1 ('control'), of seeds from natural population of *F. angustifolia* (in red), natural population of *F. excelsior* (in blue) and putative hybrid population (in grey) without a stratification treatment (no stratification) and after a stratification treatment.

Table 6.2 Effect of stratification treatment (Treatment), species or population (Species, Population) and trees within population (Trees(Population)) on seed germination. Data were modelled using a generalized linear model with binomial error and logit link. Interaction (Treatment*Species) has been considered. Values of Akaike's information criterion corrected (AICC) and the overall significance of the fitted models, together with coefficient estimates, are shown.

Experiment	Whole Model			Factors				
	AICC	Deviance/df	*P*	Effect	Coefficient	df	Likelihood ratio χ^2	*P*
Experiment 1 – Control	94.84	4.67	<0.001	(Intercept)	20.36	1	20.36	<0.001
				Species	6.7	2	13.4	0.001
				Treatment	6.19	1	6.19	0.013
				Species x Treatment	16.01	2	32.01	<0.001
Experiment 2 – Irish native and non-native material	287.24	0.3	<0.001	(Intercept)	6320.97	1	6320.97	<0.001
				Population	484.72	3	1454.16	<0.001
				Treatment [1]	8988.19	1	8988.19	<0.001
				Tree(Population)	50.93	45	2291.61	<0.001
Experiment 2 (empty seeds)	290.95	3.59	<0.001	(Intercept)	564.92	1	564.92	<0.001
				Population	6.34	3	19.01	<0.001
Experiment 2 (insect infested trees)	380.29	4.56	<0.001	(Intercept)	139.64	1	139.64	<0.001
				Population	37.19	3	111.56	<0.001

[1] The interaction between Population and Treatment was not part of the most parsimonious model according to the AICC criterion.

Table 6.3 Significance of the pairwise comparisons following the generalized linear model for Experiment 1(Control). Means were separated using Fisher's protected least significant difference with effects declared significant at $P<0.05$ (***).

		Control			Stratification		
		F. angustifolia	*F. excelsior*	Hybrid population	*F. angustifolia*	*F. excelsior*	Hybrid population
Control	*F. angustifolia*	-					
	F. excelsior	***	-				
	Hybrid population	***	***	-			
Stratification	*F. angustifolia*	***	***	ns	-		
	F. excelsior	ns	***	ns	ns	-	
	Hybrid population	ns	***	ns	ns	ns	-

6.3.2 Experiment 2: Irish plantation seed compared to native seed

The best-fit model that we choose for our analysis of seed from plantation and native Irish trees had an AICC of 287.24 and included population, treatment and trees within population effects (Table 6.2). All the effects were significant ($P<0.05$).

6.3.2.a Germination without and with stratification treatment

Only one seed from the plantation mother trees germinated in the soil without stratification treatment, out of a total of 1152 seed. Neither the native *F. excelsior* nor the plantation seeds germinated without the stratification treatment that was highly significant ($P<0.001$; Table 6.2 and Figure 6.2).

The population x species interaction was also significant probably mostly due to changes observed for the Plantation-Clonee (Figure 6.2) which had a reduced germination success (mean 22.1 ± 5.0) compared to the other populations. The mean germination success of seeds from the Plantation-Kildalkey trees (56.1 ± 11.0), although lower, was not significantly different from Native-Clonee (72.0 ± 4.33) and Native-Kildalkey (77.2 ± 6.5) trees. We also observed a significant effect of trees within population (Table 6.2) indicating a high degree of heterogeneity in germination success within population.

The correlation between the percentage of *F. angustifolia* genotype for each plantation individual and their percentage of germination was not significant. Indeed, after classification of the mother trees of the plantations as *F. excelsior* or hybrid type, the germination rates observed after stratification treatment were very similar (Supplementary material Table S 6.1). For the Kildalkey plantation, individuals classified as *F. excelsior* that represented 66.6% of the trees sampled, showed a germination rate of 55.9% compared to 56.2% for the individuals classified as hybrid type. For the Clonee plantation, a small difference was also observed between the two possible types (23.7% for *F. excelsior* and 20.8% for hybrid type).

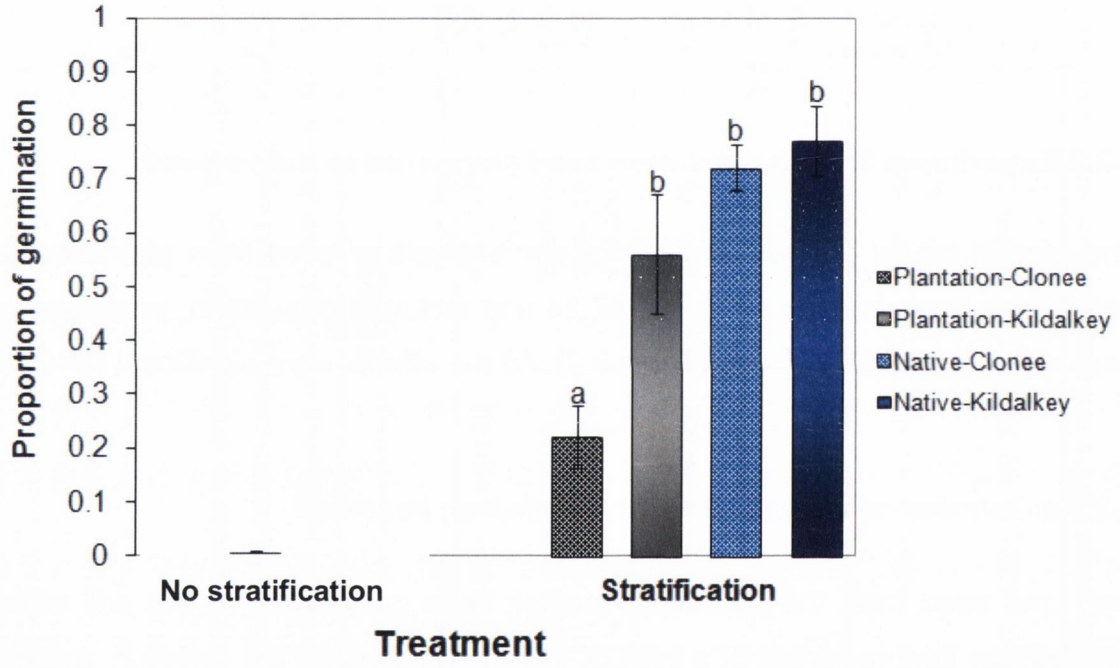

Figure 6.2 Germination rates (means ± SE if >5%), for Experiment 2 (Irish native and non-native material), of seeds from native Irish *F. excelsior* (in blue) and the two plantation populations (in grey) without stratification treatment (no stratification) or after stratification treatment. Different letters above the bars indicate significant difference ($P<0.05$) in final germination percentages.

6.3.2.b Seed quality

The best-fit model only included populations as a main effect (which was highly significant) for the two variables, percentage of empty seeds and percentage of seeds infected by insects (Table 6.2). There was no significant difference in the

percentage of empty seeds for the Clonee plantation (mean=4.7% ± 1.4) compared to the two native populations (Native-Clonee mean=5.5% ± 3.0; Native-Kildalkey mean=5.5% ± 1.2). However, our results showed that the Kildalkey plantation (mean=16.0% ± 2.3) had a higher percentage of empty seeds than the other populations (Figure 6.3).

The plantations had significantly higher number of seeds infested by insects (54.8% ± 5.9 and 32.6% ± 2.9 for Clonee and Kildalkey plantations, respectively) than the native tree populations (8.8% ± 1.6 and 8.9% ± 2.6 for the Clonee and Kildalkey native populations, respectively; Figure 6.3). In addition, the two plantations were significantly different from each other ($P<0.05$, Figure 6.3). We pooled together the data from percentage of empty and infested seeds to form one variable (damaged seeds); the Kildalkey plantation had a significantly ($P<0.05$) lower number of damaged seeds (48.6% ± 12.1) than Clonee (59.6% ± 19.4). For the Clonee plantation, the percentage of damaged seeds was more important for seeds from mothers trees classified as hybrid types (65.4%) compared to 51.4% for seeds produced by mothers classified *as F. excelsior* (Supplementary material Table S6.1). For Kildalkey however this percentage was very similar between the two types (33.4% for *F. excelsior* type compared to 31.2% for hybrid type) (Supplementary material Table S6.1). Finally, we found a significant negative correlation between the germination rate and the percentage of damaged seeds for both plantations ($P<0.05$; r=-0.49 and r=-0.52 for Clonee and Kildalkey, respectively).

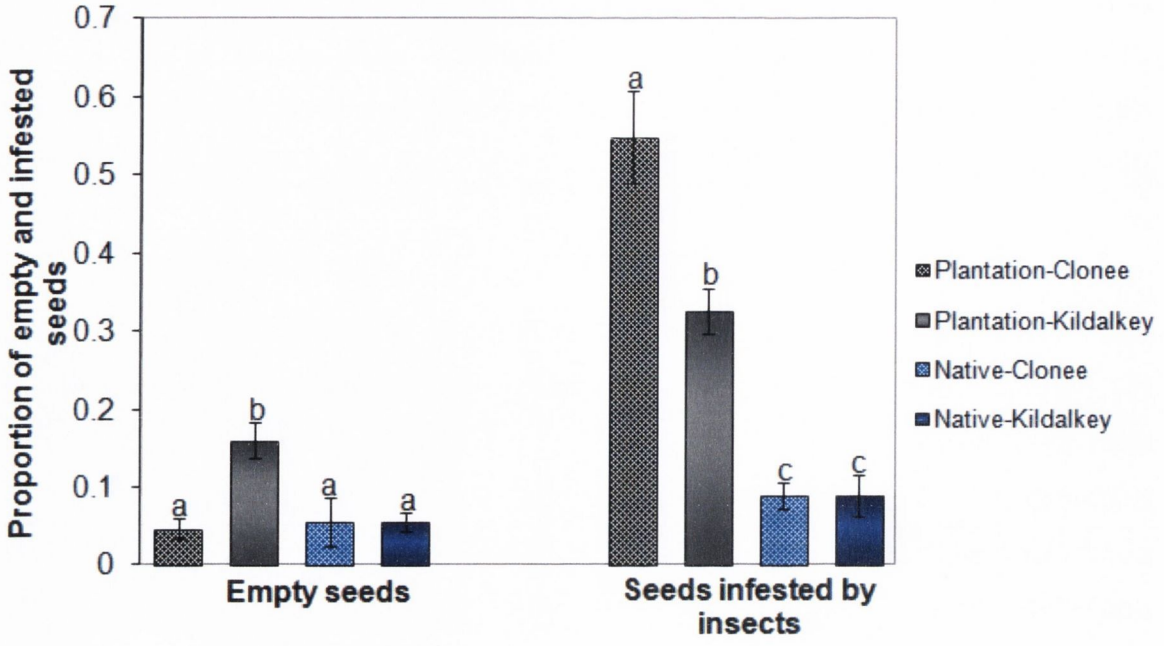

Figure 6.3 Proportion (means ± SE if >1%) of empty seeds (on the left part) and infected seeds (on the right part) of seeds from native Irish *F. excelsior* (in blue) and the two plantation populations (in grey) from Experiment 2 (Irish native and non-native material). Different letters above the bars indicate significant difference (P<0.05) in final germination percentages.

6.3.2.c Seed characteristics

None of the data for seed characteristic variables deviated from normality (results not shown). The ANOVA tests (Table 6.4) indicated that there were significant differences in SeedL, SeedW, EmbryoL and SeedL/SeedW, among populations and among trees within populations (P<0.05). The population effect was not significant (P=0.22) for the ratio of EmbryoL/SeedL. The results of the post hoc Tukey tests (Figure 6.4) showed no consistent patterns between native and plantation seeds. However, seeds from the Kildalkey plantation showed a significantly higher SeedL and EmbryoL compared with all the other populations. The reverse was found for the seeds from the native Kildalkey group, where SeedL and EmbryoL were smaller compared with all the other populations. No significant difference was observed for SeedL and EmbryoL between the Clonee plantation and the native Clonee population. For SeedW, the Plantation-Kildalkey and the Native-Clonee were similar to each other but significantly different from the Plantation-Clonee and Native-Kildalkey groups.

Table 6.4 Analysis of variance table for seed variables partitioned inot Populations (Pop) and trees within population (tree (Pop)).

Variable	Effects	df	SS	MS	F	*P*
SeedL	Pop	3	3.26E+08	1.09E+08	121.59	<0.05
	Tree (Pop)	44	2.03E+09	4.62E+07	51.66	<0.05
	Error	913	7.90E+08	8.95E+05		
SeedW	Pop	3	1.09E+07	3.62E+06	27.57	<0.05
	Tree (Pop)	44	2.37E+08	5.40E+06	41.09	<0.05
	Error	913	1.16E+08	1.31E+05		
EmbryoL	Pop	3	7.88E+07	2.63E+07	32.4	<0.05
	Tree (Pop)	44	2.15E+08	4.90E+06	6.04	<0.05
	Error	913	7.16E+08	8.11E+05		
SeedL/SeedW	Pop	3	426.48	142.16	1594.83	<0.05
	Tree (Pop)	44	84.23	1.91	21.48	<0.05
	Error	913	78.71	0.09		
EmbryoL/SeedL	Pop	3	0.02	0.01	1.46	0.22
	Tree (Pop)	44	3.3	0.08	16.95	<0.05
	Error	913	3.91	0		

The seeds from the native Kildalkey population (which had smaller SeedL) had a significantly (P<0.05) higher SeedL/SeedW ratio than the two populations from Clonee (Figure 6.4) but significantly (P<0.05) smaller ratio than the seeds from the Kildalkey plantation. In other words seeds from the native Kildalkey population tend be small and narrow. The main difference for the ratio of EmbryoL/SeedL (Figure 6.4) was observed for the seeds from the native Clonee group, which were significantly (P<0.05) smaller than all the other groups except the Kildalkey plantation, indicating that the seeds from the native Clonee group had rather small embryo compared to seed size. Pairwise comparisons of the EmbryoL/SeedL means showed that the Clonee plantation, Kildalkey plantation, and native Kildalkey population were not statistically (P>0.05) different from each other, meaning that even if we observed differences in the seed and embryo size the ratio was maintained.

6.3.2.d Seed production

The majority of the plantation trees were categorized as light seed producers according their level of fruiting, and none of the trees showed heavy seed

production (Table 6.5). In contrast, the majority of the native trees were classified in the medium fruiting category and one third or more were classified as heavy fruiting.

Table 6.5 Intensity of seed production by native and plantation trees, % of trees by category.

Seed production Intensity	Plantation-Clonee	Plantation-Kildalkey	Native-Clonee	Native-Kildalkey
Light	58.33	66.67	16.67	25.00
Medium	41.67	33.33	50.00	50.00
Heavy	0.00	0.00	33.33	25.00

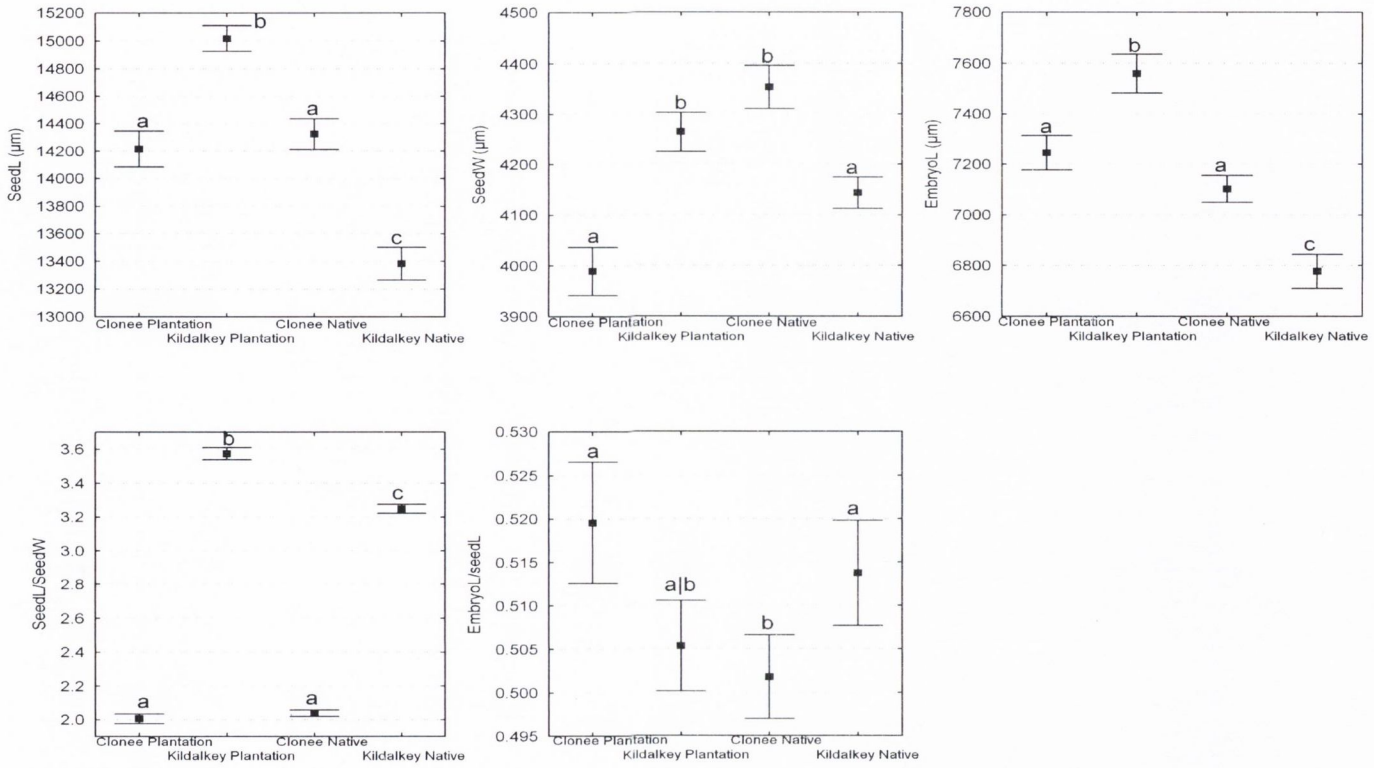

Figure 6.4 Seed characteristics (means ± SE) for the different variables (SeedL, SeedW, EmbryoL, SeedL/SeedW, EmbryoL/SeedL) of the two native populations and the two plantations. Different letters above the error bars indicate significant ($P<0.05$) differences among populations (Tukey HSD).

6.4 Discussion

We have shown that the germination requirement of seeds taken from a plantation population comprising a mixture of non-native ash genotypes and hybrids is similar to native Irish *F. excelsior* seed and that both required stratification treatment for successful germination. Our results show that seeds from plantations do not have a higher fitness than native ash for the measured characters. At the reverse, our results indicate a lower fitness of the seeds due to a general inferior quality because of insect attacks.

6.4.1 Dormancy breakdown

Seeds from *F. angustifolia* were able to germinate with or without pre-treatment but not the seeds from *F. excelsior,* the latter only germinated if a stratification period was applied (warm period followed by a cold period) (Figure 6.1). *Fraxinus excelsior* is known to have a particularly long stratification requirement (Steinbauer, 1937; Wardle, 1961; Wagner and Kafka, 1995) and our studies confirm these previous observations.

There was no noticeable reduction in germination for putative hybrid seeds compared to seeds from parental species (Experiment 1 –control, Figure 6.1). Thus, our results do not indicate that natural hybrid populations, under controlled conditions, present a reduction in fitness in terms of germination rates. We found that 13% of the seeds collected from control populations previously shown to be of hybrid origin (Fernández-Manjarrés *et al.*, 2006; Gérard *et al.*, 2006a) germinated without a stratification treatment.

Contrary to our expectation, seeds from for the two Irish plantations that contain introgressed hybrids and *F. angustifolia* according to our studies (Chapter III), could not germinate without a stratification treatment (Figure 6.2). Moreover, plantation individuals with hybrid type or *F. excelsior* type origins (Chapter III) had similar germination rates for both Clonee and Kildalkey plantations (Supplementary material Table S6.1). Introduced plantation trees in Ireland included those with hybrid or *F. angustifolia* origins, but however expressed *F.*

excelsior germination characteristics. There might be several explanations for this. Firstly, we already showed (Chapter III) that the plantation trees consisted of advanced generations of hybrids (mainly introgressed with *F. excelsior*). Secondly, previous work has shown that experimental conditions might affect seed germination characteristics but also that the conditions experienced by the mothers, i.e. maternal environment effects, are important (Alexander and Wulff, 1985; Roach and Wulff, 1987; Baskin and Baskin 1998; Donohue and Schmitt, 1998). At present, Ireland lies within the natural distribution of *F. excelsior* but further north of the natural distribution of *F. angustifolia*. It is however too early to generalise about the influence of the environment on seed dormancy.

6.4.2 Germination of plantation populations

Although Gérard *et al.* (2006a) showed that a germination test is a simple way to detect seeds of *F. angustifolia*, the authors also highlighted the finding that sample size has to be sufficient and that results might be highly dependent on damaged fruits. In the present study, heavily damaged seeds were observed for the plantation trees due to insect infection (Figure 6.3), which could have influenced germination rates. Indeed, Preece *et al.* (1995) that showed it was difficult to detect infected ash seed without removing the pericarp.. This might suggest that we were enabled to identify every seed which was no viable by eyes (i.e. without removing the pericarp).

The difference in germination rate observed between seeds from the plantations (mainly from Clonee) and the native seeds might be due to the presence of many damaged seeds from the plantation trees (exogenous selection), or to a lower viability and vigour of the seeds due to endogenous selection (Arnold, 1997; Burke and Arnold, 2001). The relatively limited number of seeds available for insects in the young plantation may explain the higher proportion of seed infested. Insect attack might change/evolve with time and we have to keep in mind that at the time of the study the plantation mother trees were much younger than the native mother trees. The increase of flower production of the plantation may influence the incidence of attack by insects in the future. However, other factors such as

genotype or interaction between genotype and environment could remain more or less stable over years, and might also influence insect attack. The time period when seeds are infested by insect is not known. The time of flowering and fruiting might be an important factor. Some of the plantation trees have shown earlier flowering during the study period and their seeds developed earlier than native ash (Chapter IV). It is conceivable that seeds from the plantation are more susceptible to insect damage by being available earlier in the season. In such a scenario, we would expect that seeds developing more in synchrony with native ash would be less susceptible to attack because the chances of any particular seed becoming infected would be much smaller in a large population than in a small population, where very few seeds would be available for infection.

6.4.3 Seed production

Seed production was lower in the plantation trees than the native trees: 58.33% and 66.67% of plantation trees showing light seed production compared to only 16.67% and 25% of native trees from Clonee and Kildalkey respectively (Table 6.5). However, on average, the plantation trees are much younger than the native trees (personal observation) and have only just reached sexual maturity (10 to 12 years). According to Pliûra and Heuertz (2003), flower and fruit production starts at 15-20 years on single ash trees and at around 30 years within stands. In fact, our results suggest that these figures are overestimated. Nevertheless, we were dealing with young trees that necessarily have low seed production. However, to draw conclusions on fruiting and seed production, it will be necessary to monitor seed production in future years.

6.4.4 Seed and embryo size

Fraxinus excelsior seeds showed morphophysiological dormancy in a study by Nikolaeva (2004) according to the Baskin and Baskin (1998) classification system. The embryos were underdeveloped at the time of seed dispersal. Conversely, *F. angustifolia* only showed physiological dormancy (Piotto, 1994) with large embryo at seed maturity. Gérard *et al.* (2006a) found that the ratio of embryo size/ seed length and the total embryo size was significantly higher in *F. angustifolia* (and

higher or intermediate in hybrid populations) compared to *F. excelsior*. We expected to observe such differences between seeds from the plantations and seeds from native trees. Contrary to our expectation, at least for embryo size, we did not observe a clear pattern between embryo sizes and germination rates. The embryo and seed size were higher for the Plantation-Kildalkey seed, but the ratio of embryo/seed size was not significantly different from the native trees. Different explanations may be considered. On the one hand, the fact that we did not observe significant differences in dormancy requirement between seeds from native and plantation trees could indicate that seeds from the plantation trees expressed mainly *F. excelsior* characteristics. On the other hand, we had within the plantation a mixture of trees of either *F. excelsior*, *F. angustifolia* or introgressed hybrid genotypes. This mixture and the reduced number of mothers (as explained in the methods section) might have masked a *F. angustifolia* effect on seed and embryo size, that was found to be clearer in the natural populations examined by Gérard *et al.* (2006a).

6.4.6 Conclusions and consequences

We investigated the fitness of seeds with regard to germination from introduced *Fraxinus* material including hybrids and seeds from native *F. excelsior*. Seeds from plantation trees (all categories combined), mainly expressed *F. excelsior* characteristics: lack of germination without stratification treatment, and a similar seed size. One of the key characters for plants to become invasive is for seeds of the potential invader to have higher fitness in term of germination capacity than the native plant with whom they compete for resources. In our case, the fitness of the seeds from plantation trees was similar, and even inferior due to insect attack, than native ash. These results suggest that the invasive potential, in term of germination, is low. Moreover, high mortality of plantation trees has been observed in some plantations but has not been measured systematically (J. Fernández-Manjarrés, personal communication).

However, since the seeds from plantation trees exhibited *F. excelsior* characteristics we can conclude that very little prevents them from germinating. It remains unknown whether the seeds from introduced plantation trees will

germinate at the same rate as native *F. excelsior* seeds and at the same time (year) under natural conditions. Therefore, the survivorship of the seedlings, which is also clearly an important component for establishment should be measured in the future.

In the present study, seeds from plantation trees were found to be much more sensitive to insect infection. Maron and Vila (2001) suggested that seed destruction by insects might be a potential mechanism to control spread of exotic plants. However, it is also unknown if the high rate of insect infested seeds will be maintained when plantation trees start to produce a larger quantity of seeds.

Acknowledgements

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Supplementary material

Table S6.1 Means and standard error of the nine different variables regarding the seed characteristics for trees from the two different plantations classified as *F. excelsior* or hybrid type.

	***F. excelsior* Plantation-Clonee (41.6%)[1]**		**Hybrids or *F. angustifolia* Plantation-Clonee (58.2%)[1]**		***F. excelsior* plantation-Kildalkey (66.6%)[1]**		**Hybrids or *F. angustifolia* plantation-Kildalkey (33.3%)[1]**	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
% germination after stratification	23.75	9.14	20.83	8.38	55.99	13.35	56.25	22.26
% empty seeds	6.10	3.27	3.70	0.82	14.96	2.65	18.15	5.11
% seeds intected by insect	45.29	10.80	61.67	5.90	33.36	4.27	31.19	2.90
% damaged seeds in total	51.39	10.30	65.37	5.94	48.33	4.62	49.33	5.87
SeedL (µm)	14012.01	856.00	14851.64	763.10	14634.17	599.21	14963.90	489.17
SeedW (µm)	3860.68	306.69	4221.94	236.72	4291.45	186.37	4206.63	98.80
EmbryoL (µm)	7520.94	82.78	7049.50	102.34	7331.21	225.91	7779.89	504.67
SeedL/SeedW	1.89	0.13	2.16	0.11	3.47	0.19	3.58	0.10
EmbryoL/seedL	0.55	0.04	0.48	0.02	0.50	0.01	0.52	0.03

[1]numbers in brackets indicate the percentage of trees characterised as either *F. excelsior* like or as hybrids and *F. angustifolia like* trees sampled as the mother trees.

Chapter VII

General discussion

The work presented in this thesis provides novel and valuable information on hybridization between *F. angustifolia* and *F. excelsior*. I examined the morphology of artificial F_1 hybrids to investigate the inheritance of species specific characters/traits and to help deduce the origin of plantation ash in Ireland. I then compared the flowering phenology of introduced ash and native ash trees, evaluated pollen-mediated gene flow between alien plantation trees and native ash, and explored the potential introgression of exotic genes. I also compared the germination capacity and dormancy requirements of introduced and native ash trees. In this final discussion chapter, I synthesize and summarize the main findings of this thesis, and outline management and direction issues for future research. Our results are not only pertinent for cases of artificial introduction of populations but also for the understanding of the dynamics of hybrids in general.

7.1 An overview of the findings

7.1.1 Morphology of artificial F_1 hybrids and inheritance patterns

Chapter II investigated the morphology of artificial F_1 hybrids, with either *F. angustifolia* or *F. excelsior* as pollen donor. F_1 hybrids presented intermediate morphology between the two parents even when the range of variation overlapped with the parental species (Chapter II, Figure 2.2). Environmental conditions associated with climate, light, precipitation, soil, relief, and altitude are known to influence leaf morphology (Givnish, 1984; Körner *et al.*, 1986; Körner *et al.*, 1989; Roderick *et al.*, 2000). In my experiment, all hybrids were grown in the same common garden, located at a site within the natural distribution of *F. excelsior.* Hybrids were distinguishable from *F. excelsior*, suggesting a genetic basis for character inheritance. Moreover, we detected that the expression of a few characters were influenced by parental effects. These findings are useful for assessing the morphology of potential hybrids introduced on Irish plantations and elsewhere as environmental conditions were under control. It allowed me to validate the use of the morphological and molecular markers chosen in this thesis for detecting hybrids. The work of Chapter 2 has been published in the International Journal of Plant Sciences (Thomasset *et al.*, 2011b).

7.1.2 Characterisation of hybrid alien ash (*Fraxinus excelsior* x *F. angustifolia*)

One of the main aims of the thesis was to identify the origin of introduced plantation ash material. By using a combination of molecular and morphological markers, clear evidence is presented for the presence of hybrids between *F. angustifolia* and *F. excelsior* in each of the two plantations that were assessed in detail. Hybrids were found to be present in proportions ranging between 28.3% and 58%, depending on the plantation, and the parameters chosen as the threshold for hybrid recognition (Chapter III, Figure 3.2). About 10% of introduced plantation trees were classified as pure *F. angustifolia*. In general, trees recognized as hybrid did not show an intermediate morphology (Chapter III, Figure 3.3), but instead showed novel characteristics. The hybrids were found to have mainly *F. excelsior* traits, indicating that they were probably introgressed with *F. excelsior* and were, hence, cryptic hybrids that would be difficult to recognize based on morphological markers alone. The ash plantations were not uniform in their composition and instead of considering them as a 'population' they may be better considered as artificial 'hybrid swarms'. Moreover, several introduced plantation trees with unusual morphology were found to be *F. excelsior* using molecular markers. So we must conclude that it is unsafe to assume that any tree with unusual morphology is a hybrid. In addition, the two plantations contained trees of different origins.

In addition, by combining the molecular and morphological data in one discriminant analysis (Chapter III, Figure 3.1C), the power of hybrid detection was increased and important information regarding the cryptic morphology of some types of hybrids was gained. Usually in studies about hybridization and species delimitation, molecular markers (Valbuena-Carabanã *et al.*, 2005; Currat *et al.*, 2008; Burgarella *et al.*, 2009; Curtu *et al.*, 2009; Lepais *et al.*, 2009) or morphological markers (Kremer *et al.*, 2002; Vervaeke *et al.*, 2004) are used on their own, and if used in the same study they are generally not combined in one analysis (Kelleher *et al.*, 2005; de Conceição *et al.*, 2008).

7.1.3 Overlapping flowering period

In Chapter III, which examined the morphology of the introduced plantation trees in Ireland, I found that there was a large variation in phenotypes, and a low but significant genetic population differentiation among the native Irish ash trees (AMOVA differentiation parameter Φ_{CT}=0.0211 P< 0.001). Based on the results of the morphological and molecular analyses, it was possible to classify the plantation trees as *F excelsior*, *F. angustifolia* or hybrid types. Consequently, it was then possible to investigate the flowering phenology of the different ash taxa in the plantations and native populations (Chapter IV).

Fraxinus excelsior and *F. angustifolia* are wind pollinated species (Wallander, 2001), whose pollen can move freely over large distances. It is clear that flowering time could potentially play an important role as a pre-zygotic barrier preventing crosses between native and plantation trees (Levin, 1978; Rathcke and Lacey, 1985; Arnold, 1997). Asynchrony of flowering periods between the two species should also limit possibilities for crosses between plantation and native Irish ash trees. *Fraxinus angustifolia* is known to flower earlier (from mid-December to the end of January) than *F. excelsior* in France (Raquin *et al.*, 2002b; Jato *et al.*, 2004; Gérard *et al.*, 2006b; Gérard *et al.*, 2006c). However, results of the flowering phenology study over two years of observations indicated that flowering time does not constitute a strong barrier to gene flow in the present case (Chapter IV, Figure 4.4).

Individual variation in flowering time was observed within plantations and within native trees. It was possible to classify trees as 'early', 'intermediate', or 'late' flowering, according to their date of flowering onset. This classification was stable over the two years, even though the cold weather observed in the second year seemed to have delayed flowering onset in one of the plantation (Chapter IV, Table 4.4).

The two plantations differed in terms of flowering time. Kildalkey had an earlier flowering time (Chapter IV) and also a higher percentage of hybrids (Chapter III). Cold and dry weather influenced the flowering period, especially trees with an early flowering time, by delaying them. These results indicated that the weather conditions of each year, in combination with genotypic effects, will have an impact on the hybridization rate by influencing the flowering period overlap. Exceptionally hard and cold winters which can delay flowering time in *F. angustifolia* (Jato *et al.*, 2004) might increase the synchronisation of the flowering period and hence the level of gene flow between the hybrid and the native ashes, if flowers are not lost to frosts. In fact, extremely cold winters are probably one of the ecological barriers that prevent *F. angustifolia* from establishing farther north than its present distribution (Fernández-Manjarrés, personal. communication.)

Gérard *et al.* (2006b) desmostrated that flowering phenology could be used as a good indicator for hybrid recognition. In this thesis the majority of the plantation trees with early flowering were identified as hybrids or *F. angustifolia*. However, individuals categorised as hybrid (from Chapter III) showed early flowering, but as well an intermediate onset flowering. Indeed several hybrids individuals were grouped with the native Irish (chapter IV, Figure 4.5). Based hybrid recognition only on phenological data will lead to a underestimation of the number of hybrids. Hence, phenology is a powerful tool for taxon differentiation, but, as with molecular markers, they need to be used in conjunction with each other.

At the time of the study, not all the surveyed plantation trees flowered (only about 50%, although figures varied across years and plantations; Chapter IV, Figure 4. 2). This could be due to the young age of the plantations, or to the fact that trees are planted very close to each other. Within a few years, trees will be thinned, decreasing competition between them, and possibly stimulating more trees to flower. The quantity of pollen produced is an important factor for possible hybridization (Ellstrand, 1992). Therefore, since the plantation trees are likely to start to produce more pollen in the near future, we would expect altered hybridization potential of the alien plants compared to now. It would be interesting to perform another survey in approximately five to ten years time.

7.1.4 Vigour and growth of introduced trees

After the foresters planted the alien trees in the Irish plantations, one of the first indicators of the potential forestry problem, linked to the origin of the trees, was that the trees showed very bad stem-form, generally poor vigour and extensive canker, especially when grown in wet conditions. Since it is known that late frost in spring is mainly responsible for forking in *F. excelsior* (Kerr and Boswell, 2004), and that a test of provenances (Cundall *et al.*, 2003) has shown that trees which tend to flush earlier had also poor growth, I measured the vegetative development of the buds of plantation trees (Chapter IV), compared to the native ones. In general, plantation trees were found to flush earlier than native trees, indicating a possible relationship between forking of plantation trees and time of vegetative onset.

Foresters originally hypothesized that trees with poor growth habits were hybrids or *F. angustifolia* with *F. excelsior.* Surprisingly, using a Spearman correlation (data not shown), the correlation between the percentage of *F. angustifolia* genotype of each tree and size (DBH) was not as expected. Indeed, for the Kildalkey plantation, no correlation was found, indicating that smaller trees were no more likely to be *F. angustifolia* or hybrid types than *F. excelsior*; but for the Clonee plantation a positive and significant correlation (Spearman correlation $r=0.22$, $P<0.01$), was found indicating that hybrid trees with higher percentage of *F. angustifolia* genotype tend to be bigger than trees with high *F. excelsior* percentage. These results suggest that some trees with *F. angustifolia* background might grow well in Ireland, and that, in contrast, provenances of *F. excelsior* from the continent might not be adapted to the Irish environment. To avoid this problem, and especially in forestry, governmental agencies now advise the planting of local provenances.

It is clear that more studies are needed to explain the poor vigour and growth of plantation trees. On one hand, it could be due to the presence of hybrids and *F. angustifolia* among introduced plantations, as originally suspected by foresters, but on the other hand, it may also result from sourcing provenances of *F. excelsior* that are ill–adapted to Irish conditions.

7.1.5 Pollen mediated gene flow between native and alien populations of *F. excelsior* and paternity analysis

Synchrony of flowering period between plant populations is an essential condition for gene flow to occur. However, other pre-zygotic barriers besides flowering asynchrony (Coyne and Orr, 2004; Lowe and Abbott, 2004), such as infertility, could prevent gene flow between plantation trees and native Irish trees. However, *F. excelsior* and *F. angustifolia* are known to be inter-fertile (Raquin *et al.*, 2002a; Raquin *et al.*, 2002b; Fernández-Manjarrés *et al.*, 2006), and their hybrids found in natural populations in France are able to reproduce with their parental species (Gérard *et al.*, 2006b; Gérard *et al.*, 2006c). As demonstrated in Chapter V, plantation trees were found to receive pollen from native trees, but also to act as pollen donors for the native trees. When plantation trees were classified as *F. excelsior*, *F. angustifolia*, or hybrid (Chapter III), LOD scores for paternity analyses indicated that trees assigned as putative hybrids are able to pollinate native trees. Indeed, for Clonee and Kildalkey respectively, 7 out of 18 and 6 out of 10 pollen donors identified as potential fathers of native mother trees had previously been identified as hybrids. Hence, the observations from the dynamics of natural hybrid zones (in France) are somewhat repeated in this case of an artificial introduction in Ireland.

Plantation distances between identified pollen donors and mother trees were high (Chapter V). The maximum observed pollination distance was around 1033m, while the maximum sampled distance between two mother trees was around 1500m. Pollen of wind pollinated trees is known to travel over long distances (Streiff *et al.*, 1999; Burczyk *et al.*, 2004b; Gérard *et al.*, 2006c; Goto *et al.*, 2006; Slavov *et al.*, 2009). Given the results, pollen might have come from outside the study area, but pollen produced from the plantation might also have travelled from far outside the sampling area. In *F. excelsior*, the probability of pollen dispersal has been found to decrease rapidly with the distance from pollen sources (Gérard *et al.*, 2006c; Bâcles and Ennos, 2008). However, Bâcles and Ennos (2008) observed gene flow between two populations which were 2900m apart suggesting that long distance pollination events are possible.

A low but significant structure of the pollen cloud was observed that did not appear to be due to isolation by distance, but more likely due to the differences in flowering time of the surveyed trees (Chapter V, Figure 5.4). This indicates that trees tend to receive pollen from trees having the same flowering period or from plants that started flowering earlier. The number of effective fathers increased during the flowering season (Chapter V, Table 5.4), however Figure 5.4 showed that for Clonee, which was the smallest plantation, the late native pollen cloud remains differentiated. These results might suggest that the increased number of effective fathers was due to the income of external pollen from the study area. The structure of the pollen cloud due to different flowering times indicated that for some years, and more likely for small plantations, the risk of contamination of the native ash by alien trees might be reduced.

7.1.6 Fitness of introduced plantation trees as determined by seed germination capacity, dormancy requirement and quality

Several authors have hypothesized that hybrids will be less fit than their parents (Mayr, 1963; Barton and Hewitt, 1985; Burke *et al.*, 1998). However, other studies aimed at comparing fitness between hybrids and parents have shown the fitness of hybrids to range from less fit to more fit than their parents (Arnold and Hodges, 1995; Arnold, 1997). In Chapter VI, I have shown that seeds from plantation trees were found to express predominantly *F. excelsior* germination characters: deep dormancy, and similar seed size. If seeds from plantation trees were to outcompete the seeds from native *F. excelsior*, in any case, they need to show higher fitness. On the contrary, seeds from introduced plantation trees showed lower fitness characteristics due to insect attacks and a somewhat lower germination rate. Indeed, seeds from plantations were found to be more damaged by insects than seeds produced by native trees. This could be an important factor influencing germination rate and success of seed produced by plantation trees. Higher damage caused by insects to the seeds from non-native trees can have several explanations. It might be due to the low fruiting rate of the plantation trees compared with that of the native trees (insects infected seeds that they found available). Insects could also have a preference for seeds produced from young

trees. These two factors might change with time, but some factors could be related to the adaptive traits of the plantation trees. For instance, the fact that plantation trees tend to flower slightly earlier than native trees means that their seeds will be available earlier for insect infestation. To evaluate the fitness of the plantation trees, more studies will be needed, especially when trees have reached the age of full maturity.

7.2 Implications for control and management of alien plants

7.2.1 Risk assessments

Even though hybridization is not the same as introgression (the latter is a consequence of the former), the results of the present work indicate that the likelihood of gene transfer from introduced plantations to native Irish ash trees is real. This is largely due to the presence of advanced generation hybrids (Chapter III), overlap in the flowering time (Chapter IV), pollen-mediated gene flow (Chapter V), and viability of the seeds (Chapter VI).

In general, the larger the distance between two individuals, the lower the probability that they will mate (Adams and Burczyk, 2000; Smouse and Sork, 2004; Sork and Smouse, 2006). Pollen of *Fraxinus* spp. is able to disperse over large distances (Chapter V; Bâcles *et al.*, 2005; Bâcles and Ennos, 2008). Furthermore, plantations were generally planted near native trees, thereby greatly increasing the chance of gene flow between them. At both the Clonee and Kildalkey sites, *F. excelsior* trees were present in the hedgerows at a maximum distance of 5 metres from the plantation.

The risk of hybridization and rate of introgression of exotic species with native trees depends on the phylogenetic distance between the species involved. *Fraxinus angustifolia* and *F. excelsior* are closely related (Wallander, 2008; Hinsinger, 2010). Besides, several plantation trees are already advanced generations of natural hybrids between them, so pre-zygotic and post-zygotic barriers are likely to be low and may not prevent introgression.

Another source of concern is raised at the intraspecific scale. Indeed, some plantation trees are known to be *F. excelsior,* but from alien provenances. Those trees identified as *F. excelsior* showed, in some cases, poor vigour and bad growth form. Reproductive barriers with local *F. excelsior* populations are not expected in these, and thus high levels of gene flow are likely to occur. In both cases (hybridization at interspecific level or intraspecific levels) there is a high risk of losing local adaptive traits to specific site requirements (Keller *et al.*, 2000), and a risk of disruption of co-adaptive gene complexes.

Around the world, there has been numerous reports in which the introduction of exotic species resulted in a potential invasion and contamination of native populations (Dawson *et al.*, 1996; Ellstrand *et al.*, 1999; Vanden Broeck *et al.*, 2005; Weir and Grant, 2005; Prentis *et al.*, 2007; Burgess *et al.*, 2008). Guidelines should be drawn up to minimise undesirable effects that might result from introgression between plantation and native ash. These guidelines are discussed in the next section.

7.2.2 Management strategies

7.2.2.a Management of introduced plantation trees

Allendorf (2001) stated that for conservation purposes, it is important to distinguish between natural hybrids and anthropogenic hybrids. In Ireland, trees planted in plantations were not artificial hybrids, as often occurs for industrial plantations (Barbour *et al.*, 2003; Potts *et al.*, 2003b; Vanden Broeck *et al.*, 2003a; Barbour *et al.*, 2007; Barbour *et al.*, 2008a), and therefore do not properly fit the definition of anthropogenic hybrids (Allendorf *et al.*, 2001). Plantations were determined (Chapter III) to be composed of a mix of naturally advanced generations of hybrids between *F. angustifolia* and *F. excelsior*, and *F. excelsior* x *F. angustifolia* trees (although in a smaller proportion). Although the hybrids were originally from natural crosses, they were imported and planted far outside their natural distribution (Thomasset *et al.*, 2011b). To my knowledge, the present situation in Ireland appears to be unique, and precise guidelines for management will be difficult to determine.

The mixed nature of the plantations, with different species and hybrid generations presents great challenges for management. From the forester's point of view, the fact that trees do not grow well and are ill-adapted to Irish conditions is a serious economic problem, and also a potential threat to the genetic stocks of Irish native ash. Because it is clear that those plantations will not yield a final commercial crop, the government has introduced a scheme that allows farmers to remove the stock and to replace it with a new crop under the reconstitution grant. For many farmers, such an option is not attractive as they still incur costs associated with removing and replanting trees. In fact, foresters have expressed the wish to be able to identify and therefore target specifically hybrid trees and *F. angustifolia* trees in their plantations, rather than replacing the whole crop. However, the present results indicate that the recognition of hybrids, based only on morphological characters will result in an underestimation of the number of hybrids.

In general, trees were planted in high density, close to each other, and have now reached the size where efficient pruning and thinning is essential (personal observation). First, it will be necessary to prioritise the removal of trees with bad stem form and poor growth, as these are the characters that foresters want to eliminate. Next, foresters should target trees with early flowering for removal, as these might include a majority of hybrids (Chapter IV), however trees with early flowering are potentially the one will represent less risk of interbreeding with native. So removing plantation trees with intermediate and late flowering with unusual morphology (for example presence of brown buds, reduced numbers of leaflets, or low number of teeth) might reduce more efficiently the risk of gene flow. These steps will reduce the quantity and risk of gene flow among native and non-native material, although it will not eradicate it completely. Genetic analyses will help the decision-making, but add further costs as the work is not cheap. Moreover, farmers and foresters will have to spend considerable time on plantations to identify and characterize each tree, on top of having good knowledge about flowering period and morphological characters of *Fraxinus*.

7.2.2.b Policy for future breeding programmes

In Ireland, a programme for genetic improvement of ash trees has been initiated in recent years with a series of collaborative projects involving Teagasc, Coillte and the British and Irish Hardwood Improvement Programme (BIHIP). Management advice for open-pollinated plantations is usually to prevent mating among relatives and to locate plantations far from native resources to minimise gene flow. Adams and Burczyk (2000) reported that, in order to prevent gene flow, it is important to control the size and isolation of the plantation. Potts *et al.* (2001) recommend that an isolation distance between the native source and the exotic plantation of about 1000m is necessary to prevent geneflow. However, in the case of wind pollinated species such as *Fraxinus*, this distance appears to be too small. Indeed, in Chapter V, we observed a cross between trees 1200 m apart and Bâcles and Ennos (2008) even observed an effective dispersal at 2900 m between two neighbouring populations. To collect seeds for a programme of genetic improvement or seed bank collection, it should be advised that seeds from native *F. excelsior* should be collected from trees located at least 10 km away from introduced plantations. For this purpose, a map indicating the location of plantations with suspect alien ash material could be very useful.

In the long term, it is possible to consider managing seed dispersal and seedling growth. First, all plantations were surrounded by an agricultural landscape. Seed dispersal might hence be reduced, as seeds (and or their seedlings) that disperse into fields will be in majority removed or destroyed by agricultural practices. Seedlings will have the opportunity to grow mainly in the hedgerows along fields. Although it is possible to consider removal treatments such as frequent cutting and removing stems by hand, such measures are labour and cost intensive. Moreover, since it is not possible to distinguish between seedlings from native crosses and seedlings issued from the plantations, all of the seedlings will have to be removed. This could eventually create new problems, especially regarding the regeneration of natural Irish stocks. Ecologically, it could be argued that it is best not to intervene with the spread of alien ash trees/genes because ill-adapted trees would be expected to be removed by natural selection. Moreover, so far seeds from the

plantation (Chapter VI) have shown high rates of damage by insects which could reduce the risk of spread. Well-adapted material could also potentially offer novel genetic variation on which natural selection can capitalise.

7.2.3 Future climate change and consequences for Irish plantations

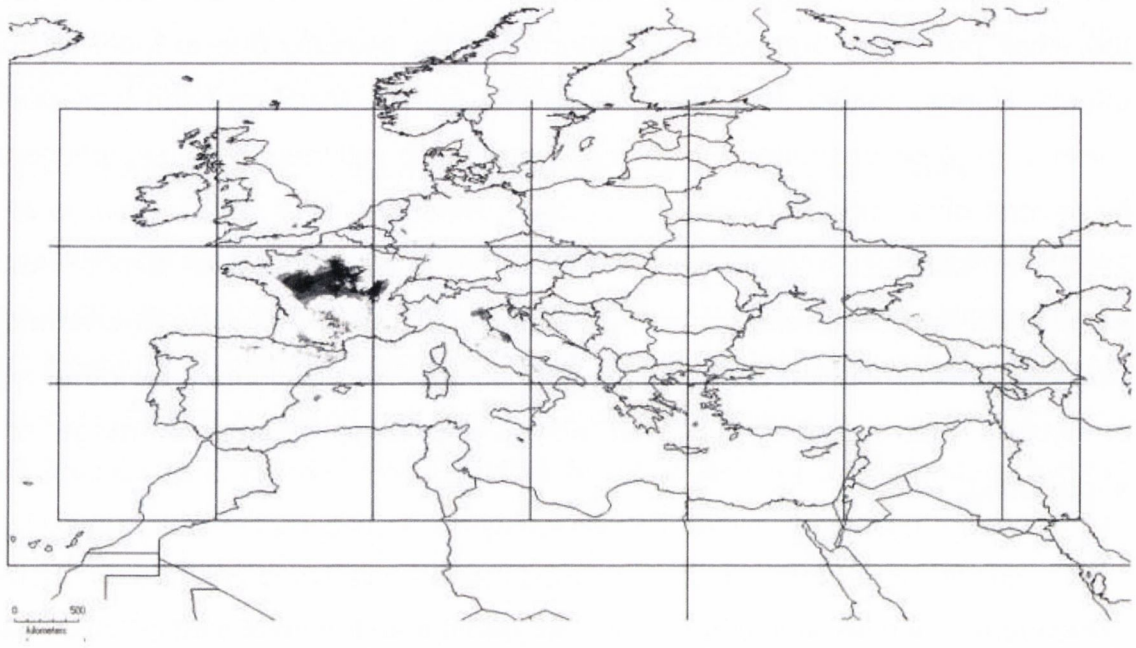
Foresters tend to prevent hybridization and introgression to preserve wild resources, but a conservation programme might on the contrary see hybridization as a way to promote adaptation as discussed above. Given the extent of introduced plants in Ireland, one central question is whether the future Irish climate will change to a climate more suitable for alien ash. In this scenario, hybrid individuals and introduced *F. angustifolia* will have increased chances of establishment, and could also be well adapted to future climatic conditions. Alien ash could be thus seen not as a threat, but as a source of novel genes that could help *F. excelsior* to adapt to changing environmental pressures.

To investigate whether the future Irish climate will be more suitable for *F. angustifolia,* a simulation study of the present and potential niche distribution of *F. angustifolia* from the hybrid zones of the Loire and Saône valleys was undertaken (for details of material and methods see: Thomasset *et al.*, 2011b), using maximum entropy models (Maxent - Phillips *et al.*, 2006). The model was first run to simulate the distribution of hybrids for the present climate and then run a second time to project the present day distribution onto a global warming scenario. The global warming model used in this study was a high resolution mid to strong case scenario of doubled CO_2 ($2xCO_2$, CCM3 model) and other greenhouse gases from pre-industrial times to the year 2100 (Duffy *et al.*, 2003), downloaded from www.diva-gis.org/climate.htm, representing a 2 to 3°C increment in global mean temperatures.

Results of this study indicated that under the current climatic conditions, the potential habitat for hybrids is relatively restricted (Figure 8.1A). However, a projection of the hybrid habitat under this future global warming scenario suggested that c variables suitable for the development of *F. angustifolia* and hybrids would be widespread in northwestern coastal Europe, including the

southeast of Britain. Nevertheless, a potential suitable habitat for *F. angustifolia* or hybrids would not be significantly present in Ireland (Figure 8.1 B). Note, however, the small potentially suitable area around Dublin, possibly due to local warming effects. Hence, neither the Loire type climate nor the Mediterranean type climate seem to be likely scenarios for Ireland, a projection validated by observations and by several other models (Giorgi *et al.*, 2004; Räisänen *et al.*, 2004; Alcamo *et al.*, 2007). In conclusion, the likelihood of an overall favourable climate for *F. angustifolia* in Ireland remains low, at least under the current state of knowledge. However, this simulation is not sufficient to summarise adequately the other types of hybrids that are likely to exist across the large distribution range of *F. angustifolia*.

A.



B.

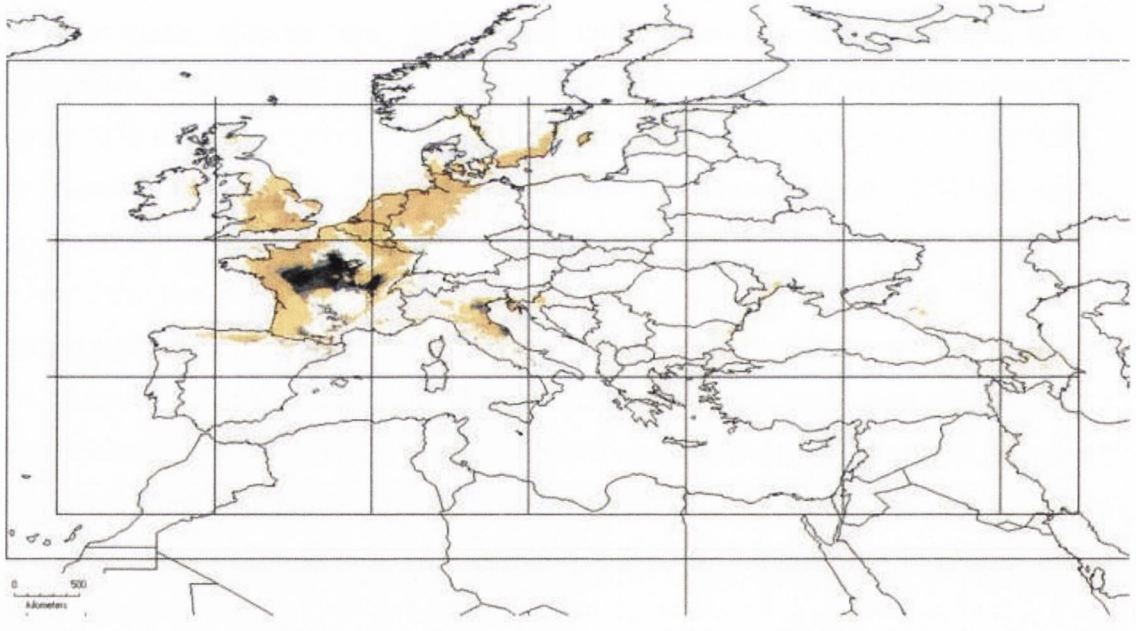


Figure 7.1 Potential niche distributions of *Fraxinus* hybrid zones under current conditions (grey scale) and a future climate scenario (mustard colours). **A.** Maxent simulation of the potential niche distribution of *F. angustifolia* x *F. excelsior* hybrid populations under current climate conditions (average 1950-2000, see text for details), AUC of the simulation=0.997; **B.** Maxent distribution of the potential niche of the hybrid zones under a global warming scenario of doubled CO_2 (from Thomasset *et al.*, 2011b)

7.3 Conclusions and further directions

In this thesis, I aimed to provide a contribution to understanding hybridization in ash and in particular, to improve our knowledge about gene flow and introgression among alien and native ash in Ireland. Evidence gathered from several sources (phenology, parentage studies and seed fitness assessment) indicates that the potential of introgression is real.

However, many questions remain as yet unresolved. For example:

1) What will be the relative fitness of seedlings from crosses between alien trees and native trees in field conditions? To better understand this, factors such as seed dispersal and seedling establishment deserve consideration.

2) Are introduced plantations a continued threat to native gene pools? Regarding the large distribution of native *F. excelsior* in Ireland, it is unknown how quickly genes will introgress and whether they will have any lasting impact. Further studies on genetic diversity in Ireland might help explore this topic. It will also be necessary to monitor the progeny of the plantation trees in the future.

3) How will ash respond to climate change? The analysis on future climate change and how it will affect Ireland's environment reported in this thesis did not take into account how *F. excelsior* will respond to climate change or whether the phenology of the two species would be modified if winters are too become milder in northwestern Europe. If *F. excelsior* was to flower earlier and *F. angustifolia* later in the winter season, the risk of introgression into Irish plantations would be increased. However, this needs to be monitored in Ireland for several years before definite conclusions can be drawn.

4) What potential is there for adaptation in future environments and how is this influenced by hybridization and introgression? The development of new molecular markers (candidate genes Appendix 2) might give important insights into how the genetic basis of the selected traits might influence the fitness of the hybrids and explain the observed differences of ecological niches between *F. angustifolia* and *F. excelsior.*

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Appendix 1

15

Hybridisation, introgression and climate change: a case study of the tree genus *Fraxinus* (Oleaceae)

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Appendix I

Abstract

The distribution of potential hybrid zones depends largely on climate, habitat quality and historical biogeographic factors including dispersal and local extinctions. Global climate change can produce more favourable conditions for certain species to survive in areas that were previously unsuitable for their growth and/or their reproduction, and it may therefore change the potential for their hybridisation with closely related taxa. This chapter discusses general issues of plant hybridisation and invasiveness in the context of global climate change and presents a case study of hybrid ash trees (*Fraxinus excelsior* × *F. angustifolia*) that are mostly geographically separated in their natural range by climate but can have large hybrid zones. In general, both species are temporally separated by flowering times, which occur in early winter for *F. angustifolia* and in early spring for *F. excelsior*. In Ireland, introduced alien ash (*F. angustifolia*, *F. excelsior* × *F. angustifolia* hybrids, and non-native *F. excelsior*) can be found growing in sympatry with native *F. excelsior* populations. It is not known whether alien ash will hybridise with native populations or how climate change, principally in temperature and precipitation, will influence their hybridisation and invasiveness potential. We firstly examine the climate presently associated with known hybrid zones for ash in continental Europe and in Ireland. We then evaluate if a double CO_2 global warming scenario ($2xCO_2$, CCM3 model) would provide improved climatic conditions for hybrids in Ireland and elsewhere. We also present results of phenological observations, which show that flowering periods of alien trees in two Irish plantations overlap with adjacent native trees. Nevertheless, peak pollen release dates for the alien and native populations at these two plantation sites remain separated by at least six weeks.

15.1 Introduction: climate change and hybridisation

Climate and biodiversity have interacted closely since the first appearances of life on earth, *c.* 3.8 billion years ago (Lenton, 2004; IPCC, 2007). For example, changes in temperature, precipitation patterns or prevalence of extreme events can have an impact on species in many ways (Root et al., 2003). They may influence their distribution by altering the suitability of an area for growth and survival. They may create favourable conditions for alien species to survive in areas that were previously unsuitable for their growth and/or reproduction. Conversely, climate change might render an area unsuitable for the survival of a species. Therefore, it can influence the chance that individuals will hybridise, the chance that their progeny will survive, and the chance of introgression of genes among populations.

Appendix I

There are numerous examples of how the geographical range of a species has changed because of climate change, either over geographical timescales or over the most recent human-induced warming period (Tsukada, 1982; Mahoney, 2004; Kuchta and Tan, 2005; Schönswetter et al., 2005). For instance, fossil, palaeoecological and DNA evidence has shown how distributions of species have changed during the most recent glacial episodes (Hewitt, 2000; Kullman, 2008; Liepelt et al., 2009). Furthermore, there is evidence that species are already shifting their ranges in response to current climate change (Parmesan et al., 2005; Root et al., 2005; Lavergne et al., 2006), and that some species are consequently facing extinction (Parmesan, 2006; Pauli et al., 2006; Foden et al., 2007). There is also evidence that hybrid zones are shifting. For example, Buggs (2007) referenced examples in animals (ticks, salamanders, crickets and lizards) where climate fluctuations might have produced hybrid-zone movement. Climate change may also alter the timing of developmental events (phenology) or species' behaviour (Menzel and Fabian, 1999; Visser and Holleman, 2001; Fitter and Fitter, 2002; White et al., 2003). Such changes can have a profound effect on the reproductive biology of species and their evolution.

Climate change may also influence hybridisation among closely related species or populations within species. Hybridisation refers to the mating between individuals of two populations (be they determined as separate species or not) distinguishable by one or more heritable characters (Arnold, 1997). If hybrid progenies backcross with one or more of their parent taxa, the phenomenon is termed introgression. Introgression was first defined as 'the infiltration of germplasm from one species into another, through repeated backcrossing of hybrids with parental species' (Anderson and Hubricht, 1938).

There has been much discussion in the scientific literature about the potential role of hybridisation in evolution. Some biologists believe that hybridisation has no major long-term effect on the evolution of species (Wagner, 1970). However, most biologists now recognise hybridisation as an important evolutionary phenomenon; it is particularly well documented in plants (Stace, 1975; Ellstrand et al., 1999; Ellstrand and Schierenbeck, 2000; Rieseberg et al., 2003; Arnold, 2004; Rieseberg and Willis, 2007). Hybridisation may lead to speciation, increased variability (in hybrid swarm situations), reinforcement of reproductive isolation, or species convergence (Harrison, 1993). Hybridisation can also provide a source of evolutionary novelty (Anderson and Stebbins, 1954). Experimental work on hybridisation in natural populations by Anderson and Hubricht (1938) and Anderson and Stebbins (1954) showed that hybridisation and introgression could be favoured by selection, and both contribute to the development of adaptation of populations to the environment.

Several reviews exist on the topic of hybridisation (Arnold, 1997; Rieseberg, 1997; Rieseberg and Carney, 1998) but few studies have attempted to discuss the

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influence of climate change on this phenomenon. This chapter aims to highlight the influence of climate change (mainly temperature and precipitation) on hybridisation and introgression and provides a case study demonstrating the influence of climate change on the distribution and phenology of two closely related species within the genus *Fraxinus* (Oleaceae) in western Europe.

15.2 Consequences of climate change on hybridisation

15.2.1 Hybrid zone and climate change

Hybridisation can result in a diverse range of evolutionary outcomes. Interbreeding between two different taxa can lead to hybrid-zone formation, introgression, hybrid speciation or species' isolation and divergence. Hybrid zones can occur when previously allopatric taxa or populations come into contact (Lowe et al., 2004). Therefore, climate change can bring previously isolated populations together in geographical space. Hybrid zones can be short-lived (ephemeral) when the hybrids, and genes transferred via them, are removed by selection and have no lasting effect on the parental populations via introgression. However, hybrid zones can also be long-lasting and of more consequence to the evolution of the plant populations because there is permanent introgression of genes among species (Anderson and Hubricht, 1938).

A consequence of hybridisation may be the disappearance of the hybrid zone by fusion of different species into one, a process also called 'speciation in reverse' (Taylor et al., 2006). Hybridisation may also lead to the formation of new species, and this process has been documented for the origin of many species, especially in plants (Rieseberg, 1997; Otto and Whitton, 2000; Ramsey and Schemske, 2002). Hybrids that are isolated by reproductive barriers can become fertile new species that are reproductively isolated from their parental species (Avise, 2004). There are two main mechanisms of such hybrid speciation. The first one that can be detected easily is allopolyploidy (Levin, 2002). Winge (1917) was the first to report that hybridisation, accompanied by duplication of chromosome sets, can create a new stable species in plants. The hybrid population could then become isolated from the parental taxa. In the second mechanism, hybridisation can create new species without a change in ploidy level, and this is known as homoploid hybrid speciation. Some annual species of the genus *Helianthus* provide well-studied examples of this mode of speciation (Rieseberg, 2006), as do species of *Tragopogon* (Soltis and Soltis, 2009).

Hybridisation may lead to the extinction of one of the parental species, particularly in the case of rare species with small population size (Levin et al., 1996).

It may also be a mechanism of facilitating the invasion of introduced species. Pollen-mediated gene flow from exotic plant species can result in the introgression of exotic genes into native gene pools and lead to the dilution or contamination of 'pure' native species (Raybould and Gray, 1994; Rhymer and Simberloff, 1996; Schierenbeck et al., 2005; Chapman and Burke, 2006; Currat et al., 2008). Such gene flow might be a real threat for many native plant species around the world and could be exacerbated by climate change, especially if hybrids are more competitive and reproductively successful (Vila et al., 2000).

However, interbreeding between distinct species or populations can result in enhanced genetic diversity (Rieseberg et al., 1999) and novel genetic combinations (Anderson, 1954; Arnold, 2004; Seehausen, 2004). Hybrids can have phenotypes that are extreme variants to those of either parental line, a phenomenon called transgressive segregation (Rieseberg et al., 1999). By generating individuals that exceed parental phenotypic values, transgressive segregation could give hybrid individuals the opportunity to access new ecological niches such as those that may be created through changing climatic conditions. Furthermore, when hybrids are fertile, repeated backcrossing can result in introgression and the exchange of genetic material from one species to another (Anderson and Stebbins, 1954). This may increase genetic diversity, providing raw material for novel adaptations required for new environments. Favourable alleles may spread efficiently from one species or population to another through introgression (Rieseberg and Burke, 2001). Therefore small populations, which often show a low genetic diversity due to the effects of genetic drift and inbreeding (Young et al., 1996), may well benefit from influxes of new genetic material through hybridisation and introgression. In the context of global warming, high genetic diversity may help species to respond to climatic variation and adapt to the new conditions. For example, Savolainen et al. (2007) showed that the potential for tree adaptation to climate change depends on genetic variation, dispersal and establishment rate.

15.2.2 Influence of climate change on barriers to hybridisation

The degree of gene flow between two populations, or the probability of hybrid formation between two taxa, is influenced by reproductive isolation mechanisms (Levin, 1978). These can be divided into two types, based on whether they act before (prezygotic) or after (postzygotic) fertilisation (Rieseberg and Carney, 1998).

Postzygotic barriers act after zygote formation by decreasing viability or fertility of the hybrids. They can occur via several mechanisms, including genomic incompatibility, hybrid inviability or sterility. Hybrid inviability also includes situations where hybrids are viable but ill-adapted for survival. Postzygotic mechanisms can be either intrinsic or extrinsic (Rieseberg and Carney, 1998). Intrinsic postzygotic mechanisms are the consequence of genetic incompatibility or negative epistasis

between alleles from different species (Dobzhansky, 1937). Intrinsic postzygotic isolation can occur at different levels: non-viability of the first-generation hybrids (F1), sterility of F1 hybrids and hybrid depression that can affect viability and fertility beyond the first hybrid generation (Coyne and Orr, 2004). Such factors are not likely to be greatly influenced by climate change. However, extrinsic postzygotic isolation, or exogenous selection, could be influenced significantly by climate. In such cases, hybrids can show phenotypes which are ill-adapted to the parental environment (Coyne and Orr, 2004; Lowe et al., 2004). Under future climatic scenarios, the ecological tolerance of a species might change. For example, Ross and Harrison (2006) showed that egg viability and survival over different winter climate regimes plays an important role in determining the distribution of two cricket species (*Gryllus firmus* Scudder S. H., 1902, and *G. pennsylvanicus* Burmeister, 1838) and the position of their hybrid zone.

Premating or prezygotic isolation mechanisms limit hybrid formation before fertilisation and can, again, be due to intrinsic and extrinsic factors. An incompatibility of reproductive organs (mechanical isolation) can act as a barrier. For example, within many insects, the male and female copulatory organs of closely related species do not fit together, preventing sperm transfer (Sota and Volger, 2001). Intrinsic isolation may also be due to gametic isolation, either by gametic incompatibility (e.g. intrinsic defects causing physiological or biochemical recognition of gametes during fertilisation - Lewis and Crowe, 1958), or by gametic competition, also, in plants, called pollen competition (see Howard, 1999).

Prezygotic barriers to hybridisation are arguably more likely to be influenced by climate change. They can be temporal (including phenological) or ecological in origin. Temporal isolation is related to a time delay of reproductive events. It can refer to reproductive seasons as well as the timing of the release of gametes. In plant species, this mechanism involves different flowering periods (Broeck et al., 2003; Gerard et al., 2006a, 2006b; Pascarella, 2007; Donnelly et al., Chapter 8) or a delay in the different steps of flowering: flowering peak or period of pollination (Schluter, 2001; Weis and Kossler, 2004). Species with overlapping flowering time have a greater opportunity to interact. The timing of reproductive events is thus an important mechanism for maintaining species coexistence in diverse plant communities (Rathcke and Lacey, 1985). Recent studies have shown a change of flowering time (mainly earlier spring phenology) in several angiosperms, which is significantly correlated with global warming (Fitter and Fitter, 2002; Menzel et al., 2006).

15.3 Detection of hybridisation

While some studies have used only molecular markers to identify and characterise hybridisation (Khasa et al., 2005; Valbuena-Carabanã et al., 2005; Coyer et al.,

2007; Mebert, 2008; Wachowiak and Prus-Glowacki, 2008), it has often been necessary to combine chromosome, molecular DNA and morphological markers for that purpose (Perron and Bousquet, 1997; Hodkinson et al., 2002; Kelleher et al., 2005; Ebina and Otho, 2006; Curtu et al., 2007; Conceição et al., 2008). Hybrids with a level of ploidy different from their parents can be detected readily by examination of their chromosome number (Carroll and Borrill, 1965). Molecular cytogenetic approaches such as fluorescent in-situ hybridisation (FISH) and genome in-situ hybridisation (GISH) have also proven useful in this respect (Leitch and Bennett, 1997; Hodkinson et al., 2002).

When chromosome studies are not suitable or convenient, hybrids have generally been identified based on morphological or ecological attributes (Stace, 1975). However, Allendorf et al. (2001) argued that detection of hybrids based on morphological traits can be difficult. Hybrids do not necessarily present intermediate morphology, because they often express a mosaic of parental phenotypes. Moreover, in the presence of a population of hybrids with varying numbers of generations of backcrossing with parental species, or among hybrids (hybrid swarms), distinguishing between hybrids and parental taxa becomes a challenging task.

Molecular DNA markers can provide fast and efficient tools to study hybridisation and are often the method of choice for hybridisation investigations. A variety of methods including allozymes, restriction fragment length polymorphism (RFLP), microsatellites (SSRs) or DNA sequence analysis are now available to study species' boundaries and hybrids (Lowe et al., 2004). While uniparentally inherited markers (i.e. chloroplast DNA or mitochondrial DNA) can be used to study differential parental contribution, biparental inherited markers can be used to observe genetic differentiation between species or to examine the genetic profile of hybrids which combine the specific markers of their putative parents.

15.4 Natural hybridisation in the genus *Fraxinus*: a case study

15.4.1 Hybridisation in *Fraxinus*

The genus *Fraxinus* L. is one of 24 genera in the olive family, Oleaceae (Wallander and Albert, 2000). *Fraxinus excelsior* L. (common ash) and *F. angustifolia* Vahl. (narrow-leaved ash) are native tree species of western Europe, with important economic value for the production of timber (Horgan et al., 2004). Despite the fact that they are generally geographically isolated because of different climate preferences, they are also known to form viable hybrids (Gerard et al., 2006c) and their distributions overlap in several areas, potentially producing different hybrid zones (Fig 15.1). As climate is predicted to change towards warmer conditions, the most southern *Fraxinus* species, *F. angustifolia*, could, for example, have the potential

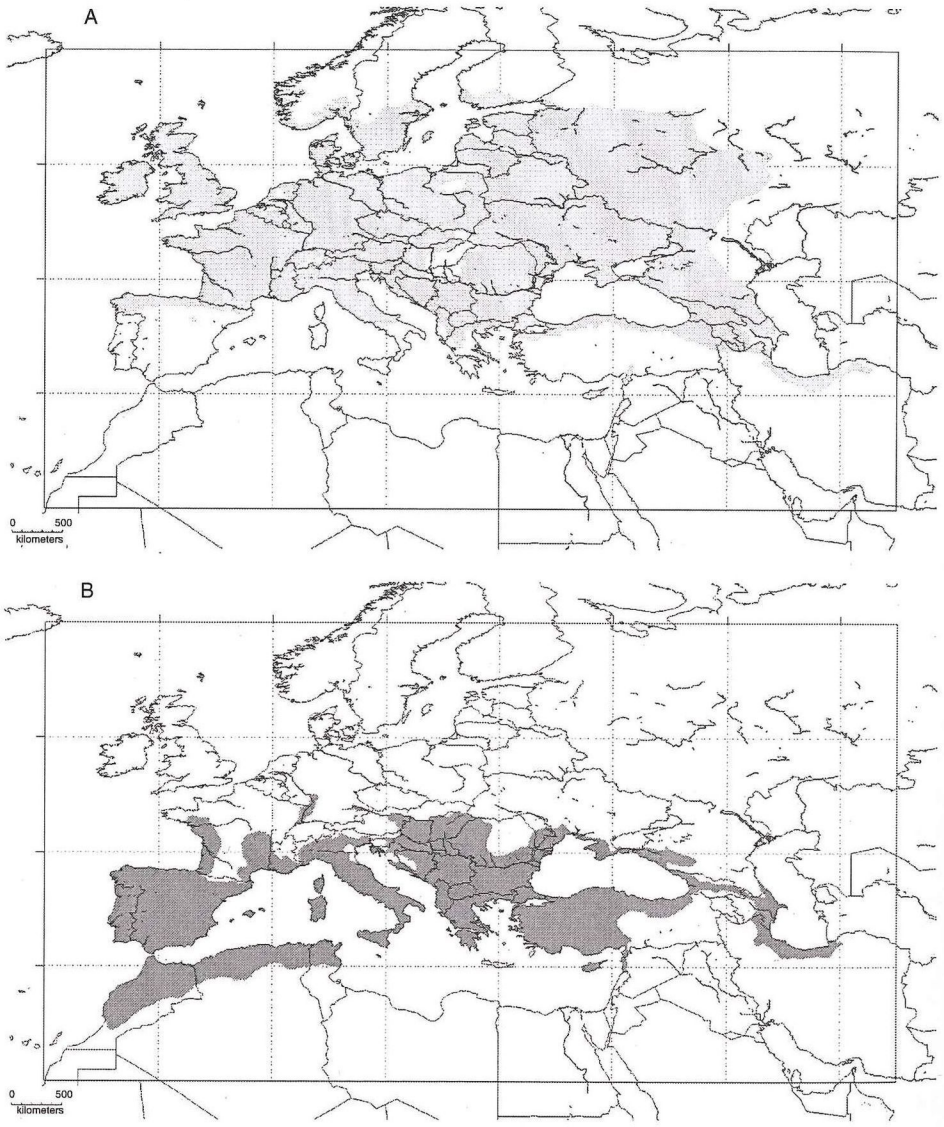

Figure 15.1 General distribution of *F. excelsior* and *F. angustifolia*. (A) Distribution map of *F. excelsior*, plotted with the shape file provided with permission by EUFORGEN. (B) Approximate distribution of *F. angustifolia*, based on records of our own research, literature and public databases (www.gbif.org).

to shift its range northwards and increase the potential hybridisation zone with *F. excelsior*.

Here we review our current knowledge about hybridisation in ash and analyse present and future climate data at the hybrid zones of *F. angustifolia* and

F. excelsior to predict the potential impact of climate warming on these species. We focus on the potential future climate of Ireland under a global warming scenario ($2xCO_2$ climatic conditions, CCM3 model) to evaluate whether accidentally introduced hybrids could find a climate resembling that of their geographical origin. Finally, we present data on the phenology of the two species and their hybrids to assess how much their flowering periods currently overlap in Ireland when they are grown together artificially in plantations.

15.4.2 Extent of hybridisation and introgression in *Fraxinus*

Fraxinus excelsior is mainly present in central and northern Europe and is gradually replaced towards the Mediterranean basin by *F. angustifolia* (Fig 15.1). The present-day distribution of these two species indicates a contact zone where hybridisation has been suspected for many years (Picard, 1983). In France, the extent of hybridisation between the two species has been analysed, and evidence for hybridisation between them obtained from: (1) plants showing intermediate morphological characteristics (Rameau et al., 1989); (2) experimental crosses which show that the two parental species are compatible (Morand-Prieur et al., 2002; Raquin et al., 2002); (3) molecular markers (Jeandroz et al., 1996; Heuertz et al., 2001; Heuertz et al., 2004); and (4) a combination of molecular and physiological (based on seed dormancy) markers (Gerard et al., 2006c).

Molecular analyses show that *Fraxinus* chloroplast DNA haplotypes largely overlap in western Europe, confirming the occurrence of historical interspecies gene flow (Heuertz et al., 2006). The lack of a clear separation between plastid haplotypes according to species suggests that hybridisation and introgression have been widespread. Analyses with nuclear microsatellites and multivariate morphological characters suggested differential patterns of hybridisation in the Loire and the Saône valleys in France (Fernández-Manjarrés et al., 2006). The mild climatic conditions in the Loire appear to promote introgression of morphological characters and molecular markers of *F. angustifolia* into *F. excelsior*, while the most continental areas of the Saône appear to allow mostly molecular introgression between species.

15.4.3 Climate and hybridisation *in Fraxinus*

The distribution of the two species and their hybrids is linked to ecological variables; climate is a particularly important factor in determining hybridisation potential (Fernández-Manjarrés et al., 2006). Within a sympatric population in the Loire valley, Gerard et al. (2006a) assessed the role of floral phenology in restricting gene flow at a local scale. Reproductive events mainly occurred between co-flowering trees, and pollen flow appeared to be asymmetric. For early-flowering hybrids, male and female reproductive success was high, and they were producing more flowers and fruits than later-flowering hybrids. Moreover, the authors found

relatively high selfing rates, indicating that hybrids may have reproductive assurance resulting in higher fitness in this type of intermediate ecotone.

Physiological studies also show that the two species have different responses and tolerances to summer temperature stresses, as *F. excelsior* relies on malate (Patonnier et al., 1999; Marigo et al., 2000) and *F. angustifolia* on mannitol accumulation (Oddo et al., 2002) to cope with water deficiencies. However, the growth of *F. angustifolia* depends greatly on water availability, a feature not seen as markedly in *F. excelsior* (Marigo et al., 2000). In winter, frost damages the flowers of *Fraxinus* and limits its most northerly distribution. However, *F. angustifolia* can survive as an adult tree in colder areas than its native range, as it is often seen in parks throughout Europe, although it seldom sets and disperses seeds successfully. Indeed, the capability of *F. angustifolia* to produce hybrids with *F. excelsior* in hybrid zones is variable across years, as frost patterns can induce a late second flowering period of *F. angustifolia*, allowing the pollen of this species to potentially sire seeds of *F. excelsior* (personal observation).

15.4.4 Alien ash in northwest Europe

Ireland is a good place to study the potential expansion (invasion) of *F. angustifolia* to more northerly and western regions of Europe. *Fraxinus angustifolia* is not native to Ireland but has been planted either deliberately or accidentally, principally by foresters and horticulturalists. There are therefore several study sites with a mix of ash species that can be used to study the potential impact that invading *F. angustifolia* or hybrid populations will have on Irish *F. excelsior* as a result of range shifts arising from global warming. These sites came into existence by accident, because the Irish government introduced substantial planting grants in 1992 for farmers to plant hardwood trees. Today, there are at least 100 afforested sites in which suspected alien ash trees were planted between 1992 and 2000.

Given the extent of introduced plants in Ireland, one central question regarding the potential invasiveness of hybrid material is whether future Irish climate will change to resemble the climate of continental *F. angustifolia*. If that is the case, hybrid individuals and any introduced *F. angustifolia* will have increased chances of establishing, hybridising and introgressing with native Irish ash. To investigate this, we simulated the present and potential niche distribution of *F. angustifolia* from the hybrid zones of the Loire and Saône valleys using maximum entropy models (Maxent - Phillips et al., 2006) of species distributions. A large-scale analysis of *F. angustifolia* and *F. excelsior* (Fernández-Manjarrés et al., submitted) indicates that six variables are highly explanatory of their distribution: (1) temperature of the coldest month; (2) temperature of the warmest month; (3) annual temperature variance; (4) precipitation of the warmest quarter; (5) precipitation of the coldest quarter; and (6) precipitation variability.

We used 706 geolocalised localities obtained from our own research and the public database GBIF (www.gibf.org) for which the six climate variables data listed above were extracted. The climate database (Hijmans et al., 2005) has a spatial resolution of 2.5 minutes and contains climate data obtained from several world meteorological stations covering the period 1950–2000. Adequacy of the distribution model produced by Maxent was verified using the area under the curve (AUC) of a receiver operating characteristic (ROC) plot (Fielding and Bell, 1997). Firstly, the Maxent model was run with default settings (50% of the data used at random to train the model, convergence threshold 10–5, maximum iterations 500, and automatic regularisation value) to simulate the distribution of hybrids for the present climate. Next, this present-day distribution was projected by Maxent onto a global warming scenario. The global warming model used was a high-resolution mid to strong case scenario of doubled CO_2 ($2xCO_2$, CCM3 model) and other greenhouse gases from pre-industrial times to the year 2100 (e.g. CO_2 concentration of 710 ppmv and CH_4 concentration of 2538 ppbv) (Duffy et al., 2003) downloaded from www.diva-gis.org/climate.htm. This represents a 2–3 °C increment in global mean temperatures.

Simulation results for current climate for the two hybrid zones (Loire and Saône) indicated that the potential habitat for hybrids was relatively restricted in these areas (Fig 15.2A). However, projection of the hybrid habitat under the future global warming scenario suggested that weather variables suitable for the development of *F. angustifolia* and hybrids would be widespread in northwestern coastal Europe, including the southeast of Britain. Nevertheless, a potential suitable habitat for *F. angustifolia* or hybrids would not be significantly present in Ireland (Fig 15.2B); but note the small area of suitability around Dublin, possibly due to local warming effects.

Hence, neither the Loire-type climate nor the Mediterranean-type climate seems to be a likely scenario for Ireland, a projection validated with observations and several other models (Giorgi et al., 2004; Räisänen et al., 2004; Alcamo et al., 2007). For example, temperatures are increasing more in winter than in summer and mean winter precipitation is increasing in most of the Atlantic and northern Europe (Jones and Moberg, 2003). Hence, the likelihood of an overall favourable climate for *F. angustifolia* in Ireland remains low, at least under the current state of knowledge.

The present analysis, however, does not take into account how *F. excelsior* will respond to climate change, or if phenology of the two species would be modified if winters become milder in northwestern Europe. If *F. excelsior* flowers earlier and *F. angustifolia* flowers later in the winter season, the risk of introgression into Irish plantations would be increased. Most field observations show that during mild winters, *F. angustifolia* completes its flowering in late December/early January, long before the spring flowering of *F.* excelsior (Jato et al., 2004). However, this

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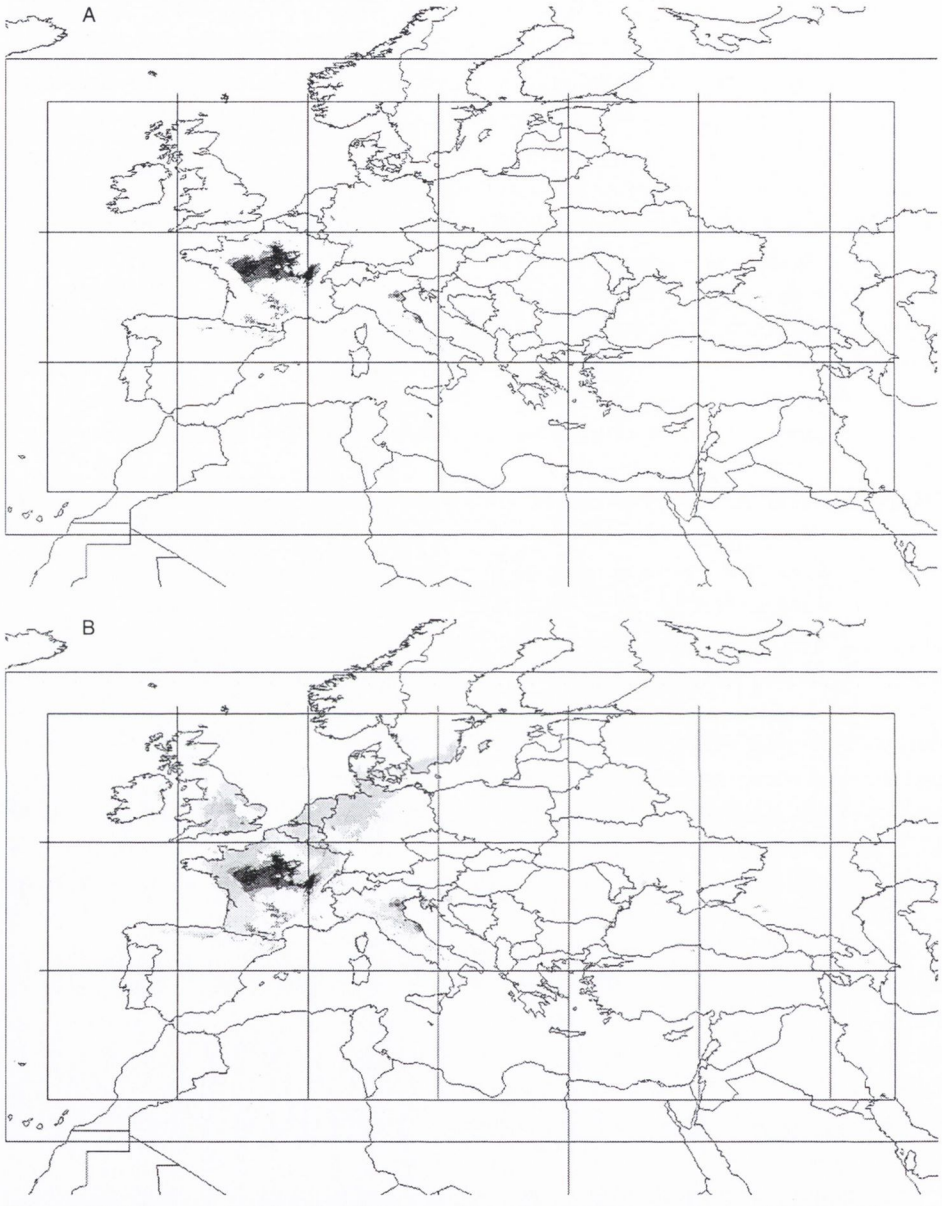

Figure 15.2 Potential niche distributions of *Fraxinus* under current conditions and a future climate scenario. (A) Maxent simulation of the potential niche distribution of *F. angustifolia* hybrid populations under current climate conditions (average 1950–2000, see text for details); AUC of the simulation = 0.997. (B) Maxent distribution of the potential niche of *F. angustifolia* under a global warming scenario of doubled CO_2. *See colour plate section.*

needs to be monitored in Ireland for a number of years before definite conclusions can be drawn. Similarly, our analysis would be insufficient to summarise adequately the other types of hybrids that are likely to exist across the large distribution range; but to the best of our knowledge, the provenances introduced to Ireland are likely to be from the Saône and Alsace region.

15.4.5 Phenology and climate change in *Fraxinus*

We also wanted to evaluate the risk of hybridisation and introgression of non-native *Fraxinus* with native ash populations under current conditions. We wanted to know if prezygotic barriers to hybridisation existed between *F. excelsior* and non-*excelsior* types. Highly appropriate study sites for such research are available in Ireland because plantations containing non-native *F. angustifolia* and their hybrids exist that are adjacent to native *F. excelsior* woodlands. These trees are now mature enough to flower.

We recorded the phenology of trees during spring 2008 at two alien ash sites, Greenan in County Wicklow (8 ha planted in 1996) and Kildalkey in County Meath (20 ha planted in 1999). Thirty-two trees (Greenan) and 24 trees (Kildalkey) that had previously been selected on the basis of their morphological characteristics as '*excelsior* type', '*angustifolia* type' or 'hybrid type' were visited to record flowering phenology. Recordings were also made on 30 native *F. excelsior* trees growing around each plantation. Observations of flowering phenology were recorded from January to May 2008, every 8–10 days. Flower phenology was scored using a scale of 0 to 5 (0 for the dormant flower bud stage to 5 for the formation of seed stage – Fig 15.3).

At the Greenan site, 93% of native trees showed full flowering, whereas 62% of the alien trees flowered. Our observations showed that the native trees flowered later than the alien ash. The beginning of the pollen release was earlier for the selected hybrid type and *angustifolia* type trees inside the plantation compared to the native trees (Fig 15.4A). However, the trees that were selected as *excelsior* type showed a pattern similar to the native trees. The peak time of flowering for the selected trees of hybrid type and *angustifolia* type was around 10 March, whereas for native *F. excelsior* the peak was at the end of March (Fig 15.4A). A considerable overlap was found to exist in the flowering times of the native trees and the imported trees.

At the Kildalkey site, the proportion of flowering for the native trees was 97%, compared to 63% for the imported trees. As at Greenan, we observed that the beginning of the pollen release and the moment where the female became receptive was earlier in the selected hybrid type trees than in the native trees (Fig 15.4B). However, in one out of 30 native *F. excelsior* trees, the flower buds started to flush at the end of February, producing a fully exposed inflorescence (stage 2.5 – Fig 15.3).

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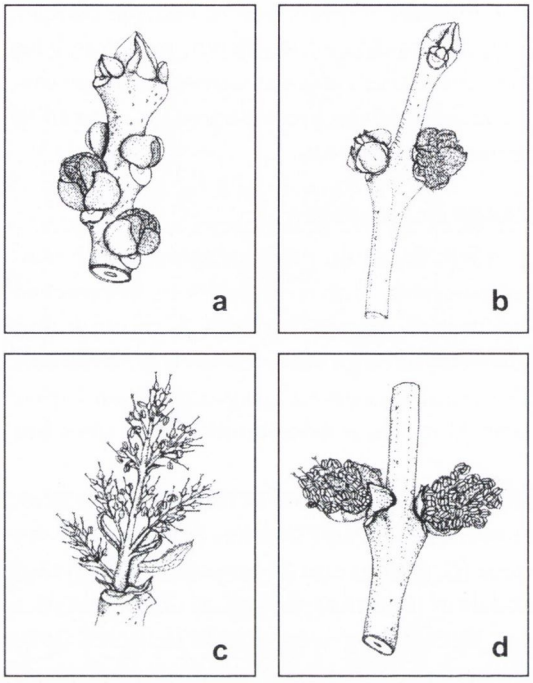

Figure 15.3 Stages and score notation of *Fraxinus* inflorescences and flowers: (a) stage 1 and 2 – flushing starts and first inflorescences are visible but are mainly covered by the bracts; (b) stage 2.5 – bracts fallen, completely visible inflorescences; (c) stage 3 – first pollen release and stigmas are receptive; (d) stage 4 – end of pollen release.

The timing of peak flowering for the alien trees and the native trees of *F. excelsior* was approximately the same (i.e. from the end of March to the middle of April).

Therefore the flowering periods (when pollen is released and stigmas are receptive) of the alien *angustifolia* type trees and native trees were found to overlap for approximately six weeks. This indicated the potential for pollen transfer and interbreeding between the native trees and the aliens. Our results do not project what will happen under future climate scenarios but do demonstrate that synchronous flowering can occur with a high proportion of the material under current conditions.

Fitter and Fitter (2002) showed that of six pairs of species, all of which form natural hybrids, four showed more synchronous flowering under present-day conditions than in the past, promoting the probability of hybridisation. Change in spring phenology in response to climate warming across the northern hemisphere, including Ireland, is especially well documented (Parmesan and Yohe, 2003; Root et al., 2003; Donnelly et al., 2006). In temperate climates, temperature is one of the

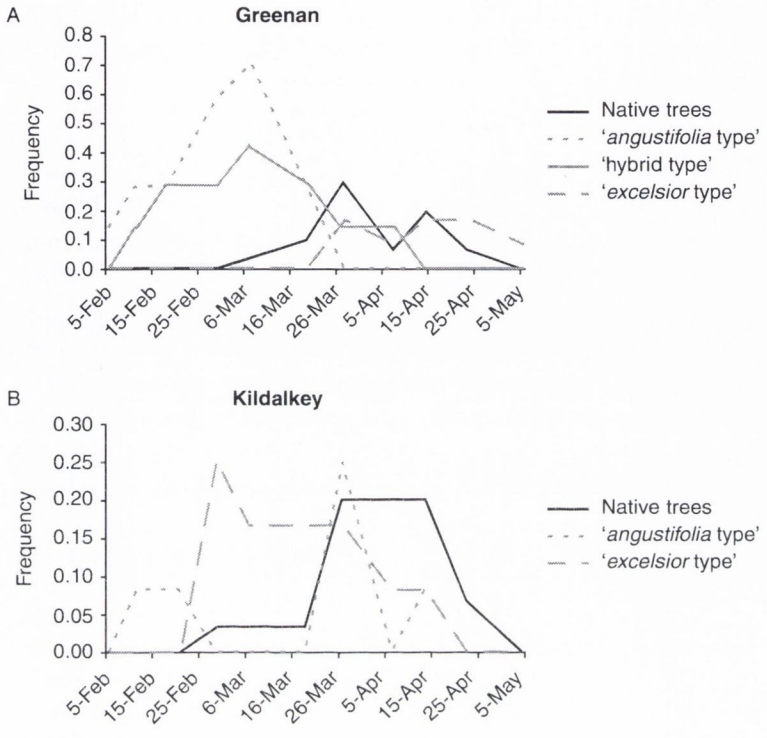

Figure 15.4 Frequency of trees reaching flowering stage 2.5 at (A) the Greenan site and (B) the Kildalkey site. Stage 2.5 represents the stage just before pollen release and receptivity of the stigmas.

most important factors influencing the timing of bud dormancy release and tree flushing (Jato et al., 2004; Vitasse et al., 2009). For *F. angustifolia*, Jato et al. (2004) have shown that temperature during the months prior to flowering determines the date of the pollen season. Maximum temperatures in December are critical to accumulate the required amount of heat to break dormancy, and temperatures below 0 °C in December will lead to a delay in flowering time. In *F. excelsior* the timing of spring leaf flush is also influenced by winter chilling and warm temperature (Cottignies, 1986; Jouve et al., 2007). Given predicted increases in winter temperature under global warming, *F. excelsior* could have an earlier start to its flowering. In Ireland, under future climatic predictions, both species may start flowering earlier, but the period of overlap found between the alien trees and the native trees may still be maintained. However, *F. excelsior* was not found to flower significantly earlier during the last 30 years in a study by van Vliet et al. (2002). Moreover, *F. angustifolia* is highly sensitive to frost (Jato et al., 2004), and an early start will expose it to increased risk of frost damage. In our case, frost damage was

observed on some early-flowering alien trees, and the buds were dying before pollen was released (data not shown). If winter frosts become more prevalent, hybridisation between native trees and alien trees may become less likely.

15.5 Conclusions

Few studies have examined the impact of climate change on hybridisation and introgression. We have reviewed several ways in which climate can influence hybridisation in plants. The most significant impacts are likely to involve range shifts of parental species/populations and changes in phenology. We have presented a case study of the tree genus *Fraxinus* showing that the future climate in Ireland is not likely to be more favourable than the present to *F. angustifolia* and its hybrids with *F. excelsior*. However, the evolution of the phenology of *F. excelsior* and the alien trees is unknown under future climatic conditions in Ireland. Overlap in flowering time was found between alien and native trees, leading to the opportunity for interbreeding. More studies on gene flow using molecular markers and seed germination capacity are in progress.

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Appendix 2

Appendix 2

A set of primers for candidate genes potentially involved in ecotypic variation in *Fraxinus excelsior*, *F. angustifolia* and their hybrids

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Abstract

From a total of 8030 ESTs, we developed 12 primer pairs for 11 genes potentially involved in ecotypic variation in *Fraxinus excelsior*, *F. angustifolia* and their hybrids. The candidate genes are for functional traits associated with abiotic stress (e.g. drought resistance, water regulation, cold acclimatization) and flowering phenology (altering, inhibiting or promoting flowering time). Four out of our twelve primer sets are useful for detecting polymorphism among and within species.

Introduction

Fraxinus angustifolia (Vahl) and *F. excelsior* L. grow in sympatry in several locations within Europe and are known to hybridize (Jeandroz *et al.*, 1996; Raquin *et al.*, 2002a; Fernández-Manjarrés *et al.*, 2006). The two ash species show pronounced ecological differentiation with different climatic and edaphic requirements (Patonnier *et al.*, 1999; Marigo *et al.*, 2000; Thomasset *et al.*, 2011b). Physiological studies have shown that *F. excelsior* and *F. angustifolia* respond differently to temperature stress during the summer: *F. angustifolia* relies on malate to respond to water deficiency (Marigo *et al.*, 2000 1999), whereas *F. excelsior* tends to rely more on the accumulation of mannitol (Oddo *et al.*, 2002). *Fraxinus angustifolia* is frequently located close to waterways and is resistant to flooding (Jaeger *et al.*, 2009). In contrast *F. excelsior* is mostly located in areas with well-drained soils or on slopes. They have different leaf and bud morphology (Fernández-Manjarrés *et al.*, 2006; Thomasset *et al.*, 2011a) and have differing flowering time: *F. excelsior* flowers later than *F. angustifolia* (Raquin *et al.*, 2002a; Jato *et al.*, 2004).

DNA markers are required to help understand the adaptation of *Fraxinus* to their specific habitats and niches and to improve our knowledge of *F. excelsior* x *F. angustifolia* hybrid zones. Moreover, the markers are required to evaluate adaptive genetic variation that is of use to applications in forest ecology and management. Ultimately, such information can also be of use to breeders who attempt to modify germplasm for forestry.

In this paper, we identified 11 candidate genes directly associated with abiotic stress resistance and flowering phenology. We designed 12 pairs of primers to amplify these regions. We tested the utility of the markers on DNA extracted from *F. angustifolia*, *F. excelsior* and their hybrids from different populations across Europe.

Materials and methods

To identify putative candidate genes, we searched GenBank for genes already known to be associated with flowering phenology, drought tolerance (including those involved in the regulatory pathway of the mannitol and malate) and other abiotic and biotic stress responses. We selected over 40 candidate genes using nucleotide sequences mainly from *Arabidopsis thaliana*. These putative gene sequences were downloaded from GenBank.

An EST (expressed sequence tag) bank has been developed from dormant and swelling bud tissues of *F. excelsior* in the EC Project *'EVOLTREE'* by the CBIB (Centre de Bioinformatique de Bordeaux). A total of 8030 ESTs were obtained from this resource for comparison to our candidate genes.

We ran a tBLASTn search of our candidate genes against the EST database using the blast tool available on BioEdit version 5.0.6. After initial searches, we narrowed our candidates to 21 genes. The EST sequences were then used to design primers for amplification of these regions in *Fraxinus* using the primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were chosen to yield amplification products of up to 1000 basepairs (bp).

Amplification of the markers was first assessed on one *F. excelsior* individual and annealing temperatures were optimised. Primer annealing temperature was assessed using a temperature gradient between 55 and 65°C. PCR products were evaluated using 1.2% agarose gel, nine of our primer pairs did not produce specific or reliable amplification and were discarded for further analysis. The twelve primer pairs that remained showed specific amplification for 11 genes (Table 1) and these were tested on *F. angustifolia* individuals to see if they were transferable to this species.

Appendix II

Table 1 Eleven candidate genes potentially involved in adaptive variation in *F. excelsior* and *F. angustifolia.*

Gene	Candidate for	Annealing temperature (°c)	Primer sequences
Supressor_Frigida(SUF4)	Flowering phenology	59	F: CAGGTCCACAAGGAGCAAGT R: TGTTGTGACATTGGTGCAGA
PhytochromeB	Flowering phenology	59	F: GCCGGGGATGAGAGATAGAT R: GATAAATGCAGTGCGCCATA
Calmodulin	Flowering phenology	59	F: GAAGCGCATAATGTCGGAAT R: TCATCATCTTCTTGAATTCTTCG
ABA HYPERSENSITIVE 1 (ABH1)	Flowering phenology	59	F: TGGAAAGAGAAGTGCGTTTGT R: TGACCGTACCATATCTCTCCAA
GA20ox (Gibberellins)	Flowering phenology	62	F: AGGGTGTTTCATTGGATTGG R: CATGGATAAAGTGGGGGATG
Abscisic acid insensitivity 1B	Flowering phenology	62	F: AACAGCTTCAACAGCAGTTCTC R: GACATGGCCAAGACACCTC
OSMOTIN1	Abiotic stress response	59	F: TAGTGTCCCTTTGGGCAATC R: CGTCAACGTTTGAGATGTCG
OSMOTIN2	Abiotic stress response	59	F: TTGCCCTTAATCAGCCAAAT R: GATCACGGGCAGAAGACAAC
Mannitol-dehydrogenase-transporter	Abiotic stress response	59	F: CAGTTGGATTCACCAAAACAAT R: CACTTGAAGAAAGTGCCAAACA
Mannitol-dehydrogenase	Abiotic stress response	59	F: TAGCTTGCCGAAGAAGATGC R: AGGTTCCGAACAGAAGCTCA
Malate-dehydrogenase	Abiotic stress response	62	F: GTTAGGGCCAAGACATTCTACG R: AACACCCAAAGATGTGCAAAAT
Dehydrin	Abiotic stress response	55	F: TCTGGAGAACACAAGACTCAGG R: TTTATCCAGGAACCCCTTCTC

F: Forward; R: Reverse

We amplified fragments of the 11 chosen genes for 16 *F. angustifolia* and 16 *F. excelsior* from different provenances across Europe (Table 2). Total DNAs were extracted using dried leaf material and the DNeasy® 96 Plant Kit (QIAGEN) as outlined in Thomasset *et al.* (2011a). PCR used a total mix of 20 µL, containing 2 µL of DNA (between 10 to 50 ng.µl^{-1}), 100 µM dNTPs, 1xPCR buffer + 2 mM $MgCl_2$ (MP Biomedicals), 0.5 µM of the forward and reverse primers and 1 unit of *Taq* DNA polymerase (MP Biomedicals). PCR conditions were as follows: 94°C for 4 min, 35 cycles of 1 min at 94°C, 45 s with annealing temperatures indicated in Table 1 and 1 min at 72°C, with a final elongation of 7 min at 72°C. PCR products were tested on a 1.2% agarose gel using 5 µL of the PCR reaction. PCR products were sequenced using a BigDye terminator V3.1 kit (Applied Biosystem) and an ABI3730XL sequencer.

The trace files of the sequencing products were edited and assembled using CodonCode Aligner 3.5. Alignment of sequences and detection of the polymorphic sites were done with Genalys Version 2.0 ß. To evaluate the number of haplotypes heterozygote genotypes were considered as new haplotypes. A principal coordinates analysis (PCoA) on the haplotypes matrix of pairwise genetic distance (Nei 1978) was performed using GenAlEx 6.3 (Peakall and Smouse, 2006). Analysis was done without the characters that have missing data since GenAlEx codes the ambiguous codes as a single character state .

Table 2 Details of the different populations of *F. excelsior* and *F. angustifolia* used for sequencing.

Species	Country	Population	*n*	X	Y
F. excelsior	France	La Romagne	8	49°40'	4°19'
	Ireland	Clonee	8	53°28'	6°28'
F. angustifolia	Croatia	Jaska	3	45°60'	15°71'
		Kotoriba	3	46°34'	16°81'
		Djurdenovac	2	45°59'	18°12'
	France	Mas Larrieu	4	42°35'	3°01'
	Portugal	River Tanha	4	41°25'	7°67'

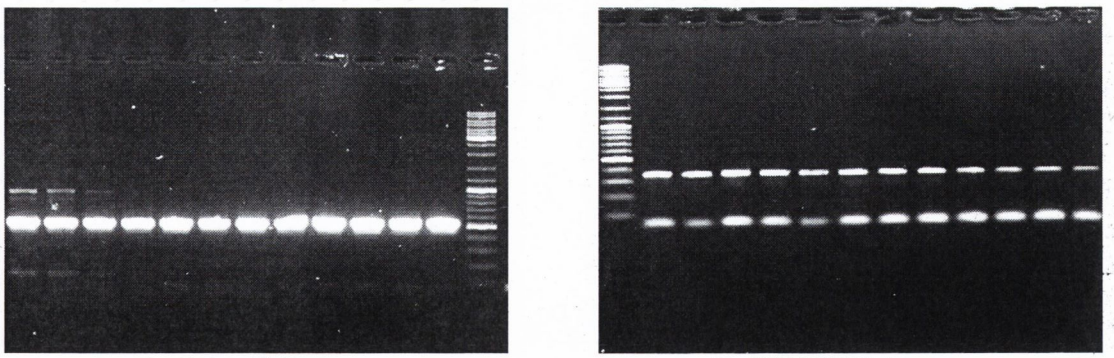
Results

PCR optimization and determination of the annealing temperature

Out of the initial 21 primers, six primers showed non-specific amplification with two or more bands (Figure 7.1) and increase of the temperature did not improve their specificity. We never observed any amplification for three of the primer pairs.

Figure 1 Example of non-specific amplification for one of the primer pairs after a PCR gradient from 55℃ (on the left) to 65℃ (on the right).

However, 12 primer pairs showed highly specific and strong amplification on *F. excelsior* (Figure 2). After choosing the annealing temperature which was the best for each marker (Table 1), the primers were tested on one individual of *F. angustifolia*. The amplification of the 12 primers was specific as well. This demonstrates transferability of the markers between species.



A. Calmodulin B. OSMOTIN 1

Figure 2 Picture of two agarose gels for PCR optimization.
PCR amplification was done on one *F. excelsior* individuals from a gradient ranging from 55℃ (on the left) to 65℃ (on the right). A. Calmodulin primers. B. OSMOTIN1 primers.

Sequencing results

Out of 12 candidate gene loci, five yielded forward and backward sequences that could be assembled in CodonCode Aligner 3.5. The sequences of five loci were aligned and scored for single nucleotide polymorphisms (SNPs) segregating within and between species using Genalys Version 2.0 ß. Insertion-deletion (indel) polymorphisms were not detected In any of the sequence alignments. One of the genes (OSMOTIN 2) did not show any SNPs. A total of 16 DNA polymorphisms were identified within the four target genes, ranging from one to 10 (Table 3). The number of haplotypes and type of polymorphic markers within species for each marker are presented in Table 3.

Table 3 Distribution of polymorphisms within the analysed sequence regions and number of haplotype for four genes for which multiple polymorphisms were detected,

		F. angustifolia	*F. excelsior*
Gene	No. and type of polymorphisms	No. of haplotypes detected	No. of haplotypes detected
Mannitol-dehydrogenase	1SNP G/C/S	3	1
Mannitol-dehydrogenase-transporter	10 SNPs A/G/R	8	10
	C/T/Y		
	A/M		
	A/R		
	A/R		
	C/S		
	G/S		
	A/C/M		
	A/R		
	G/K		
	C/S		
Calmodulin	2 SNPs A/G/R	2	4
	G/T		
OSMOTIN1	3 SNPs C/T/Y	3	1
	A/T		
	A/G/R		

The principal coordinates analysis based on the genetic distance matrix extracted six coordinates; with only the first three coordinates with an Eigenvalues superior to one. The first three factors in combination possess the highest cumulative variance of nearly 76.52%. The results of the PCoA (Figure 3) show that several *F.*

angustifolia did not cluster with *F. excelsior*, however four *F. angustifolia* individuals clustered with *F. excelsior* individuals. The grouping of the PCoA is different that the species grouping. A parallel evolution of sites because of selection migh be responsible for such results However, there is insufficient resolution to draw conclusions from that. A range of *F. excelsior* and *F. angustifolia* accessions has been amplified and we hope these markers will be used in future studies.

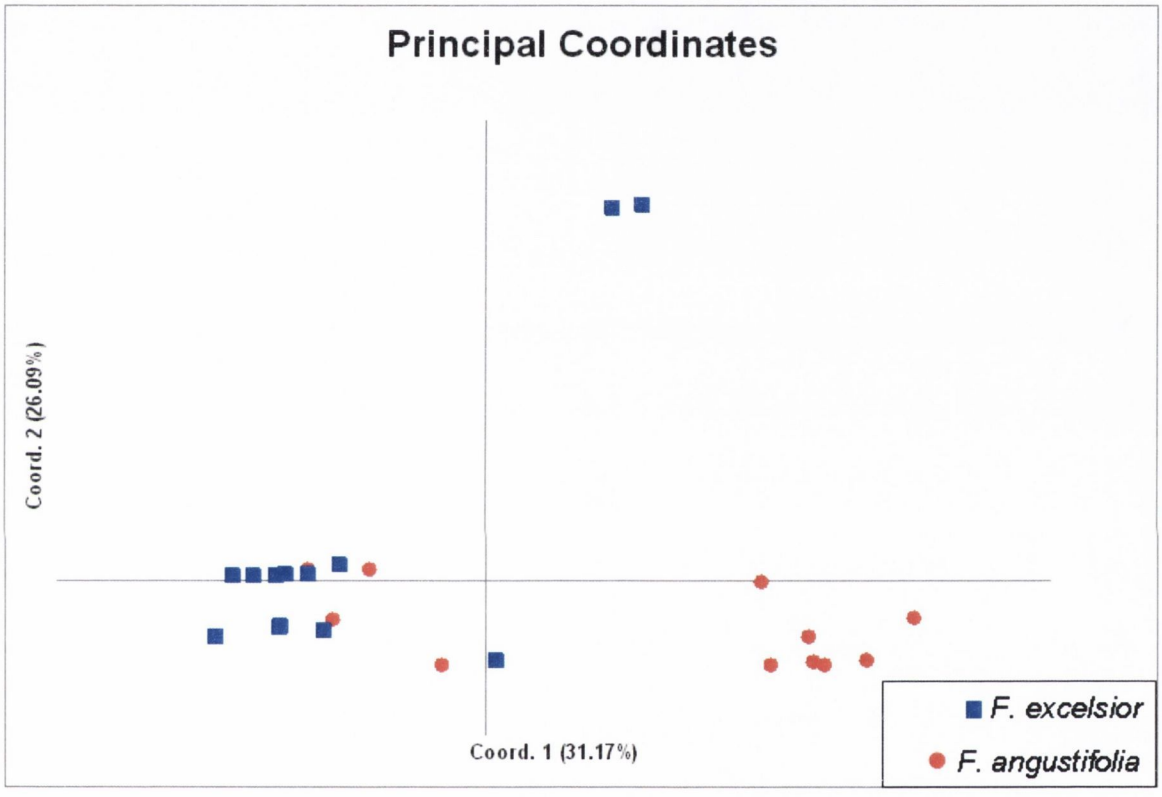


Figure 3 Plot of the scores of the PCoA for each of the individuals of *F. excelsior* and *F. angustifolia*.

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