Report

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Quality of Life and Management of Living Resources

FINAL REPORT (2001-2005)

INTRA AND INTERSPECIFIC GENE FLOW IN OAKS AS MECHANISMS PROMOTING GENETIC DIVERSITY AND ADAPTIVE POTENTIAL

OAKFLOW

Research programme	Quality of life and management of living resources (1.1.1.)
Key action	Sustainable agriculture, fisheries and forestry (1.1.1.-5.)
Action line	Multifonctional management of forests (1.1.1.-5.3.1) The protection and conservation of the forest ecosystem

CONTENT

EXECUTIVE SUMMARY

Gene dispersion processes are key mechanisms that shape the genetic structure and diversity of forest stands. Gene dispersal occurs by means of both natural migration of pollen and seed as well as via the artificial transfer of seeds and plants. These processes affect the genetic structure of forest stands and thereby influence their ability to adapt to future changes in the environment. Within the OAKFLOW project natural and artificial gene flow, including interspecific geneflow, was monitored in white oaks (mainly *Q. petraea* and *Q.robur*) in different purposely installed study plots across Europe. Three main objectives were set to the project:

- *To trace and quantify gene flow and hybridisation in terms of distances and rates*
- *To evaluate genetic and ecological consequences of gene flow and hybridisation on the adaptation of oak stands*
- *To evaluate impacts of gene flow on management rules and silvicultural regimes of oak stands*

In regards to the three main objectives of the OAKFLOW project, the following main conclusions can be drawn:

(1) An overall assessment of pollen and seed flow was obtained from the different intensive study plots (ISP) that were installed across Europe under various ecological conditions. Within the size of the plots varying between 4.5 to 50 ha, the pollen dispersal curve followed a negative power exponential function, with mean dispersal distances varying between 47 to 870 meters (and standard deviation from 40 to 2400 meters). However, in more than 60% of the pollination events (from 39% to 88% depending on the ISP), the male parents could not be assigned, suggesting that most of the pollen originates from outside the Intensive Study Plots. These results support the general view that pollen flow is the result of two processes: local dispersion (that has been addressed in OAKFLOW) and long-distance transport, the ratio between both processes amounting to approximately 40/60. Seed dispersal distances varied between 10 to 155 meters, and seed dispersal from outside the ISP was much lower than for pollen (on average 39 % , varying between 15% and 48%.

(2) Hybridisation occurred in all ISP, regardless of the density or the census numbers of the different species. The average proportion of hybrids over all ISPs amounted to 17 % (from 1% to 41%). This is however a lower limit of hybridization rates, as it accounted only

for those mating events where both parents could be assigned by parentage analysis. These figures do not take into account pollen donors from outside the study plots, as they could not be identified. In contrast to previous papers, there was no preferential direction of hybridisation between *Q. robur* and *Q. petraea.*

(3) In several case studies distributed across Europe, past artificial seed transfer could be identified by chloroplast DNA fingerprints and their spatial structure in the studied stands. These results suggest that the method could be used for certification purposes, if autochthony of the stand is to be used as a certification criteria

(4) Genetic maps for *Q. robur* were constructed in two full sib pedigrees, that were further used for QTL detection. Strong colinearity was found with the map of *Castanea sativa*, suggesting that the different genera of Fagaceae may share the same genetic system. These results open new perspectives for genetic and genomic research in the Fagaceae

(5) Quantitative Traits Loci (QTLs) were detected for traits showing interspecific adaptive differentiation between *Quercus petraea* and *Quercus robur* : leaf morphology and pubescence, water use efficiency, response to waterlogging. The percentage of phenotypic variation explained by the QTLs remains moderate to low (from 0 % to 12% in general). Interestingly for water use efficiency (as assessed by carbon isotope discrimination ($\delta^{13}C$)), the proportion of phenotypic variance explained by one QTL amounted to 20 - 25% (on linkage group 11)

(6) Differentially molecular screening methods were used to identify genes that exhibit different expression profiling between the two species under stressed conditions: hypoxia and osmotic stress. The corresponding genes were sequenced and mapped on the *Quercus robur* map.

(7) Results obtained in the project were shared an discussed with end-users (forest managers, conservation agencies). Stakeholders were invited to all project meetings and directed the project towards their needs and most important practical problems. Outcomes of the project , mainly of the gene flow studies, were discussed in regards to two main topics ‘Natural regeneration in common oak stands’ and ‘Design and management of seed and conservation stands’, taking into account the comments and expressions of interest of stakeholders

1 Introduction

Gene dispersion processes are key mechanisms that shape the genetic structure and diversity of forest stands. Gene dispersal occurs by means of both natural migration of pollen and seed as well as via the artificial transfer of seeds and plants. These processes affect the genetic structure of forest stands and thereby influence their ability to adapt to future changes in the environment. Thus, the success of natural regeneration may be influenced through effects of gene dispersal on genetic quality and diversity. Similarly, gene dispersal via artificial transfers of populations will alter the genetic composition of stands. Oaks are particularly susceptible to the effects of gene dispersal as they have the capacity for interspecific hybridisation. Hybridisation among white oaks can occur between two temperate species as well as between a temperate and a Mediterranean species providing they come into contact by gene dispersal. Although gene flow may have beneficial effects by enlarging the genetic diversity of a species it may also act detrimentally by introducing undesirable genes into autochthonous populations.

Changes in population genetics due to gene flow and hybridisation are set to increase in the near future. The 20th Century has witnessed a huge modification to the genetic composition of forests due to the intensive use of plantations. These artificial changes will be reinforced by the natural colonization of abandoned farm land by forest trees and by the northward migration of species induced by the climatic changes. Out of all the European forest tree species, the genetic composition and future adaptation of oak species are likely to be the most affected by these changes. This is not only because they occupy a large proportion of European forests, but also because oaks are highly interfertile and interspecific hybridisation is common, not only amongst temperate species (e.g. *Quercus robur* and *Q. petraea*), but also between temperate and Mediterranean species (e.g. *Q. pubescens* and *Q. faginea*). Due to the influence of global climate change, Mediterranean oak species will probably be able to colonise more northerly latitudes giving opportunities for hybridisation with local temperate species and the possibility of generating new genetic combinations. Furthermore pioneer species quite rapidly colonise abandoned farm land through natural seed dispersal, and the availability of new niches for colonization by native and alien oak species is likely to provide a dynamic forum for such hybridisation events. Indeed, hybridisation events between alien and native species, due to introductions, range changes and the availability of new niches, are responsible for a large number speciation and introgression event. And the consequences of dynamic genomic

recombinations following hybridisation to plant adaptation and evolution are only just being realised.

Thus the future composition of European oak forests will be faced with new challenges due to the interaction between natural dispersal processes and human introductions of non-native material. Until recently the assessment of genetic diversity in populations has usually been addressed by monitoring levels and distribution of genetic variation. However, levels of diversity are usually not modified by human interference in the short term but delayed for a few generations. In contrast, changes to regeneration regimes and other silvicultural treatments have immediate impacts on dispersal mechanisms; and as a result, such modifications to gene flow processes are expected to cause more immediate change to the distribution of genetic diversity. Rather than focusing on the changes in the distribution and levels of diversity, this project addressed key biological mechanisms that may interact with human interferences to generate and modify diversity in these scenarios.

In the original proposal OAKFLOW has set three major objectives:

- ***To trace and quantify gene flow and hybridisation in terms of distances and rates***
- ***To evaluate genetic and ecological consequences of gene flow and hybridisation on the adaptation of oak stands***
- ***To evaluate impacts of gene flow on management rules and silvicultural regimes of oak stands***

The final report is a synthesis of the main results obtained within OAKFLOW along these 3 main objectives.

(1) Effective natural pollen and seed movements were detected in the ISPs (Intensive study sites) that were installed throughout Europe from Scandinavia to Spain. Parentage analysis based on microsatellite markers was implemented and the data were analysed with standardized methods. Gene flow was quantified as distances and rates of dispersal and illustrated in the form of dispersal curves.

(2) Genetic and genomic modifications due to gene flow were assessed by two complementary approaches. First a systematic screen of the genome was implemented to identify regions of the genome that are “permeable” to interspecific gene flow vs those regions that maintain strong species differentiation. Second a targeted approached aimed at the discovery of genes or QTL (Quantitative Trait Loci) involved in ecophysiological differences between the two main oak species (*Quercus petraea* and *Q. robur*)

(3) The amount of gene flow can lead to practical decisions and silviculture recommendations concerning the management of seed and conservation stands. As a

synthesis and critical analysis of the two previous parts of the final report, practical consequences of the results obtained are discussed in the last part of the scientific report.

2 Quantification of intra and interspecific gene flow

Gene dispersal occurs in oak trees populations by means of gene flow via pollen and seed. This gene flow affects the genetic structure of forest stands, potentially evokes hybridisation, and therefore might influence their ability to adapt to future changes in the environment.

The aim of this part of the oakflow project was to trace and quantify gene flow and hybridisation in the White oak species complex (mostly *Quercus robur* (pedunculate oak) and *Q. petraea* (sessile oak)) in terms of distances and rates. Effective natural contemporary pollen and seed movements were estimated in study plots distributed across Europe.

2.1 Pollen Flow

2.1.1 Material and methods

2.1.1.1 Intensive studied plots (ISP)

During the European project Fairoak ("Synthetic maps of gene diversity and provenance performance for utilization and conservation of oak genetic resources in Europe", 1996-1999), several white oak stands were selected in the countries of the different project partners. The selection followed criteria established during the first coordination meeting:

- Mixed stand comprising *Q. petraea* and *Q. robur* in approximately equal proportions. This intermixing may be spatially subdivided in three zones: 2 pure zones and one zone where the two species are confronted to each other. In a Mediterranean species *Q. pubescens* or *Q. pyrenaica* and other subspecies of *Q. petraea* (*Q. virgiliana, Q. dalechampi*) were also part of the oak complex.
- Stand of natural origin ;
- Adult trees (more than 120 years old) ;
- Population size for each species should be close to 200. This is the real size and not a sample size. This means that, on an experimental area, the sampling was exhaustive on the study area

According to these criteria, study stands were sampled by each participant of the Fairoak project. The project Oakflow included some of these stands and new ones were also added,

with *Quercus robur* and *petraea* whenever possible and alternative *Quercus* species if either of the two main species was not present. All adult trees were labelled, mapped and genotyped for a set of DNA markers (nuclear microsatellites). Fourteen such Intensive Study Plots were available, among which 9 could be analysed with common standardized procedures. The final report is based on these nine ISPs (Table 1). Results of the others are available in the intermediate progress reports.

2.1.1.2 Sampling strategy of trap trees

Trap trees are mother trees on which acorns are collected for paternity analysis. The sampling strategy (number of trap trees and number of acorns per trap tree) resulted from a compromise between the resources available and the scientific objectives of the study.

There were two main objectives:

1- construction of pollen dispersion curve

2- estimation of mating success of different male and female parents

Objective 1 required to collect acorns on many trap trees in order to account for the tree-to-tree variation of pollen dispersal, whereas more acorns on a limited number of trap trees had to be collected for objective 2. Compromises had to be established by each partner depending on technical and biological constraints: availability of material, levels of seed crop, genotyping capacity, etc...

Most of the oak stands were mixed and there were usually three different areas (two pure species zones and one mixed zone), the partners tried to select the trap trees in these three areas. Since the estimation of hybridisation rates was one of our goal, and interspecific crosses occur less frequently than intraspecific crosses, more trap trees were selected in the mixed area than in the pure areas where possible.

2.1.1.3 Paternity analysis

Fourteen stands were available and nine could be analysed with standardized procedures (France, Italy, Holland, Switzerland (two years), Spain, Great Britain, Denmark, Sweden and Hungary) **.** All adult trees of the study area in the ISP and a sample of acorns collected on identified mothers were genotyped at 4–8 nuclear microsatellite markers. Spatial coordinates of adult trees were available (Table 1 and 2).

Table 1: **Description of Intensive Study Sites (ISPs)**

ISP : Partner # Country	Stand name	Size	Number of *Q.petraea* trees	Number of *Q. robur* trees	Nb Trees of intermediate morphology	Tree density
P1 France	Petite Charnie	5	166	183	5	70.8
P2 Italy	Tatti	50	184	70**	41	5.9
P3 Holland	Meinweg 1998/2002	4.5	182	187	-	82
P4 Spain	Islands	*	50***	189	-	-
P5 Great Britain	Dalkeith	23	704	50	-	32.8
P8 Denmark	Velling	5	120	245	-	73.0
P10 Sweden	Båtfors	10	-	631	-	63.1
P11 Switzerland	Bueren	9	76	349	17	49.1
P12 Hungary	Sopron	5.1	48/21****	210	151	84.3

* 5 *Q. robur* islands (500 m, 11.24 ha, 1 ha , 4 ha, 4.4 ha), 1 *Q. faginea* 26.8 ha
** *Quercus pubescens*
*** *Q. faginea*
**** 12 *Q. petraea* + 32 *Q. virgiliana* + 4 *Q. dalechampii* (considered together) / 21 *Q. pubescens*

Table 2: Sampling design for paternity analysis

ISP : Partner# Country	Nb of loci	Nb of genotyped parents	Number of sampled mothers /	Mean number of offspring per mother	Total number of offspring analysed
P1 France	6	354	13	76	992
P2 Italy	5	295	30	28	841
P3 Holland 98	5	369	5	41	700
P3 Holland 02	5	369	3	147	440
P4 Spain	6	239	11	24	264
P5 Great Britain	6	754	44	26	1136
P8 Denmark	8	365	33	17	553
P10 Sweden	6	631	47	23	1061
P11 Switzerland	5	434	19	81	1543
P12 Hungary	4	430	20	20	382

▪ *Building and evaluating paternity statistical test with the software Famoz (Gerber et al., 2003)*

The capacity of the marker systems of each stand to be used for paternity and parentage analyses was evaluated by calculating exclusion probabilities and identity probabilitites.

▪ *Determining thresholds*

Simulations were conducted to identify threshold levels for parent assignments. In a first analysis, offspring (100.000 for each simulation) were created using firstly parent genotypes and secondly populations allele frequencies to build tests for each stand. Simulations were computed either with no mistyping or with a non-null but small value (0.001%). These simulations were used to build a statistical test, defining a lod-score threshold (likelihood ratio) and a delta threshold (difference between the most likely father lod-score and the next most likely males, see Marshall et al. 1998) to assign fathers to offspring inside the stands, ie among the genotyped parents.

The estimation of the gene flow, i.e. the percentage of offspring with no father assigned, eg with father assumed to be outside the stand, was deduced from father assignment using three tests, one based on lod-score, the second on delta and the third on a combination of both lod-score and delta; in case of ex-aequo fathers for an offpsring, no father was assigned.

▪ *Paternity test simulation*

For each stand, simulations were made to mimic the experimental design (number of mother, number of offspring per mother), use the gene flow estimation to create offspring with a father from the genotyped parents or with a father from "outside" the stand. The percentage of correct father choice, the percentage of paternity correctly assigned among the assigned paternities were calculated with or without mistyping and with the three types of tests (lod-score, delta or both): the test providing the highest percentages was used in the final paternity analysis. The β error (cryptic gene flow, or false assignment, assign a genotyped father when the true father is outside the stand) and the α error (false rejection, assign no father when the true father is inside the stand) were computed.

▪ *Father assignment and pollen dispersal analyses*

Father assignments were obtained after the application of the most efficient test on each offspring of each stand.

- the number of different fathers involved in reproduction and their reproductive success (number of offspring per father) was calculated for each stand;

- the percentage of acorns with a father assigned was available for each mother of each stand;

- the percentage of hybrid acorns was available for each mother of each stand;

- the mother-father distances, ie effective pollination distances, were calculated for each stand, observed dispersal curves were deduced. These curves were fitted with a one dimensional power exponential function:

$$f(x) = \frac{b}{a\,\Gamma(1/b)} \exp\left(-\left(\frac{x}{a}\right)^{b}\right)$$

where *x* is the pollen dispersal distance, *Γ()* is the gamma function, and *a* and *b* are distance and shape parameters (if *b*=1 *f(x)* is an exponential function, if *b*=2, *f(x)* is a Gaussian function and if *b*<1, the function is fat-tailed) (Clark 1998).

The mean distance δ travelled by the pollen is expressed by:

$$\delta = \frac{a\,\Gamma(2/b)}{\Gamma(1/b)}$$

The two parameters δ and *b,* were estimated using a maximum likelihood method. 95% likelihood-profile confidence intervals for both parameters were calculated by plotting contour plots of the likelihood function (Oddou-Muratorio *et al.* 2005).

2.1.2 Results

2.1.2.1 Quality of markers

The nine oak populations studied exhibited high exclusion probabilities, reaching almost 100% (Table 3). These high values are a prerequisite to allow valuable paternity and parentage analyses. Identity probabilities were very low, but four stands had between 13 and 44 pairs of identical individuals. These identical genotypes may be the result of forest

management, i.e. when coppicing creates genetic clones that can sometimes be separated by some meters after a long time and, thus, are considered as two different trees. The highest number of identical pairs corresponds also to the smallest number of loci genotyped (in the case P12), indicating maybe only a lack of discrimination power, even if the expected identity probability is null (Table 3).

Table 3: Marker polymorphisms for each stand. UIP: unbiased identity probability, NIP: number of identical pairs, OIP: Observed identity probability.

ISP : Partner# Country	Nb of loci	Exclusion probabilities			UIP	NIP	OIP
		single parent	paternity	parent pair			
P1 France	6	0.998243	0.999910	1.000000	0.00	1	0.00002
P2 Italy	5	0.987387	0.998713	0.999989	0.00	1	0.00002
P3 Holland	5	0.975451	0.997031	0.999960	0.00	7	0.00010
P4 Spain	6	0.997946	0.999893	1.000000	0.00	0	0.00000
P5 Great Britain	6	0.993198	0.999390	0.999997	0.00	25	0.00009
P8 Denmark	8	0.997005	0.999892	1.000000	0.00	0	0.00000
P10 Sweden	6	0.993038	0.999507	0.999998	0.00	29	0.00015
P11 Switzerland	5	0.991458	0.999099	0.999996	0.00	13	0.00011
P12 Hungary	4	0.988654	0.998455	0.999987	0.00	44	0.00048

2.1.2.2 Assignment test quality

For each stand, we decided to use for paternity assignment the most successful test in terms of percentages of correct father choice (Cfc) and of father correctly assigned among the assigned paternities (Pca). Eventually, tests with no mistyping and using either fathers' delta-scores or both delta- and lod-scores for assignment proved to be the most reliable (Table 4). The mean Cfc was 87.34% with a standard deviation of 9.72, the mean Pca was 86.19% with a standard deviation of 11.57: the quality of the tests was variable from one experiment to the other but most of the time higher than 80%. α and β errors were less than 5% except in two cases (P2 and P5 for α, P10 and P11 for β). The observed gene flow, that is the percentage of pollen grains from outside the studied plot, or the percentage of acorns with no assigned father among the genotyped parents was deduced from the decisions made with the tests applied to the different datafiles.

Table 4: Results of test simulations (highest values) and corresponding type of test used to assign fathers in each stand. Test: fathers assigned according to their delta scores (delta) or both to their lod-scores and delta-scores (l & d). Sim (%) the most likely father is the true father when simulating offspring (100,000) with an identified genotyped father. Ogf (%) observed experimental pollen flow according to the test. Cfc (%) correct father choice rate (father correctly assigned either inside or outside the studied stand). Pca (%) paternity correctly assigned among the assigned paternities. Cgf (%) cryptic gene flow or β error (assign a genotyped father when the true father is outside the stand, false assignment). α: α error (%) (assign no father when the true father is inside the stand, false rejection)

ISP :Partner# Country	Test	Sim	Ogf	Cfc	Pca	Cgf	α
P1 France	l & d	97.88	66.94	98.84	98.87	0.07	0.41
P2 Italy	delta	71.43	39.24	74.88	63.05	14.76	2.12
P3 Holland 98	l & d	88.46	69.86	90.24	88.38	3.46	2.41
P3 Holland 02	l & d	88.52	64.77	90.36	89.88	2.46	3.54
P4 Spain	delta	98.50	81.06	99.43	98.87	0.31	0.00
P5 GreatBritain	l & d	82.52	40.93	81.38	73.99	8.22	4.82
P8 Denmark	delta	98.72	20.94	99.11	98.40	0.04	0.14
P10 Sweden	l & d	64.97	77.57	75.16	81.66	4.35	10.62
P11 Switzerland	l & d	79.45	53.14	85.12	85.81	1.28	9.79
P12 Hungary	l & d	75.38	87.69	78.88	83.00	3.97	3.49

2.1.2.3 Pollen flow

The mean pollen flow observed, eg the pollen originating from outside the ISP, over all stands is 60% with a standard deviation of 21 (Table 5). This pollen flow is highly variable from one stand to the other: 21% of pollen comes from outside the population in Denmark whereas 88% is observed in Hungary.

Table 5: Pollen flow results

ISP : Partner # Country	Size (ha)	Nb of loci	Nb of genotyped parents	Nb of offspring	Nb of offspring with a father assigned	% of offspring with a father assigned	% pollen flow
P1 France	5	6	354	992	328	33.06	66.94
P2 Italy	50	5	295	841	511	60.76	39.24
P3 Holland 98	23	5	369	700	211	30.14	69.86
P3 Holland 02	9	5	369	440	155	35.23	64.77
P4 Spain	4.5	6	239	264	50	18.94	81.06
P5 Great Britain	5	6	754	1136	671	59.07	40.93
P8 Denmark	4.5	8	365	554	438	79.06	20.94
P10 Sweden	10	6	631	1061	238	22.43	77.57
P11 Switzerland	*	5	496	1543	723	46.86	53.14
P12 Hungary	5.1	4	430	382	47	12.31	87.69

* 5 *Q. robur* islands (500 m, 11.24 ha, 1 ha , 4 ha, 4.4 ha), 1 *Q. faginea* 26.8 ha

2.1.2.4. Father assignment

On average, 130 different fathers contributed to the reproduction in the different stands, but with a high variability (standard deviation of 79, 21 (P4) to 286 (P5) fathers, Table 6). The 130 male parents however represent only a subset of all male trees contributing to the matings, as all acorns produced were not collected. The reproductive success of these fathers (number of offspring per father) according to distance follows a L-shaped distributions in the ten situations, with few fathers siring a high number of offspring and a greater number with few offspring (many fathers exhibit only one offspring whatever the stand considered). The efficient number of fathers is hence reduced, representing on average less than 50% of the number of different fathers, but again with an important variability (from 26 (P11) to 80% (P12)). In ISPs P2 and P8, assignments per mother reach almost a 100% and then decrease slowly. The same trend is observed in the other stands but with lowest global percentages.

Table 6: Father assignment results - No/Nm: number of mothers and mean number of offspring per mother in the experiments. Nf: number of different fathers assigned. No/Nf: number of offspring per father, (minimum, maximum, mean and standard deviation (sdev)). Ne: effective umber of fathers ($1/\Sigma(f_i^2)$, where f_i is the relative reproductive success of each father). Ne/Nf : %. Nf/Nm: number of fathers assigned per mother and standard deviation

ISP : Partner # Country	No/Nm	Nf	No/Nf			Ne	Ne/Nf	Nf/Nm	
			min-max	mean	sdev			mean	sdev
P1 France	13 / 76	120	1-21	2.73	3.42	47.02	39.18	25.23	10.65
P2 Italy	30 / 28	159	1-36	3.21	4.65	51.55	32.42	17.03	12.65
P3 Holland 98	5-41	92	1-18	2.29	2.77	37.70	40.98	42.20	16.24
P3 Holland 02	3 / 147	68	1-16	2.28	2.57	30.14	44.32	51.67	24.91
P4 Spain	9/24	21	1-13	2.38	2.73	9.33	44.43	4.55	3.70
P5 Great Britain	44 / 26	286	1-25	2.35	2.67	125.03	43.72	15.61	11.20
P8 Denmark	33 / 17	178	1-19	2.46	2.46	89.40	50.22	13.27	9.85
P10 Sweden	47 / 23	158	1-8	1.51	1.13	101.51	64.25	5.41	3.25
P11 Switzerland	19 / 81	175	1-55	4.13	7.08	44.65	25.51	38.05	36.46
P12 Hungary	20 / 20	39	1-4	1.21	0.61	31.11	79.77	3.92	2.39

2.2.1.5 Hybridisation rate

Hybridisation involving *Quercus petraea* and *Q. robur* could be observed in the five stands where the two species co-occurred (P1, P3, P5, P8, P11, see Table 7). In all cases except P5, the crosses involved more often a sessile mother and a pedunculate father than the opposite. The percentage of hybrid acorns was high in P8, P3 (but two times higher in 1998 than in 2002) and P5. The general trend is that some mothers contributed more to

hybridisation than others, the species of these mothers varied from one stand to the other. A high number of hybridising mothers were observed in P5, P8 and to a lesser extend in P2. In P5, the highest hybridising mothers were pedunculate. On average, selfing represented 2.3% of crosses, with a standard deviation of 1.6.

Table 7: Hybridisation results, type of crosses observed. Percentages of intraspecific and hybrid crossing: *Qp* (*Q petraea*), *Qr* (*Q. robur*), hybrid crosses: female ♀ × male ♂. h: percentage of hybridisation among acorns with an assigned father. H/M: mean number of hybridisation event per mother and standard deviation. s: percentage of selfing

	Intraspecific		Hybrid (♀ × ♂)			H/M		
ISP : Partner # country	*Qp* × *Qp*	*Qr* × *Qr*	*Qp* × *Qr*	*Qr* × *Qp*	h	mean	sdev	s
P1 France	47.26	44.21	5.79	1.52	6.94	1.85	4.65	4.57
P2 Italy*	*75.93*	*13.31*	*2.74*	*4.11*	*7.37*	*1.17*	*2.47*	2.54
P3 Holland 98	5.69	63.03	28.91	2.37	33.17	13.20	16.30	3.32
P3 Holland 02	0.65	81.94	14.19	3.22	17.76	9.00	11.36	0.00
P4 Spain	-	100	-	-	-	-	-	4.00
P5 Great Britain	67.96	2.68	8.35	21.01	29.80	4.58	8.76	2.24
P8 Denmark	28.08	30.14	21.69	20.09	41.03	5.55	4.89	2.28
P10 Sweden	-	100	-	-	-	-	-	0.84
P11 Switzerland	67.51	19.24	1.45	0.18	1.04	0.47	0.90	3.04
P12 Hungary**	*2.17*	*69.57*	*0.00*	*2.17*	*1.20*	*0.08*	*0.29*	0.00

* *Qp*=*Q petraea*, Qr=*Q. pubescens*,

***Qp*=*Q. petraea* + *Q. virgiliana* + 4 *Q. dalechampii* , Qr=*Q. robur*

2.2.1.6 Pollen dispersal curves

The distribution of mother–father distances observed were plotted for each stand, and the respective fit with a power exponential function was computed (Table 8). The distances were obviously largely dependent on the size of the stand. In Spain (P4), some very long dispersal events (almost 1 km) were observed between the oak "islands", while the rest of observations were local, with no fit possible in this case. In all stands except P12, the mean dispersal distances observed were significantly smaller than the one obtained with a random assignment of fathers to the acorns, indicating a restricted spatial dispersion of pollen.

Except for P3 in 1998 and for P12, the dispersal distances were adequately fitted by the exponential power distribution. The mean distances δ travelled by pollen exhibited rather limited confidence intervals (Table 8). Two stands (P2 and P10) had fat-tailed dispersal curves (b<1) as well as the gathering of all (except P4) dispersal data.

Table 8: Pollination distances observed after the paternity analyses. *P* (%): probability of having observed a smaller mean pollination distance value by picking random fathers. Fit of the observed data to a one-dimensional exponential power distribution with parameters δ and *b*, δ: mean dispersal distance travelled by the pollen (in meters) *b*: shape parameter

ISP : Partner # Country	Pollination distances					Exponential power fit [CI]	
	min	max	mean	sdev	P	δ (m)	*b*
P1 France	0.00	216.33	46.49	39.82	0	46.45 [41.43,52.17]	1.41 [1.09,1.83]
P2 Italy	0.00	835.77	222.96	212.86	0	225.78 [198.52,260.85]	0.70 [0.55,0.90]
P3 Holland 98	0.00	263.58	67.08	52.66	0	66.82 [58.52,76.32]	1.74 [1.24,2.46]
P3 Holland 02	19.21	232.86	77.16	51.97	0	76.66 [66.63,87.50]	2.78 [1.69,5]
P4 Spain	0.00	9976.74	867.30	2377.27	0	-	-
P5 Great Britain	0.00	643.85	164.59	128.18	0	164.88 [149.95,181.21]	1.01 [0.81,1.25]
P8 Denmark	0.00	224.32	55.19	46.11	0	55.26 [50.09,60.91]	1.50 [1.17,1.94]
P10 Sweden	0.00	841.27	178.62	188.72	0	179.68 [149.14,221.60]	0.71 [0.53,0.95
P11 Switzerland	0.00	346.07	71.41	60.91	0	71.56 [66.34,77.36]	1.42 [1.17,1.74]
P12 Hungary	4.89	248.48	96.37	74.73	46	105.72 [78.03,127.70	4.84 [0.97,5]
All stands (except P4)						117.4 [109.9,123.2]	0.7 [0.64, 0.72]

2.1.3. Discussion

In this study, we compared eleven different pollen dispersal situations in nine White oak (*Quercus* spp.) ISPs spread all over Europe. The nuclear microsatellite markers used exhibited a high polymorphism (exclusion probabilities reaching almost a 100%, identity probabilities reaching 0%), allowing reliable paternity analyses. Pollen dispersal curves and hybridisation trends were obtained after the paternity assignments.

When assigning fathers to acorns (using a maximum likelihood ratio method), the correct decisions were made with a rate usually higher than 80%, and type I and II errors were mostly less than 5%, according to simulated paternity tests.

Fathers were assigned on 12 to 79% of the total number of acorns genotyped depending on the stand. The corresponding pollen flow therefore comprised between 88 and 21%. A high variability was observed between stands, the mean value of pollen coming from outside the stands being 60%, a value consistent with previous observations for this kind of wind pollinated tree species.

The number of different fathers that contributed to reproduction was also highly variable among stands (from 21 to 286). Individual fathers' reproductive success had a L-shaped distribution in all stands (few fathers siring a large number of offspring but many siring few, especially only one offspring). On average, the effective number of fathers represents less than half the number of trees in the stands, with, again, some extreme values. The number of fathers assigned per mother tree showed the same trend in all stands, with a smooth decrease from one mother to the next. However, some stands had mothers with almost all acorns having a father assigned whether smaller rates are observed elsewhere.

When *Quercus petraea* and *Q. robur* were co-occurring in stands, hybridisation events could be detected among acorns. In all stands except P5 (Great Britain), *Q. petraea* trees were sired by pollen from *Q. robur* more often than the opposite. In P5, the stand was composed of 93% *Q. petraea* trees, but *Q. robur* mothers exhibited significantly more hybridisation events than *Q. petraea* mothers. We observed that some mothers showed more hybrid acorns than others in the different stands. One explanation could be that some mother trees could be themselve hybrids (P1, Lepais *et al.* unpublished data). Three stands (P3-1998, P5 and P8) exhibited a global percentage of hybrid acorns greater or equal to 30%.

The distances between mothers and fathers assigned were used to draw pollen dispersal distribution. The mean distances travelled by pollen inside the stands were always (except for P12) significantly smaller than a distance that would be obtained with a random choice of fathers: closely positioned trees are more likely to pollinate one another than those standing at greater distances.

In seven cases out of the ten, a power exponential function described the data in a convenient way, with only two stands displaying a fat-tailed distribution (b<1). Combining all

data (except P4), however, resulted in a fat-tailed distribution with the mean distance travelled by pollen of about 120 m.

Our data show a general trend in effective within-stand pollen dispersal of wind-borne White oak pollen. Stand-specific and overall pollen dispersal curves largely confirm the dispersal curves of other wind-pollinated tree species. But as the data for external gene flow and that from the large-scale analysis in the Spanish plot indicate, plot-related pollen flow only accounts for a fraction – though prevalent – of effective pollen dispersal. Alternative approaches using assignment tests at a landscape level could further elucidate how genes are transferred also among stands at longer distances.

2.2 Seed Flow

2.2.1 Material and methods

2.2.1.1 Intensive studied plots

In 7 different stands (see Table 9), seedlings were sampled following different spatial strategies. They spatial coordinates were recorded and the trees were genotyped for the same loci as the one used for parents. Seedlings were sampled within the natural regeneration and were distributed in different ways across the ISP areas (either as patches or over the whole area of the ISP, Table 9)

Table 9: Stand details for parentage analysis

ISP : Partner # Country	Number of loci	Number of genotyped fathers	Number of sampled seedlings	Sampling strategy
P1 France	6	354	165	grid
P2 Italy	5	295	387	2 patches
P4 Spain	6	239	65	3 patches
P5 Great Britain	6	754	191	1 patch
P8 Denmark	8	365	84	regular
P10 Sweden	6	631	175	regular
P12 Hungary	4	430	75	2 patches

2.2.1.2 Parentage analyses

The software FaMoz was used for the parentage analyses. As for paternity analyses, test thresholds for assigning a single parent or a parent pair to a seedling were determined thanks to simulation (two sets of 100,000 simulated offspring with either genotyped parents or genotypes created using allele frequencies) for both lod-scores and delta-scores. Test mimicking the true data were built to evaluate the quality of assignments compared to true relationships, using either single parent and parent pairs lod-scores, delta-scores or both, with a null mistyping rate or with a small non-null value (0.001%). The tests providing the

highest correct assignment rates and the smallest type I and II errors were selected for the final analyses of datasets in each stand.

2.2.1.3 Parent assignments, gene flow and dispersal curves

After the parentage test, each seedling was assigned either zero, one or two genotyped parent. The nuclear microsatellite markers used in this study do not allow to distinguish mother- from father-trees. Since acorns are heavy seeds and oaks are wind pollinated, we assumed that when a single parent was assigned, it was the mother. Total gene flow was then split into seed and pollen flow: seed flow was deduced from seedling with no parent assigned and pollen flow was calculated as the percentage of seedling with one single parent assigned plus the seedling with no parent assigned. Total gene flow can be seen as the mean between seed and pollen flow.

When both parents were assigned, the pollen dispersal corresponds to the distance between the parent trees and the closest tree determined the seed dispersal distance. We could thus trace seed dispersal distribution by cumulating the distance between seedling and their single parent and the distance between seedling and the closest parent when a parent pair could be assigned. The pollen dispersal distribution could be deduced from distance between parents, when a sufficient number of parent pair assignment was available. We fitted the dispersal data, when possible, with a one dimensional power exponential function, shape parameter (*b*) and mean distance travelled by seed or pollen (δ) were estimated with their confidence intervals as before (see paternity analyses).

2.2.2 Results

2.2.2.1 Assignment test quality

The stands analysed for seedling data exhibited all high single parent and parent pair exclusion probabilities, reaching almost all values close to a 100% (Table 3). For four out of seven stands analysed, according to simulations, the most reliable parentage tests were the one based on lod-scores with a null mistyping, three cases involved tests with a non null mistyping (Table 10). According to test simulations, the correct single parent choice rate was 70.7% on average (from 53.8% for P2 to 89.9% for P8) and the correct parent pair choice rate was higher, 78.8% on average (from 63.7 for P10 to 96.4 for P8). The experiment with the higher number of loci (P8, 8 loci) proved also to be the most successful for these rates. The α and β (cryptic gene flow) errors associated to these test were 15.5% and 6.2% on average, with large variations depending on the stand analysed, especially for

α (from 0.5 to 35% for α and from 0 to 13.1% for β). The observed gene flow, combining pollen and seed flow, was deduced from these tests.

Table 10: Results of test simulations (highest values) and corresponding type of test used to assign parentage in each stand. Test: parent(s) assigned according to their lod-scores with mistyping (lod+m) or without (lod). Simp (Simpp) (%) the most likely single parent (parent pair) is the true parent (parent pair) when simulating offspring (100,000) with identified genotyped parent(s). Ogf (%) observed experimental gene flow according to the test. Cpc (Cppc) (%) correct parent (parent pair) choice rate (parent (parent pair) correctly assigned either inside or outside the studied stand). Cgf (%) cryptic gene flow or β error (assign a genotyped parent when the true parent is outside the stand, false assignment). α: α error (%) (assign no parent when the true parent is inside the stand, false rejection)

ISP : Partner # country	Test	Simp	Simpp	Ogf	Cpc	Cppc	Cgf	α
P1 France	lod m	89.79	95.78	62.42	84.70	92.73	9.03	2.26
P2 Italy	lod	54.31	52.37	43.80	53.75	69.25	0.00	31.71
P4 Spain	lod	91.03	96.97	80.77	84.62	94.62	13.14	0.68
P5 Great Britain	lod	61.31	67.92	43.98	58.77	69.63	0.00	35.31
P8 Denmark	lod	93.30	97.51	72.62	89.88	96.43	9.74	0.53
P10 Sweden	lod m	49.95	37.67	64.86	60.29	63.71	4.92	19.95
P12 Hungary	lod m	59.34	54.28	69.33	62.67	65.33	6.50	18.32

2.2.2.2 Gene flow

The mean gene flow observed over all stands after the parentage analysis reached 63% with a standard deviation of 14, ranging from 44% in Italy (P2) to 81% in Spain (P4) (Ogf, Table 10 or Total gene flow Table 11). Seed and pollen flow were deduced from the percentages of seedling with 0, 1 or 2 parents assigned (Table 11). On average, among all seedling analysed, 47% (from 29 (P4) to 59% (P5)) were assigned with one single parent and only 14% (from 2 (P8) to 33% (P2)) had both a mother and a father assigned, leaving 39% of seedling with no assigned parent at all. Thus, while mean seed flow deduced from these assignments was close to 40% (from 20 (P2) to 66% (P4)), pollen flow in the seedling experiments was very high, 86% on average (from 67 (P2) to almost 98% (P8)).

Table 11: Seedlings parent(s) assignments results. Single parent and parent pair assignments achieved and gene flow observed in the different stands

ISP : Partner # Country	Number of seedlings	Seedling assigned with		Seed flow (%)	Pollen flow (%)	Total gene flow
		one parent	two parents			
P1 France	165	94 (57%)	15 (9%)	33.94	90.91	62.42
P2 Italy	387	183 (47%)	126 (33%)	20.16	67.44	43.80
P4 Spain	65	19 (29%)	3 (5%)	66.15	95.38	80.77
P5 Great Britain	191	112 (59%)	51 (27%)	14.66	73.30	43.98
P8 Denmark	84	42 (50%)	2 (2%)	47.62	97.62	72.62
P10 Sweden	175	63 (36%)	30 (17%)	46.86	82.86	64.86
P12 Hungary	75	38 (51%)	4 (5%)	44.00	94.67	69.33
mean		47%	14%	39.06	86.03	62.54
st dev		11%	12%	17.66	11.82	14.03

2.2.2.3 Parent assignments

The number of parent trees that contributed to seedling production among the sample of genotyped parents is given in Table 12. The number of parents assigned to seedlings depends on the number of seedling analysed, the highest seedling sample corresponds also to the highest number of parents. However, according to the reproductive success profile of parents, the efficient number of parents varies among stands. Seedlings in P5 and P10 were produced by the same number of parents, but since more trees in P5 leave more seedlings than in P10, where reproductive success is more balanced the effective number of parent is higher in P10. On average, 56% of the number of assigned parents participated in efficient reproduction over the seven experiments. The L-shaped structure of parent reproductive success is a general trend.

Table 12: Reproductive success of parental trees. Np: number of different parents assigned, No/Np, number of seedlings per parent, Ne: efficient number of parents ($1/\Sigma(f_i^2)$), where f_i is the relative reproductive success of each parent).

ISP : Partner # country	Number of seedlings	Np	No/Np			Ne	Ne/Np %
			min-max	mean	sdev		
P1 France	165	98	1-5	1.27	0.71	74.64	76.16
P2 Italy	387	154	1-24	2.83	3.36	63.99	41.55
P4 Spain	65	18	1-6	1.39	1.20	10.59	58.83
P5 Great Britain	191	74	1-33	2.89	4.91	19.27	26.04
P8 Denmark	84	38	1-4	1.21	0.70	28.60	75.26
P10 Sweden	175	75	1-16	1.64	1.97	30.94	41.25
P12 Hungary	75	29	1-4	1.58	0.90	22.04	76.00

2.2.2.4 Dispersal distances

Dispersal distances were deduced from single parent and parent pair assignments and dispersal curves were drawn and fitted with one dimensional exponential power functions when a sufficient number of observations was available.

When only a single parent could be assigned to seedlings the mean parent-seedling distance was significantly restricted in 6 over 7 stands compared to distances obtained with random assignment of parent to the same seedlings (Table 13). The mean distance of seedlings to their single parents was slightly higher when sessile parent trees (*Quercus petraea*) were involved compared to pedunculate parent trees (P1, P5, P8), but remains in the same order of magnitude. Some very long distance dispersal were observed in the Spanish experiment (more than 10km, P4).

Table 13: Dispersal distances (single parent assignments). *Q.p.*: *Quercus petraea*, *Q.r.*: *Q. robur* Mean d: mean seedling-single parent distance. P(x<d): probability of having observed a smaller mean seedling-single parent distance with a random assignment of parent to seedlings.

ISP : Partner # Country	Single parents			Mean d (m)	P(x<d)	Mean d (m)	
	Q.p.	*Q.r.*	Other sp.			*Q.p.*	*Q.r.*
P1 France	51	38	5	58.78	0.00	60.63	56.16
P2 Italy	145	11*	7	249.45	0.00	241.00	214.29*
P4 Spain	0	19	0	1381.33	0.00	-	
P5 Great Britain	88	24	0	185.69	0.00	186.83	181.50
P8 Denmark	11	31	0	54.56	0.00	64.00	51.21
P10 Sweden	0	63	0	185.65	0.00	-	
P12 Hungary	9**	8	21	139.36	5.20	63.06**	125.61

**Q. pubescens*

*** Q. petraea + Q. virgiliana + Q. dalechampii*

The assignment success was lower with parent pairs, fewer events could be observed in each stand (Table 14). However, hybridisations were detected (3.2% of hybridisation over all seedlings from stands with mixed species) - especially in P5 - and selfing as well (1.9% over all stands and seedlings). Seed and pollen dispersal distances were deduced from parent pair assignments assuming that the mother tree was the closest, the pollen having travelled between the two trees of the pair. The mean pollen dispersal distance was significantly smaller than expected with a random assignment of fathers in 4 stands over 6 (Pp, Table 14). The mean ratio of pollen over seed dispersal distances was significantly higher than expected with random parents assignment in P1 and P5, where pollen was dispersed 17 times (respectively 5 times) more than seed. This ratio was much greater for sessile (25) than for pedunculate (9) parent pairs in P1, the opposite trend is observed in P5. However, these values are estimated on a small number of observation and should therefore be taken with caution.

Table 14: Dispersal distances (parent pair assignments). hyb: number of hybrids *Q.p.*×*Q.r.* Self: number of selfing events. Pp: probability of having observed a higher mean pollen distance with parents assigned randomly. Pr: probability of having observed a smaller mean pollen/seed distance ratio with parents assigned randomly

	Pairs				Mean distances (m)			Pollen/seed ratio			
Partner # / country	*Q.p.*	*Q.r.*	hyb	self	seed	pollen	Pp(%)	mean	Pr(%)	*Q.p.*	*Q.r.*
P1 France	9	3	1	1	9.72	81.34	1	17.43	0	24.58	9.29
P2 Italy	89	2*	6	12	108.82	206.35	0	10.92	6	5.65	8.35*
P4 Spain	-	3	-	3	-	-	-	-		-	-
P5 Great Britain	24	3	24	3	154.55	110.48	0	4.85	1	3.73	5.11
P8 Denmark	0	2	0	0	66.34	86.03	42	2.76	34	-	-
P10 Sweden	-	30	-	2	110.47	178.65	0	5.82	16	-	-
P12 Hungary	0	2	0	1	37.39	119.83	69	4.35	38	-	3.40

**Q. pubescens*

When a sufficient number of observations were available, the seed and pollen dispersal distributions were fitted with a one dimensional exponential power function. Estimations of mean distance travelled by seed or pollen (δ) and of shape parameters (*b*) are given in Table 15.

Table 15: One-dimensional exponential power fit of seed and pollen dispersal distances inferred from parentage analyses. δ: mean distance travelled by seed or pollen, *b*: shape parameter.

Partner # / country	Seed dispersal exponential power fit [CI 95%]		Pollen dispersal exponential power fit [CI 95%]	
	δ (m)	*b*	δ (m)	*b*
P1 France	53.11	0.80	85.47	3.39
	[41.17, 71.36]	[0.50, 1.35]	[50.14, 175.32]	[0.28, #]
P2 Italy	193.31	0.59		
	[162.50, 237.37]	[0.47, 0.76]	*	*
P4 Spain	-	-	-	-
P5 Great Britain	143.16	5.62	112.98	0.51
	[131.05, 154.56]	[3.91, 8.72]	[72.92, #]	[0.10, 1.08]
P8 Denmark	54.74	1.16	-	-
	[37.93, 88.88]	[0.5, 6.06]		
P10 Sweden	160.71	0.98	160.98	0.69
	[125.76, 212.69]	[0.64, 1.50]	[106.02, #]	[0.10, 2.41]
P12 Hungary	-	-	-	-
All stands (except P4)	153.69	0.86	191.76	0.35
	[140.60, 169.40]	[0.74, 1.01]	[151.57, #]	[0.1, 0.57]

* no possible fit
# no convergence
- low number of data

Except in Italy (P2) the shape parameters of seed dispersal were not smaller than 1, indicating light tailed dispersions for acorns. The curve obtained with all dispersal data gathered together (Table 15) indicate the same trend, with the value of 1 being part of the 95% confidence interval for the seed dispersal shape parameter. Pollen dispersal could be studied on less stands due to smaller parent pair assignment success. However, when gathering all pollen dispersal data available, a fat tailed distribution was observed with a mean distance travelled by pollen of 192 m (154 m for acorns) and a shape parameter significantly smaller than one (0.35 to be compared to 0.86 for seed).

2.2.3 Discussion

We compared seven different seed and pollen dispersal situations in white oak stands spread all over Europe. The high polymorphism of the nuclear microsatellite markers used (single parent and parent pairs exclusion probabilities reaching almost 100%) allowed us to expect correct assignment of parent(s) to seedlings. According to simulations, after maximum likelihood ratio tests, single parent (parent pairs) were assigned correctly to

seedlings with a rate averaging 71% (79%), with quite variable associated type I and II errors according to the stand considered.

On average, 39% of seedling had no parent, 47% had a single parent (assumed to be the mother) and only 14% had a mother and a father assigned. Seed and pollen flow were deduced from these assignments: acorns came from outside the studied plot in almost 40% of the cases while pollen flow reached a high value, 86% on average, representing a mean overall gene flow of 63%.

As in the paternity analysis, the reproductive success of assigned parents in all stands follow L-shape distributions with few individuals siring many offspring and many individuals siring few offspring, often a single one. Globally, this distribution trend results in a slightly more than one over two assigned parents efficiently reproducing through seedling.

The mean seed dispersal distances deduced from single parent assignments appeared significantly restricted in almost all stands, as was the pollen dispersal when a sufficient number of parent pair assignment events could be recorded.

Hybridisation corresponds to 3% of the total number of seedling analysed in stands with mixed species, representing less reproducing event than in the paternity analyses. Selfing was almost 2%, a value equal to the one detected in paternity analyses (2.3%).

The seed and pollen dispersal curves observed and fitted to a one dimensional exponential power function were deduced from parent assignments when a sufficient number of observations were available. In both paternity and parentage analyses, pollen exhibited a fat tailed distribution, with a global mean distance travelled by pollen higher in parentage (192m) than in paternity analyses (117m). Seed were dispersed according to a light tailed distribution (with a global mean dispersal of 154m), so even if acorn immigration was high, dispersal modelled inside the stands gave less importance to long distance than pollen, a sound result for a species with wind-dispersed pollen and acorns subjected to barochory.

2.2.4 Conclusion and perspectives

Our data show a general trend in effective within-stand pollen dispersal of wind-borne white oak pollen. Stand-specific and overall pollen dispersal curves largely confirm the dispersal curves of other wind-pollinated tree species. But as the data for external gene flow and that from the large-scale analysis in the Spanish plot indicate, plot-related pollen flow only accounts for a fraction – though prevalent – of effective pollen dispersal. Seed flow was apparently also very high according to parentage analysis in several stands (40% of acorn

immigration), and higher than expected for a species with large seeds: mechanisms other than dispersal due to gravity (barochory) must occur in oaks. The dispersal action of jays could be one explanation (Bossema 1979) and might account for the very long dispersal events (several km) detected in one stand of our study.

Gene flow, combining seed and pollen dispersal in the parentage analyses, was in the same order of magnitude than pollen flow in the paternity analysis. However, pollen flow appeared much higher when analysing the seedling stage than the acorn level (86% to be compared to 60%). Some mechanisms must act from seed to seedling stages to increase apparent gene flow and favour for instance immigrants. One hypothesis, that should be studied, could be that pathogens or herbivores associated to mother-trees are more adapted to seedlings related to the mother-tree than to immigrants, favouring the settlement of the latter (Janzen-Connel hypothesis (Janzen, 1970; Connell 1971) explaining species diversity in tropical forests adapted to an intraspefic level). Alternative approaches using assignment tests at a landscape level could further elucidate how genes are transferred also among stands.

2.3 Past artificial seed transfer

Long distance transfer of oak seeds has occurred in the past in Europe. These transfers were made mainly from southern to northern latitudes. For example Slavonian oaks (Croatia) have been used for afforestation in Germany and France in the last century (Kleinschmit, 1993). New genes were therefore imported that have likely been dispersed into native populations by pollen flow. Artificial seed transfer were retrospectively tracked by using cpDNA fingerprints in two different spatial scale, local and regional. Suspicion about historical transfer came from the intensive survey of cpDNA polymorphism across Europe made within the FAIROAK project. Within OAKFLOW, detailed analysis were conducted in those areas to confirm or invalidate earlier suspicions

2.3.1 Past artificial transfer at the local scale

At le local level, case studies were investigated in detail in the following known forest stands: Forest of Montecorrona in Italy, Forest of Manhartzberg in Austria, Forêt de Compiègne in France, Dean Forest in England, The Veluwe Forest in the Netherlands, Forest of Ujszentmargita and Kerecsend in Hungary. The results are illustrated here with two examples illustrating how cpDNA analysis confirmed the historical suspicion about the past artificial introduction of foreign material.

2.3.1.1 Case study in The Veluwe (Netherlands) (Buiteveld and Koelewijn, 2006)

The objective of this study was to characterize, retrospectively, artificial seed transfer in The Veluwe. Chloroplast DNA variation was assessed in planted and autochthonous oak stands in the Veluwe. The hypothesis was that the putative autochthonous stands at the Veluwe are monotypic, while the planted stands diverge from this natural pattern and are polytypic (cf. Petit et al., 1997; König et al., 2002). However, if planted stands have a local seed source, it is expected that they show a similar cpDNA composition and diversity as the autochthonous surrounding stands. Lastly, if autochthonous stands are geographically structured at a regional scale, spatial genetic structure and a positive association between genetic and geographic distance (isolation by distance) is expected. On the contrary, among planted stands, no or substantial less spatial structure is expected.

The following results were obtained:

(1) Five haplotypes (haplotype 1, 7, 10, 11 and 12) were detected in the planted stands at The Veluwe (Table 1). All five haplotypes belong to the most common ones across Europe. Four of these (haplotype 1, 10, 11 and 12) are considered to be autochthonous for the Netherlands. The most frequent haplotypes, both in presence as well as in average frequency, were 1 (23 out of 28 stands; average frequency 31%) and 10 (22 out of 28 stands; average frequency 29%). In 9 out of the 28 stands haplotype 7 was detected. Haplotype 7 is very rare in The Netherlands (only found in the southeast) and probably introduced (König et al., 2002). This haplotype occurred in a low frequency (5%) in the stands. None of the planted stands were monotypic (fixed for one haplotype). Four stands consisted of a mixture of 2 haplotypes, with one of them being dominant. The other stands were composed of more than 2 haplotypes. A significant difference between planting period and haplotype frequency was observed ($\chi^2_8 = 54.18$, $P < 0.001$). Haplotype 1 was more frequently planted before 1900, while haplotype 11 was planted more frequently between 1900 and 1948. In three stands planted before 1900 a dominant haplotype was detected (80 – 94% of the trees possessing the dominant type).

(2) In the autochthonous oak stands at the Veluwe four haplotypes (1, 10, 11 and 12) were detected. Haplotype 7 was not present in the autochthonous material in this region of The Netherlands. The predominant haplotypes in the autochthonous stands at The Veluwe are haplotype 10 and 12 with frequencies of 47% and 33%, respectively. Twenty-three stands (46%) out of the 50 autochthonous stands were monotypic. The geographical distribution indicates some spatial structure. Haplotype 10 and 12 are common in the northern part of

the Veluwe, while haplotype 1 is mainly restricted to the southeast part. Haplotype 11, which is known for its scattered distribution, occurs only in one stand in the middle of the Veluwe. No difference in haplotype frequency between the two oak species was observed (Table 2) ($\chi^2_3 = 3.48$, P = 0.3).

In summary, the results indicated substantial human influence on the distribution of genetic diversity in native oak stands at the Veluwe and that remnants of the ancient primeval oak forests are very rare nowadays. First, a non autochthonous haplotype, originating from the Balkan, occurs in several planted stands across the Veluwe. This suggests long-distance seed transfer. Second, in contrast to old forests elsewhere in Europe the putatively autochthonous Dutch oak stands have a higher within-stand diversity. The diversity within the planted stands is even higher, suggesting the use of mixtures of (local) seed material of various origins.

2.3.1.2 Case study in Forest of Dean (England) (Cottrell et al., 2004)

In the previous FAIROAK project the results of the cpDNA analysis identified several woods which had the non-Iberian haplotype 7 in the Forest of Dean area. As part of the OAKFLOW project this area was studied in greater detail. A database listing the age of the oak woods in the Forest of Dean was obtained from Forestry Commission headquarters and a sampling strategy was devised in which five trees were sampled from fifty woods with planting dates ranging from 1720 to the present day. The aim of the work was to determine whether there was evidence for the introduction of haplotype 7 at some specific date in history and to see whether cpDNA could provide any further insight into the influence of Man on these oakwoods. The sampled woods were divided into three age categories for analysis i.e planted between 1720-1868, 1869-1941 and 1942-1943.

The results shown clearly demonstrate that haplotype 7 is present in abundance in the oldest woods that were sampled. Woods which contain a single haplotype are generally considered to have experienced minimal levels of human interference and haplotype 7 is the only haplotype to be represented in such woods planted between 1720 and 1917. Haplotype 10 only occurs in single haplotype woods established after1942 and these are known to have been planted by Man. The abundance of haplotype 7 and its presence in the oldest category of single haplotype woods suggest that its presence is the result of the natural postglacial recolonisation process and not the product of human mediated transfer. The nearest other locations where haplotype 7 occurs are in scattered individuals about

300km away on the north coast of France. If its presence is indeed not human-mediated, this represents a considerable distance for a single postglacial recolonisation event to have occurred. There are however other examples, elsewhere in Europe, where equally long distances separate of identical haplotypes.

Planting date had a major effect on the frequency of haplotypes in the Forest of Dean woods. In the two planting periods from 1720-1868 and 1869-1941 haplotype 7 was the most frequent haplotype. Haplotype 11 was extremely rare during these two planting periods and was only present in 1% of the sampled trees. The youngest woods were planted between 1942-1993 and these showed a very different distribution of haplotypes from the older woods Haplotype 10 was the most frequent and haplotype 7 was only present in 19% of the youngest woods. The frequency of haplotype 11 had also increased to 10% during this planting period.

These statistics suggest that there were major changes in species and haplotype frequencies between the last two planting periods. When the haplotype frequencies are expressed on an individual species basis it can be seen that there are also differences between the first and second planting periods. Haplotype 7 was consistently more frequent in *Q. robur* than in *Q. petraea*, although its frequency in *Q. robur* was lower in the second than the first planting period.

2.3.2. Past artificial transfer at the regional level

At the regional level, detailed investigations were conducted in Northern and Eastern Germany in the Basque region in Spain. Either earlier reports suggested large scale imports of oak seed from the Slavonian area (Kleinschmit, 1993) or results obtained within the frame of the last FAIROAK project suggested imports of foreign reproductive material.

2.3.2.1. Case study in Germany

Stands in Brandenburg (Kätzel et al., 2002)

In the Federal State of Brandenburg several forest areas have been declared ‘natural woods’ by law, with the aim to leave them for natural development. However, due to a long human influence on central European forests, a natural origin (autochthony) of the selected forests cannot be taken for granted, and in most cases history cannot be traced back very far. Therefore it appears convenient to combine investigations on stand history with genetic studies. In cooperation with the Landesforstanstalt in Eberswalde three oak stands were analysed (Fünfeichen, Kuckuckseichwald, Tauersche Eichen).

In the wood ‘Fünfeichen’ haplotype 7 was detected in 18 of 19 analysed trees, and in the wood ‘Kuckuckseichwald’ all 10 trees are carriers of haplotype 7. The origin of this haplotype is located on the Balkan Peninsula and this haplotype is also prevailing in the adjacent southeastern region from where oak presumably immigrated. Despite being rare in the analysed populations, haplotype 4 has its origin in the eastern refugium, too. Therefore it can be assumed with a high probability that these two stands have been regenerated naturally or with local material. For the wood ‘Tauersche Eichen’ however, a human influence can be assumed, since about half of the determined haplotypes were originating from the Balkan and half from the Iberian refugia. So seeds for regeneration have probably been mixed.

Stands in Northrhine-Westphalia (König and Stauber, 2004)

An interesting region for studying artificial seed transfer is the Federal State of Northrhine-Westphalia for two reasons. The woods have been exploited for a long time for charcoal production and ore melting. The arising lack of wood led to a transformation of many existing forests and the establishment of a coppice like silvicultural system called ‘Haubergswirtschaft’, favouring resprouting species, like oak. However, when the capacity for resprouting was exhausted, artificial replanting had to be done. Haplotype analysis can provide some insight if material from other regions has been used for this purpose. A second remarkable seed transfer was realized by introducing Slavonian oak. Foresters with a trained eye recognize stands of Slavonian origin. The trees have a straight stem, different branching characters than local ones, and most of them are late flushing. During summer 2003 in twelve compartments a varying number of trees (in total 105) of Slavonian and local type have been sampled and analysed during winter 2003/2004. These compartments were located in 4 forests (Schwerte / Cappenberg, City forest of Hamm / Pilsholz, Siegen / Eisern, Siegen / Freudenberg). In all twelve compartments the laboratory analyses confirmed the phenotypic assessment with regard to the allochthonous provenance of the material. The additionally analysed four single remnant trees (age between 200 and 600 years) revealed all haplotype 4, which is also the dominant haplotype in the previously coppiced stands.

2.3.2.2. Case study in the Basque region

Artificial seed dispersal has been mainly due to human activities. In Spain, plantations have been very uncommon until recently. Even now, oak plantations are very scarce and merely anecdotal, rarely exceeding a few hectares. Oak plantations data were compiled over the

last ten years, in two regions of the Basque Country (Gipuzkoa and Araba). CpDNA analysis was carried out in 5 individual samples from each of 38 *Q. robur* and 6 *Q. faginea* plantations, using 5 primer-restriction enzyme combinations. Comparison of planted haplotypes to the observed ones in natural populations of the same area clearly showed the presence of 5 “non-natural” haplotypes in these plantations. Specifically, haplotypes H1 and H7 from natural oak populations in the Iberian Peninsula are restricted to the east part of the Ebro Valley and mainly present in species such as *Q. pubescens*, *Q. faginea* and their hybrids; while haplotype H29 is predominant in the Mediterranean region (Olalde et al., 2002). Haplotypes H5 and H6, belonging to an east European maternal lineage, had not been previously found in natural populations from the Iberian Peninsula (Petit et al. 2002a). On the other hand, it has been previously shown that natural populations usually have one, at most two, haplotypes. Therefore, the great numbers of plantations showing three or more cpDNA haplotypes are a clear indication of mixed origins of the commercial seeds and/or saplings. Given that most of these plantations had origin certificates, it is clear the necessity for a stronger European regulation concerning translocation of planting materials.

3 Genetic and ecological consequences of gene flow and hybridization

3.1 Introduction.

The two most important oak species in Western European Forests, namely pedunculate oak (*Quercus robur* L) and sessile oak (*Q petraea* Mat. Liebl) are closely related and sometimes even considered to constitute merely distinct ecotypes within a large polymorphic species. Indeed the two species are sympatric over a large part of their distribution area, and are to a large extent interfertile. The two species are defined basing on their morphology (length of floral peduncle, and leaf shape).

The statistical distribution of phenotypes in the "*robur*" and "*petraea*" complex has been analysed within a variety of experiments, and in all cases the outcome was the same: the phenotypes were organised along an obvious bimodal distributions with the two modes corresponding to the "petraea" and the "robur" types with very few individuals displaying an intermediate morphotype, that could not be unambiguously assigned to one or the other phenotype. The most striking result was brought about by a European wide study that demonstrated that this clear distinction of phenotypes was present in all tested European forests, and that the two phenotypes were stable across Europe (Kremer et al. 2002), giving real sense to the species concept. Moreover, a large range of studies showed that even if the two species could be found in mixed stands, they usually segregate spatially, *Q robur* being present mainly on wet and fertile soils presenting sometimes hydromorphia, while *Q petraea* was growing in more acidic, deeper and more drought prone soils (Lévy et al. 1992).

Despite a large effort to detect specific neutral genetic markers for differentiation between the two species, none were found. For instance, (Bodénès et al. 1997) detected a significant frequency difference for only 2% of the 2800 RAPD marker they tested. Such observations, together with the demonstration of the occurrence of a significant gene flow among the two species, led to the conclusion that the genomic regions involved in species differentiation were rather small, or at least that they could not be detected with the neutral markers used until now. The strong background hypothesis was that the genes responsible for species differentiation were grouped in a small genomic region and could be exchanged between the two species in a coordinated manner, while the rest of the genome was common to the two species. One aim of the OAKFLOW project was to detect genomic regions potentially differentiating the two species by using tow complementary strategies:

1. to undertake a genome-wide scan using a large diversity of markers, computing the Nei's coefficient of genetic differentiation G_{st} and locating these regions on the linkage groups of a genetic map.

2. to detect functional markers, i.e., markers related to the known differences among the two species, basing on a QTL approach. Known differences among the two species include:

(i.) leaf morphology, based on a multi factorial analysis of several traits like petiole length, occurrence of pubescence, presence of intercalary veins (Dupouey and Badeau 1993; Kremer et al. 2002; Ponton et al. 2004);

(ii.) flower morphology, with the occurrence of a developed peduncle in *Q robur* while there is none in *Q petraea*; while this criterion is the basis for the definition of the species, it remains difficult to use due to late reproductive maturity in the species;

(iii.) water relations, and in particular water use efficiency; this latter trait is relatively easy to record from ^{13}C content in organic matter (Ponton et al. 2002; Ponton et al. 2001); *Q robur* consistently displayed a lower water use efficiency than *Q petraea*;

(iv.) tolerance to water-logging as demonstrated by the absence of *Q petraea* on temporarily water logged soils or by direct tests (Wagner and Dreyer 1997);

To identify genomic regions potentially involved in the expression of these traits, we used a Quantitative Trait Loci approach, based on a detailed linkage map established for a F1 full sib offspring of *Q robur*. The offspring seedlings were vegetatively propagated, planted in several experimental designs, and used to establish QTLs for traits related to leaf morphology, water use efficiency and related traits and tolerance to water logging. In the latter case, a preliminary experiment aimed at identifying the traits that were most relevant for the differences in tolerance in seedlings.

The occurrence of a large gene flow between the two species (preferentially from *Q petraea* to *Q robur*) should lead to the production of a significant number of hybrids displaying to some extent intermediate traits between the parental phenotypes. Nevertheless, such intermediate phenotypes are difficult to detect, as offspring usually display one of the two dominant leaf phenotypes. One of our goals was therefore to detect hybrids in natural populations using the parentage analysis developed by Gerber et al. (2003) and test their vigour in comparison to the parent species. This was done in two trials of which the first was established in a forest in Great Britain, and the second in a common garden plantation at

Nancy (France). Finally, several attempts were made to produce hybrids through controlled pollination and to plant the offspring.

3.2 Material and methods

3.2.1 Material and experiments

3.2.1.1 Mapping populations

An intraspecific hybrid famly of *Q robur* was created at INRA Pierroton by crossing two parents, the female being at Pierroton and the male from nearby Arcachon. A F1 generation was obtained, comprising 278 individuals, that were used to create a clonal bank as stool beds. At age 5, all stool beds were coppiced, and the resprouts used for vegetative propagation. The rooting procedure was carried out recurrently over several years to provide planting stock for further experiments that were used within OAKFLOW:

Population 1 comprised 174 full sibs transplanted during Spring 1999 to a field test site at Bourran, close to Bordeaux. The test comprised 1080 cuttings (on average 6 replicates per genotype), planted in 36 incomplete blocks, each block containing single vegetative replicates of 30 full sibs.

Population 2 comprised 216 full sibs propagated during 1998, (1530 cuttings, i.e., 7 copies per genotype on average) was used under a fully randomised design for leaf morphology assessment.

Population 3 comprised 207 full sibs propagated during 1998 and planted during 2000 at Bourran with an incomplete block design (183 blocks with 12 cuttings each, i.e., 2196 individuals)

Population 4 comprised 183 full sib genotypes (2 copies of each) transported to Nancy, and transplanted into 10L pots. Plants were assigned to two glasshouses (1 copy of each genotype n each). The plants were submitted to either 380 (ambient) or 680 (enhanced) vpm CO_2.

Population 5 comprised 120 full-sib genotypes (3 replicates of each) assigned to a glasshouse experiment at INRA Nancy for testing water-logging tolerance.

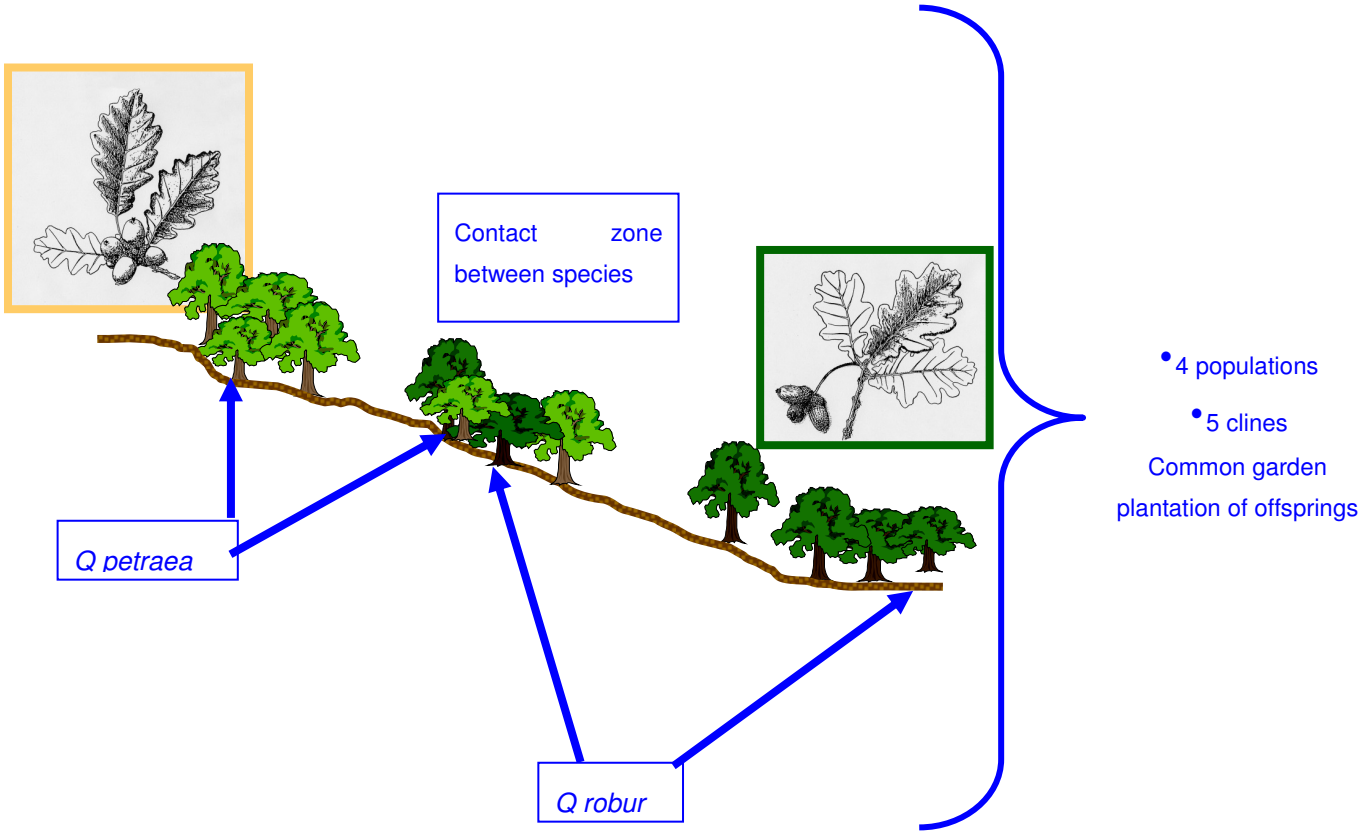
Population 6 was planted in an open field trial at Champenoux, close to Nancy. Five replicates of 257 full sib genotypes were planted in a incomplete block design with 52

blocks. The trees suffered severe transplanting stress due to the 2003 drought, and to repeated attacks by rodents, and no measurements are yet available.

3.2.1.2 Common garden experiments

Acorns were collected under individual trees in 5 forests along a North South gradient in Lorraine. In each forest, a cline ranging from bottom land with temporary water-logging up to hill tops with deep draining soils was defined. Three plots were identified: 1. bottomland with only *Q robur*; 2. intermediate with adjacent populations of *Q robur* and *Q petraea* and 3. hill tops with only *Q petraea*. The distance between the extremes was around 1-2 km. In plots 1 and 3, ten adult trees were identified and their species determined based on peduncle length and on leaf morphology, and in plot 2 10 *robur* and 10 *petraea* were identified with the same criteria (i.e., 100 *petraea* and 100 *robur* trees were identified and tagged over the five forests). Under each tree, about 100 acorns were collected. A common garden plantation was established with 12 acorns from each mother tree, resulting in 200 half sibs of 12 individuals each

Figure 1: Typical topographic cline with the distribution of the mother trees used for sampling off-springs and create a common garden plantation.



We used a randomised block design. Seedlings were planted during Spring 2001 and left growing freely until Autumn 2003 over three growing seasons. During autumn 2003, they were harvested for shoot biomass, leaf area, leaf mass to area ratio, leaf morphology, C and N contents and $\Delta^{13}C$ measurements.

In addition, the offspring from 2 of the 5 forests were tested using 6 microsatellites for their species, based on a parentage analysis: the mother's genotype being known, a probability computation (Gerber et al. 2003) allowed to assign each seedling to one or the other species or to identify it as a hybrid.

We searched (i) which traits were differentiating the two species and (ii) whether the intermediate populations (collected on plots 2) consistently differed from pure populations (plots 1 and 3) within each species.

3.2.1.3 ISPs (Intensive study plots)

As described in paragraph 2.1.1.1

3.2.1.4 Controlled environments

Measurements aiming at detecting QTLs and at comparing the intrinsic responses of species to environmental constraints were conducted in greenhouses of climate chambers with potted plants (seedlings or rooted cuttings). In greenhouses, all plants were submitted to natural irradiance and to temperature fluctuations.

3.2.2 Molecular and genetic methods

3.2.2.1 QTL detection

PCR based molecular markers and the two-way pseudo test-cross strategy are useful tools for constructing genetic maps in forest trees (Grattapaglia and Sederoff 1994). Full-sib and half-sib crosses can, therefore, be used to construct single-tree genetic linkage maps thanks to dominant PCR based molecular markers. Following this approach, three types of segregation configurations can be obtained for dominant molecular markers in the mapping population: 1) male test-cross markers, segregating in a 1:1 ratio, and inherited from the male parent; 2) female test-cross markers, segregating in a 1:1 ratio, and inherited from the female parent; and 3) intercross markers, segregating in a 3:1 ratio, and inherited from both

parental trees. Male and female test-cross markers are used to construct two independent single-tree genetic maps that are then aligned thanks to the intercross markers.

Starting in 1995, activities in genetic mapping were implemented in European white oaks at the INRA Research Centre in Bordeaux-Cestas (France). Motivations for genetic mapping in oaks were threefold: (i) the detection of genomic regions involved in species differentiation, (ii) the detection of QTLs controlling traits of adaptive significance, (ii) the comparative analysis of genomic evolution in the Fagaceae. The whole mapping project is based on three pedigrees: one full-sib family of *Quercus robur* (3P x A4), one full-sib family of *Q. petraea* (QS28 x QS21), and one interspecific F1 full-sib family *Q. robur x Q. petraea* (11P x QS29). Given the objectives of the mapping experiments, the parents of the pedigrees were not selected for any particular criteria. The *Q. robur* parent trees originated from the Southwest of France (INRA research station of Bordeaux-Cestas, and Arcachon) and the *Q. petraea* parents were from the central part of France (INRA research station of Orléans-Ardon). The controlled crosses were repeatedly done over successive years until 2004. From 200 to 1000 seeds were obtained for each cross. The young seedlings were installed in a seedbed in a nursery where they were raised as stool-beds. Starting at age 5, the full-sibs were hedged every year at ground level at the end of winter time. Following the hedging, stump sprouts developing in spring were harvested and divided in 15 to 20 cm long cuttings. These cuttings were then transplanted in field tests for phenotypic observations and further QTL detection. For the time being only the *Q. robur* intra-specific cross has been fully exploited for genetic mapping and QTL detection. The clonal test of the full-sibs has now been planted in three different sites (two near Bordeaux, South-West France and one near Nancy, North-Eastern France). The genetic mapping of *Q. robur* mapping was done on a sample of 94 offspring (pedigree 3P x A4), and the QTL detection on a sample of 278 offspring (replicated on average in five vegetative propagules).

The *Quercus robur* map was published in 1998 (Barreneche et al. 1998) (pedigree 3P x A4). Using the pseudo test-cross mapping strategy, two maps were constructed comprising 307 markers (271 RAPD, 10 SCARs, 18 SSRs, 1 minisatellite, 6 isozymes and 1 ribosomal DNA marker). Both maps provide 85 to 90% coverage of the Q. robur genome. Segregating markers could be aligned in 12 linkage groups, and the map size amounted to 893.2 cM for the paternal and 921.7 cM for the female map. This map was further upgraded by the inclusion of new SSRs (Barreneche et al. 2004) and additional AFLP and STS within

OAKFLOW (to date 854 markers (271 RAPD, 457 AFLP, 10 SCAR, 59 SSR, 49 EST, 1 minisatellite, 6 isozymes and 1 ribosomal DNA marker).

QTL approaches are based on composite interval mapping with Multi QTL (Britvin et al. 2001)

3.2.2.2 Genome scanning

A genome scanning approach was developed based on a set of 389 markers (isozymes, AFLPs, Microsatellites and SNPs) for which allelic frequencies were estimated based on pairs of populations (*Q robur* vs *Q petraea*) at close geographic distance (usually below 50 kms). Nei differentiation index G_{st} was computed for all markers across all populations. The same mapping pedigree was used to construct a consensus G_{st} map, which comprised 527 markers distributed over 980 cM. Among those, 158 were used for their Gst value and the distribution of interspecific Gst values along the linkage groups was established. See Scotti-Saintagne et al. (2004b) for details on computation procedure.

3.2.2.3 Differential gene expression analysis

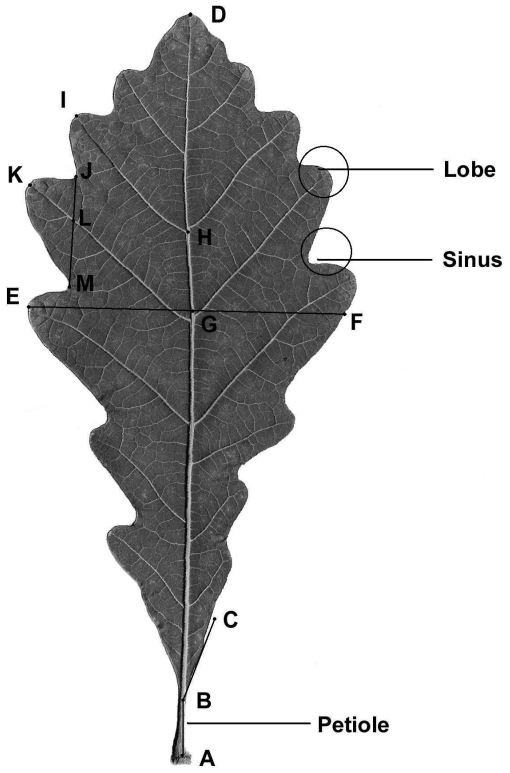
Differential gene expression studies were conducted to monitor the level of gene expression under contrasting environmental conditions or between the two study oak species (*Q. petraea* and *Q. robur*).: suppressive substractive hybridization (for genes expressed under osmotic induced stress) or cDNA –AFLPs (for genes expressed under hypoxia conditions)

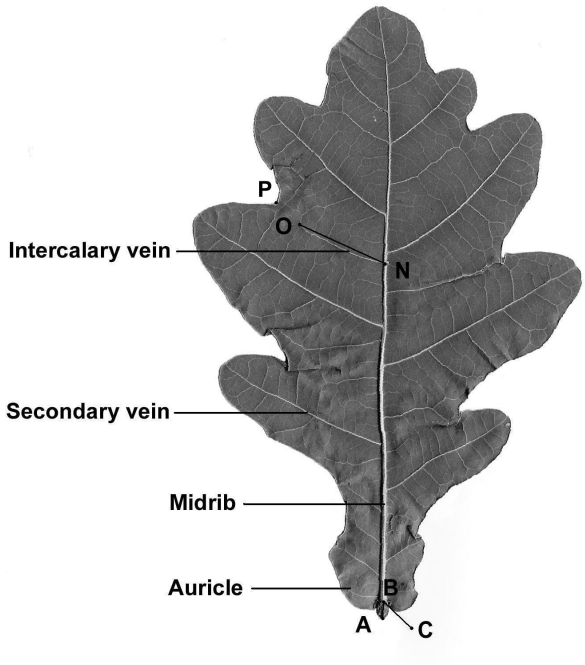
3.2.3 Biological assessments

3.2.3.1 Leaf morphological traits

Fifteen morphological traits were recorded according to the procedure described by Kremer et al. (2002). Traits included among others petiole length, number of intercalary veins, pubescence that are known to be very powerful for species differentiation (Dupouey and Badeau 1993). The analysis was applied to leaves collected from populations 1 and 2 during Summer 1999, and to seedlings from the common garden experiment to differentiate the phenotypes of the seedlings.

Figure 2 : Main morphological traits recorded on leaves of the two oak species (left, *Q petraea*, right, *Q robur*). The most discriminant traits are: the relative petiole length (AB/AD), the relative depth of the sinuses (KL), the occurrence of intercalary veins, the presence of an auricle (angle ABC) and the degree of pubescence. From Ponton et al, 2004.





3.2.3.2 Water-use-efficiency and Delta.

Water use efficiency (W) was estimated using the natural abundance of stable carbon isotopes ($^{13}C/^{12}C$ expressed as $\Delta^{13}C$) in organic matter. Plant organic material is usually depleted in ^{13}C relative to the atmosphere due to isotopic discrimination during photosynthesis as predicted by the model of Farquhar et al. (1989). The intensity of the discrimination is linearly correlated to water use efficiency according to:

$$W = C_a / 1.6\, v\, (b - \Delta)/(b-a)$$

With :

C_a: CO_2 concentration in the atmosphere

v: vapour pressure difference between leaf tissues and the air

Δ : carbon isotope discrimination

b and a: discrimination factors related to the diffusion through the stomata and to carboxylation..

During summer 2000, 2 leaves were harvested from each individual of population 1. During summer 2001, leaves were similarly harvested from 120 full sibs of population 1, and gas exchange (net CO_2 assimilation rate, A, stomatal conductance to water vapour, g_s) was recorded during 3 successive days on the same plants. Responses of g_s to water vapour deficit in the air were modelled, and the slope of the negative relationship used also for QTL analysis. Finally, during 2002, leaves from 207 full sibs (with 5 replicate clones of each) were harvested from population 2 for $\Delta^{13}C$ analysis. Leaf mass-to-area ratio, N content, chlorophyll content were recorded on the same leaves during 2001 and 2002). Detailed procedures are described in Brendel et al (2008).

The rooted cuttings from the same mapping family were transferred to Nancy during 2000, and grown in a greenhouse under two levels of atmospheric CO_2 concentration : 380 ppm (i.e., present concentrations) and 680 ppm (concentrations expected during the middle of the 21st century). Growth, leaf structure, gas exchange and $\square^{13}C$ composition were recorded on 183 genotypes (1 replicate in each treatment).

3.2.3.3 Water-logging tolerance

Three experiments were developed to analyse traits related to water logging-tolerance in oaks. The first aimed at monitoring morphological, physiological and molecular changes induced by root hypoxia in seedlings of the two oak species, in order to detect potential interspecific differences and to infer which could play a role in tolerance to water-logging.

During the second one we analysed QTLs of traits potentially involved in tolerance to water logging, using the same mapping population as above. Traits investigated included: number of adventitious roots formed, numbers of hypertrophied lenticels, and leaf chlorophyll content as indicator of stress intensity imposed to the trees.

The third aimed at analysing the intra-specific diversity of responses in the two species and to compare the degree of diversity in both cases.

Water logging was in all cases imposed by submerging potted seedlings with degassed water; oxygen content in the water was recorded with a specific electrode during the course of the experiments. The oxygen content usually dropped to values below 3 mg l-1 in the water table in close vicinity to the rhizosphere. This corresponds to a severe hypoxia.

The main traits taken into account were:

Traits detecting the intensity of the stress really supported by the plants, including leaf bleaching (by recording leaf chlorophyll content in vivo with a CCM chlorophyll meter) and leaf epinasty (records of the angle between leaves and stem).

Morphological traits corresponding to active acclimation processes like the formation of hypertrophied lenticels at the base of the trunk and on main roots or the formation of adventitious roots close to the interface with the atmosphere. Lenticels were quantified and the hypertrophy stages assessed on an ordinal scale; adventitious roots were measured at the end of experiments (numbers, cumulated length and biomass). The potential occurrence of aerenchymatic tissues in main roots and below lenticels was also investigated; such tissues could contribute to the diffusion of oxygen towards the roots suffering from hypoxia.

Activities and transcript levels of enzymes implied in several metabolic steps were quantified. Activites of Alcohol dehydrogenase were recorded as index of the degree of hypoxia. Enzymes from the fermentative pathways (expected to be induced by hypoxia), from the glycolytic pathway (expected to be depressed) and from the carbon metabolism and carbon transfer were analysed (Parelle et al, 2006). Transcipt levels in roots were quantified using an RT-PCR, but the lack of homologous sequences resulted in poor results in many cases as amplification failed. The development of a detailed EST databases based on expressed sequences from a diversity of tissues in oaks (Derory et al, 2006; Porth et al, 2005a) will provide helpful tools to circumvent these initial difficulties.

3.3 Results and discussion

3.3.1 Comparative genetic mapping in *Quercus* and *Castanea*

Populations analysed : Subset of population 1 (94 trees)
Method: Genetic mapping
Publication: Kremer et al, 2006 ; Casasoli et al., 2006

Significant efforts were made during the Oakflow project for genetic mapping in relation to QTL detection. Additionally, as genetic maps could be aligned between *Quercus robu*r and *Castanea sativa,* the mapping activities were extended to the comparative analysis of maps and QTLs in the two genera.

3.3.1.1 Assignment of linkage groups in *Quercus robur* and *Castanea sativa*

About one hundred EST sequences were selected from oak databases. Oak sequences were aligned with homologous sequences obtained from GeneBank in order to design primer pairs for amplification in the most conserved regions of the sequence and assure a good cross-amplification efficiency in chestnut. A total of 82 primer pairs were designed. A proportion of about 70% produced by PCR a single and strong band both in oak and chestnut and 51 and 45 ESTs were mapped in oak and chestnut, respectively, using SSCP and DGGE approaches (Casasoli et al. 2006). These EST derived markers, together with SSR markers previously mapped (Barreneche et al.,2004) provided 55 orthologous molecular markers that allowed the 12 linkage groups of *Q. robur* and *C. sativa* to be aligned. From two to seven common orthologous markers were mapped in the 12 homeologous pairs of linkage groups. Macro synteny and macro colinearity were well conserved between the two species. Few inversions, probably due to mapping errors, were observed. Although these data are still preliminary given the low number of common molecular markers mapped in the two species, no major chromosomal rearrangements were identified suggesting that oak and chestnut genomes are quite stable.

3.3.1.2 Comparative analysis of QTL distribution in *Quercus robur* and *Castanea sativa*

The alignment of the 12 *Q. robur* and *C. sativa* linkage groups gives rise to a logical framework defined by common orthologous markers for comparing QTL location between the two species for traist that have been monitored in both species (Bud burst, carbone isotope discrimination and height growth). Details about the definition of common genomic intervals and corresponding unique QTLs between the two species (i.e. more individual

QTLs detected several times in the same genomic region in a single species) are reported in Casasoli et al. (2006). A total number of 34 common intervals were identified between the oak and chestnut genetic linkage maps thanks to the orthologous markers. Thirteen and 10 unique QTLs were identified for timing of bud burst, five and seven unique QTLs were identified for carbon isotope discrimination, and finally, five and six unique QTLs for height growth were identified in oak and chestnut, respectively. Among these unique QTLs, nine controlling timing of bud burst and two controlling height growth were co-located between the two species. No QTL involved in carbon isotope discrimination was co-located in the oak and chestnut map. When QTL number and effects were compared for the three traits between the two species, a similar genetic architecture was observed for adaptive traits in oak and chestnut (Casasoli et al. 2006). From this simple comparison it was clear that adaptive traits are controlled by more loci of low and moderate than large effect in both species. Timing of bud flush was the trait showing the higher number of detected and stable QTLs. Despite this similar genetic architecture, most of the QTLs for bud flush were conserved, whereas only a few QTLs were conserved for height growth, and none for carbon isotope discrimination.

3.3.2 Osmotic responsive genes in oaks

Populations analysed : Subset of population 1 (94 trees)
Method : Differential molecular screening
Publication: Porth et al, 2005a and b

Gene expression was monitored in cell suspension cultures originating from Q. petrae callus (Endemann and Wilhelm, 1999). Control cell cultures (K) were grown in P24 medium containing the concentration of salts described by Teasdale (1992) (hereafter P24-S medium), whereas hyperosmotically treated cells were grown in P24 medium containing four times the concentration of salts (hereafter P24-4S medium) for 1 h (1T) or for 2 days (2T)

Osmotic-stress-induced genes were identified in the *Q. petraea* cell line grown under moderate osmotic stress conditions.Two subtraction libraries were established from callus cells cultured under hyperosmotic stress for 1 or 48 h. Thirty-three differentially expressed sequence tags (ESTs) (from 70 originally isolated) were classified according to their putative functions. At least five of these gene products may contribute to osmoticstress tolerance in oak: betaine aldehyde dehydrogenase, two trans-acting transcription factors (one abscsic acid (ABA)- responsive, the other ABA-independent), a glutathione-

*S*transferase and a heat-shock cognate protein. Seven genes were selected based on their putative function and their expression monitored in vivo. Leaf tissue from *Q. petraea* and *Q. robur* plantlets grownhydroponically under hyperosmotic conditions was harvested after 0, 1, 6, 24 or 72 h and analyzed by real-time polymerase chain reaction (PCR). We found indications of osmotic stress adaptation in *Q. petraea* based on up-regulation of genes related to protective functions, whereas down-regulation of these genes was evident in *Q. robur*. Thus, genetic markers related to adaptive traits may be useful for differentiating *Q. petraea* and *Q. robur* genotypes

3.3.3 Hypoxia responsive genes in oaks

Populations analysed : Clones of Quercus petraea *and* Quercus robur
Method : cDNA-AFLP analysis
Publication: Unpublished data

Among the 4500 cDNA-AFLP screened fragments, 3.2% were differentially expressed between stressed and non stressed conditions of *Quercus robur* and *Quercus petraea*. 130 of these fragments were sequenced and analysed on data banks. Few sequences may be clearly involved in the response of hypoxic stress and are selected for expression analysis. Validation of selected transcripts for differential expression level between stressed and non stressed samples could be done by quantitative PCR. Preliminary results on quantitative PCR (Parelle et al., 2006) allowed to quantify the transcript level of PDC (Pyruvate DeCarboxylase, enzyme of the alcoholic pathway) for both species (*Q. robur* and *Q. petraea*) and other transcripts are currently analysed (work in progress by Cécile Sulmon). The perspectives of this work are (1) to do comparative mapping for different species of Fagaceae (*Quercus, Castanea* and *Fagus*) for transcripts which show differentially expressed level between stressed and non stressed samples and (2) to assess adaptative diversity in natural populations for both species.

3.3.4 Genome scanning for interspecific differentiation

Populations analysed : Population 1 and 3
Method : Genetic mapping
Publication: Scotti-Saintagne et al. 2004b

G_{st} values displayed a L shape distribution, with largest frequencies in the lowest range of G_{st} and very low ones for larger values. Forty seven marker (12% of the total) diverged from the expected neutral distribution, and these outlier markers were more frequently

SNPs,SCARS and isozymes than AFLPs and microsatellites, which comes again in support to the hypothesis that interspecific differentiation relies more to functional (coding regions of the genome) than to neutral markers. Only 20 of the 47 identified outlier loci displayed some polymorphism and could be mapped. The loci were distributed over 9 linkage groups, and displayed also a spatial autocorrelation of Gst values whenever they were separated by less than 2 cM. This study confirmed clearly that the genomes of the two related species are very close with most markers exhibiting very low levels of interspecific differentiation and that the species divergence between *Q petraea* and *Q robur* resides mostly in functional regions of the genome. In addition, the outlier markers were spread over the whole genome and could be detected on 9 linkage groups, with nevertheless a few hotspots (markers at short genetic distance) on linkage groups 12, 2 and 4.

3.3.5. Genomic regions involved in the control of leaf morphology

Populations analysed : Population 1, 2 and 3
Method : QTL detection
Publication: Saintagne et al. 2004

This study was expected to produce important results with respect to genome location of QTLs, as leaf morphology remains the most powerful tool to discriminate species basing on their phenotype. The use of a single species cross was thought to be relevant due to the large variability in morphology within each species, which was confirmed for the studied offspring. Indeed, an important clonal effect was detected on many leaf morphology traits including the ones know to discriminate species. Moreover, QTLs for several of the traits (13 among the 15 studied), including those involved in the differentiation of the species like mid leaf pubescence, petiole length and petiole ratio, or the presence of intercalary veins were detected and were significant at genome level (with a 5% threshold. But surprisingly, the detected QTLs each explained only a small fraction of clonal variance (11-14%). In addition, the small family size may have reduced the probability of finding significant numbers of the small effect QTLs: the detected QTLs are probably an underestimate of the whole range of QTLs present in this family.

An other surprise was the fact that QTLs of the five traits segregating the best the two species were spread all over the genome, being present on at least 6 linkage groups. Contrary to expectations, no single well defined genomic region is involved in species different, but contributing genes are probably scattered around the whole genome. Some of the QTLs were clustered (for instance on linkage groups 1F and 3F, or 9F), and we do not

know whether these clusters correspond to a single locus with pleiotropic effects or to linked loci contributing to different traits.

3.3.6 Genomic regions involved in the control of water use efficiency

Populations analysed : Population 1, 3 and 4
Method : QTL detection
Publication: Brendel et al, 2008

The isotopic discrimination against ^{13}C (expressed as $\Delta^{13}C$) within the full sib family displayed during the three years of measurements a large range of values with more than 3 permil. In addition, both a large repeatability within clones and a large broad sense heritability were recorded for harvests 2000 and 2002 (Table 16). Other leaf traits displayed smaller repeatability and heritabilities, with the exception of leaf mass to area ratio (LMA) which values were almost as high as those of delta.

Table 16: Genetic effects for leaf traits related to water use efficiency calculated using a variance components model. R: repeatability; h^2_0: broad sense heritability; $h^2_{½}$: narrow sense heritability.

Trait	Year	R	h^2_0	$h^2_{½}$
$\Delta^{13}C$	2000	0.774	0.545	0.338
$\Delta^{13}C$	2002	0.879	0.743	0.475
%N	2000	0.529	0.283	0.169
%N	2002	0.493	0.279	0.166
LMA_{ln}	2002	0.732	0.519	0.320
NA_{ln}	2002	0.716	0.502	0.309
LM_{ln}	2002	0.462	0.254	0.151
LS_{ln}	2002	0.653	0.419	0.255

The QTL analysis revealed a number of QTLs for $\Delta^{13}C$ used as an estimator of water use efficiency and for related traits. Some of these QTLs were clustered mainly in hot-spots on five linkage groups (LG 2, 8, 9, 11, 12) of which two (2 and 12) were already indicated above as containing regions differentiating the two oak species. Especially for $\Delta^{13}C$, QTL were detected that explained a large part of the observed variance (>20%). In addition, on LG 11, a significant QTL for $\Delta^{13}C$ was detected repeatedly over all three years of measurements with a large explanatory power (large PEV) and a large effect. This suggests, that, in contrast to the results for morphological traits, that $\Delta^{13}C$ and water use efficiency are rather oligogenic, that is controlled by a smaller number of large effect genes.

Table 17: QTLs detected for the different traits using the Multi-Environment procedure of MultiQTL. Only QTLs detected during at least two of the three measurement years are displayed**. map**: (female of male) on which the QTL was detected; **LG**: the linkage group on which it was found; **LOD**: the LOD score of the QTL, **L**, the position of the QTL on the linkage group (cM); **PEV**: the fraction explained variance during years 2000, 2001 and 2002, respectively; **effect**: the effect on the trait of the presence or absence of the allele; $\mathbf{L_{BS}}$: the position of the QTL as estimated from bootstrap analysis and $\mathbf{P_g}$: the level of significance of the QTL. (from (Brendel et al. 2008)
Abbreviations : %N : total nitrogen content in the leaves ; LMA: leaf mass to area ratio; NA: total nitrogen per unit leaf area; LS: leaf surface.

Trait	Map	LG	L	PEV			effect			L_{BS}	p_G
				0	1	2	0	1	2		
$d^{13}C$	M	2	93	5.6	14.6	4.4	0.277	0.566	0.248	92.4±4.4	0.0004
$d^{13}C$	M	8	51.1	13.0	6.9	2.3	0.438	0.371	0.177	44.4±11.3	0.0005
$d^{13}C$	M	9	0	2.7	0.3	4.7	†0.195	†0.073	0.26	8.9±16.5	0.0006
$d^{13}C$	M	11	64.2	20.5	33.5	19.2	-0.521	-0.905	-0.513	64.0±1.3	0.0004
$d^{13}C$	M	12	61.1	3.8	3.3	2.4	†-0.23	-0.254	†-0.187	57.3±9.65	0.0007
%N	M	8	40.8	13.3	5.3	0.2	0.125	0.106	†0.017	39.9±5.55	0.0005
%N	M	11	69.4	2.4	12.6	4.4	-0.053	-0.163	-0.078	60.2±17.8	0.0004
%N	M	12	57.4	2.4	11.0	4.0	†-0.053	-0.153	†-0.074	52.3±13.7	0.0007
%N	F	1	4.9	11.1	1.7	2.4	0.112	†0.055	†0.058	10.3±15.1	0.0006
LMA	M	5	52.6	-	0.1	7.5	-	-0.005†	0.037	54.2±20.6	0.0207
LMA	M	11	51.6	-	8.6	5.6	-	0.044	0.031	52.2±14.3	0.0004
LMA	F	9	67.6	-	7.4	8.2	-	0.045	0.040	67.7±3.01	0.0006
LMA	F	2	46.4	-	8.0	4.5	-	-0.148	-0.086	33.0±17.7	0.0005
NA	M	2	92.2	-	12.0	5.0	-	0.073	0.040	91.1±12.1	0.0004
NA	M	4	79.7	-	5.4	6.6	-	-0.048†	-0.047	69.7±17.8	0.0476
NA	M	12	47.9	-	21.4	1.9	-	-0.095	-0.025†	45.6±9.9	0.0007
NA	F	2	71.2	-	0	9.0	-	†-0.001	0.052	69.5±13.6	0.0188
NA	F	6	11.5	-	11.0	7.7	-	†0.067	0.048	21.5±16.7	0.0005
LS	F	2	43.6	-	9.6	7.0	-	-0.148	-0.103	37.8±12.2	0.0005

The hotspot on LG 11 for $\Delta^{13}C$ was detected again during the glasshouse experiments independently of the CO_2 treatment (Torti 2005, Torti et al., 2008). This is probably the genomic region that would need a more detailed analysis in the future.

3.3.7 Traits and genomic regions involved in interspecific differences in tolerance to water logging .

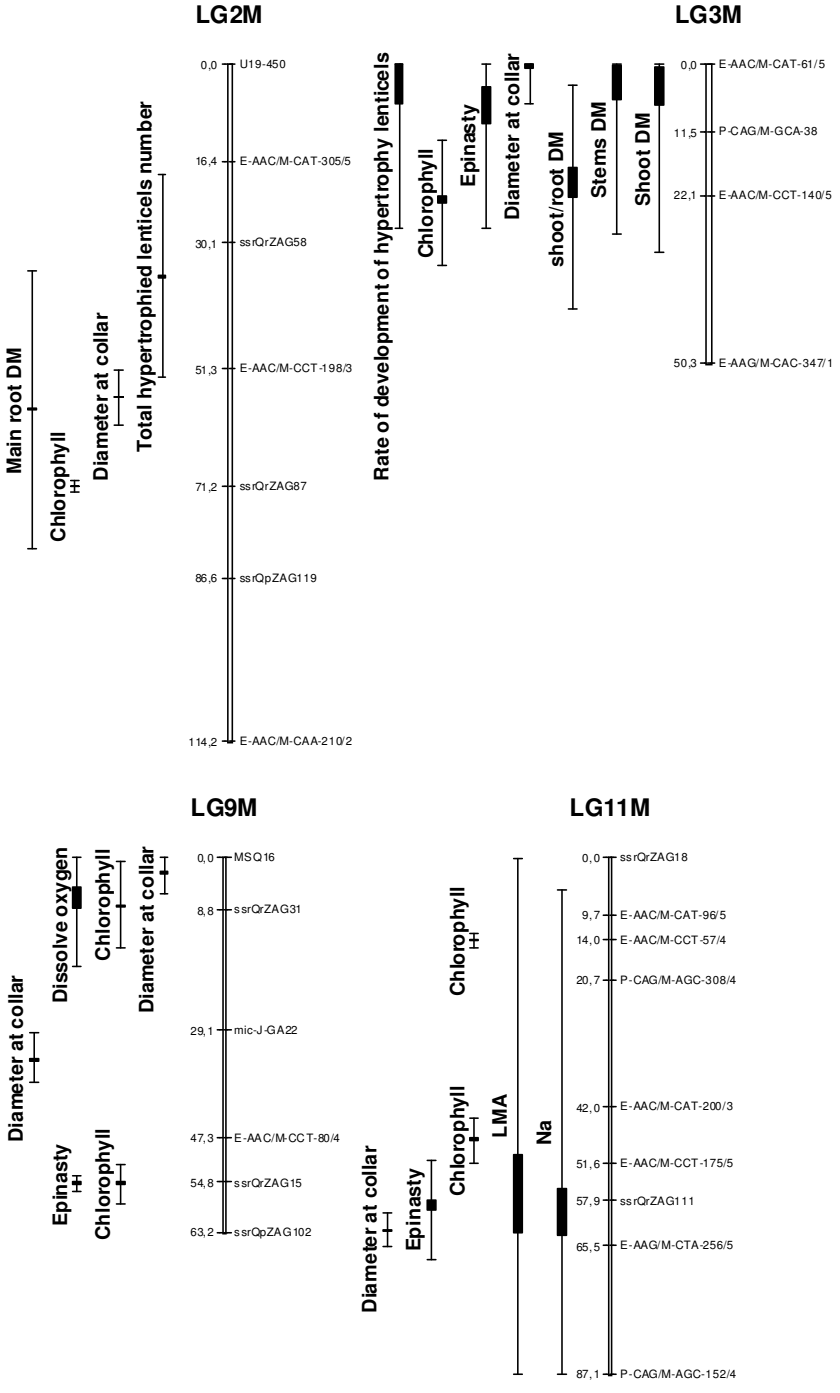
Populations analysed : Population 5
Method : QTL detection
Publication: Parelle et al, 2006 ; Parelle et al., 2007 a and b

We designed an experiment to identify morphological and physiological responses to root hypoxia that might differ between the two species. Potted seedlings were submitted during seven weeks to a water-logging treatment with 0_2 concentrations below 3 mg l^{-1} in the

vicinity of roots. The treatment induced growth cessation in both species. *Q petraea* displayed a lower tolerance as demonstrated by the larger number of seedlings suffering shoot dieback and leaf chlorosis as compared to *Q. robur*. This difference was probably related to the large number of adventitious roots and hypertrophied lenticels that were formed in *Q. robur*, compared to *Q. petraea*. In the fine roots of the two species, the activity of pyruvate decarboxylase (PDC), a key enzyme of the fermentative pathway, was stimulated after 24 h of hypoxia. Transcripts of PDC increased after 48h of hypoxia in *Q. robur* and not in *Q. petraea*. Interestingly, transcripts of haemoglobin (Hb) (possibly involved in the putative nitric oxide pathway) followed the same pattern of response than those of PDC. Enzymes of the sucrose degradation pathway displayed decreased activities after 3 weeks of waterlogging, probably due to decreased carbohydrate availability. Alcohol dehydrogenase (ADH), Susy, and pyruvate kinase (PK) activities were higher in *Q. robur* after 3 weeks of hypoxia. This study provided a set of markers characterizing the differences of tolerance between the two species which should be of use in further studies on intra and inter-specific variability.

In a second step, an experiment aiming to identify potential markers of tolerance to water-logging in this species and to assess the degree of genetic control over the corresponding traits was conducted. Quantitative trait loci (QTL) were assessed in a F1 progeny for responses to water-logging, and the relevance of the observed traits as markers of tolerance was investigated using a precise description of the time-course of their expression. Five significant QTL involved in the response to water-logging were identified (Figure 3). In particular, QTL were detected for the development of hypertrophied lenticels and for the degree of epistany but not for the formation of adventitious roots. A multi environment QTL model allowed a detailed description of the time course (7 weeks) of the allelic substitution effects of some of these QTLs. Correlation clustering identified significant clusters of QTLs at inter-trait as well as intra-trait level. Theses clusters suggest a succession the occurrence of genetically controlled response cascade to water logging.

Figure 3 : Genetic map of markers in centiMorgan and QTL position among linkage groups on which several QTL were identified at a short distance. Estimation of the position by permutation and bootstrap, and confidence intervals at 0.95.



The two oaks are sympatric oak and display different ecological requirements (in particular, *Q. robur* is more tolerant to water-logging than *Q. petraea*). This ecological divergence may play a role in the maintenance of the two species despite the lack of a tight reproductive barrier between them. Indeed, the genetic architecture of traits related to tolerance to water-logging probably differs between the two species. To gain insight in this architecture, and due to the lack of genetic markers for the tolerance to water-logging, we compared the diversity in populations of seedlings from the two species with respect to the expression of quantitative phenotypic traits induced by severe hypoxia. The ability of the two species to form hypertrophied lenticels was tested with a mastic impermeable to gases. Application of this mastic to stems of the two species during 2 months induced the formation of 3 ± 2 cm^{-2} hypertrophied lenticels independently of root hypoxia. The degree of leaf epinasty during root hypoxia (downward bending of leaves resulting from excessive growth of the upper side) was found to be an early predictor of seedling mortality, and, therefore, an efficient marker of sensitivity to water-logging stress. Four weeks of water-logging resulted in a larger degree of epinasty in *Q. petraea*, and in the formation of hypertrophied lenticels (16 ± 6 and 21 ± 9 cm^{-2}, for *Q. petraea* and *Q. robur*, respectively) and adventitious roots (2.7 ± 4.7 for *Q. petraea* and 5.2 ± 5.9 for *Q. robur*). Differences between species for these traits were due to differences in the frequencies of extreme phenotypes rather than to the existence of a general occurrence of tolerance to water-logging in the species *Q robur* , suggesting that both species share a similar genetic background for tolerance to water-logging and differ mainly in the frequency of extreme phenotypes.

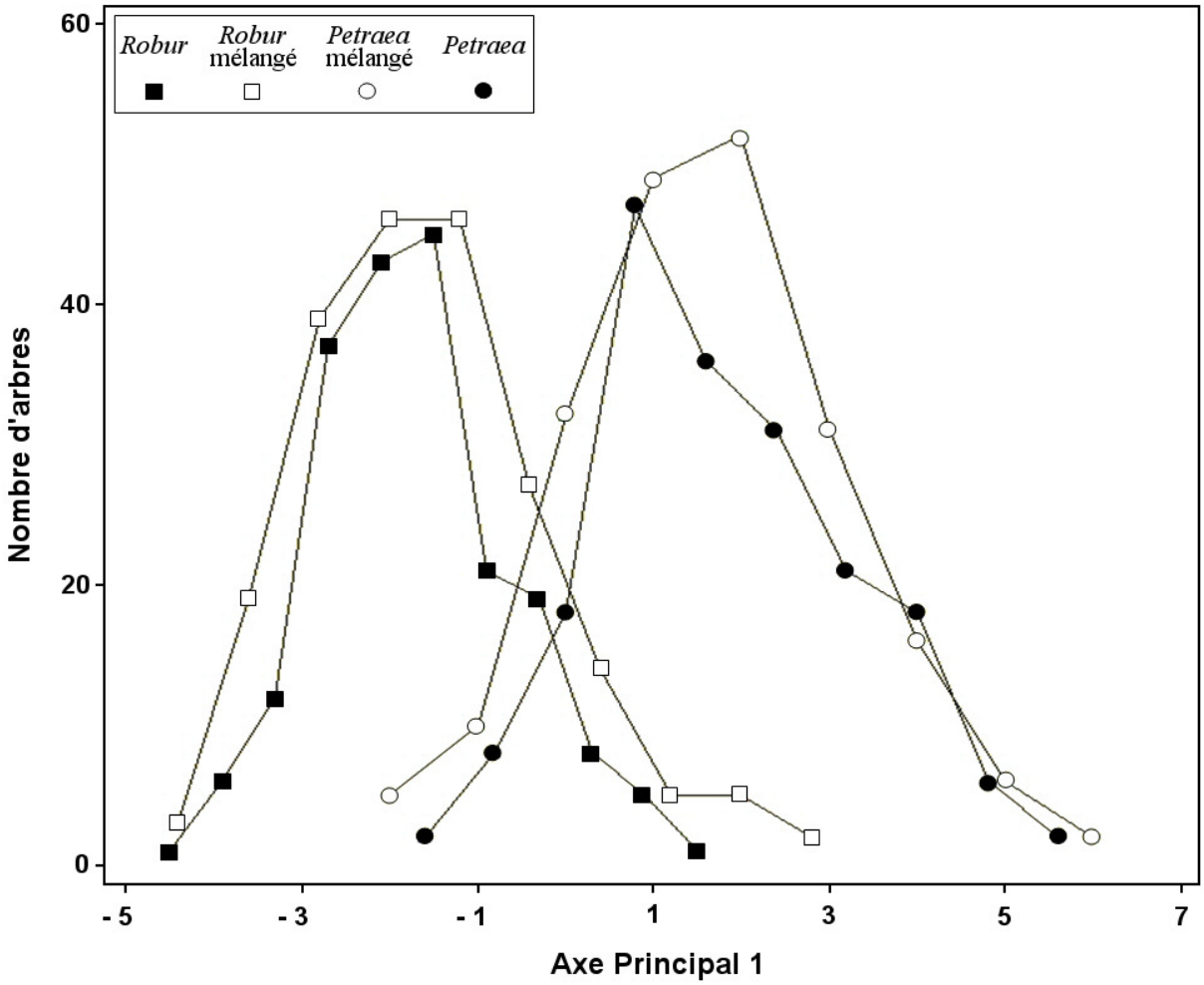
3.3.8 Inter and intra specific differences of functional traits in natural populations of oaks

Populations analysed : Common garden experiment
Method : Population comparisons
Publication: Faivre-Vuillin 2004. Faivre-Vuillin et al, 2008

The 200 families of half sibs installed in the nursery at Champenoux were harvested during Autumn 2003, and all structural and physiological parameters recorded overwinter. A few important conclusions could be drawn. The first is that the phenology of the offspring was very close to that of the mother tree, as shown by figure 4: none of the offspring from seed sampled below one species displayed a really divergent leaf phenotype. Moreover, as can

be seen in figure 4 , offspring of the populations from the pure stands were not significantly different from those of mixed stands. Only a few individuals from the mixed populations derived slightly from the maternal phenotype. These observations confirm again the now well established situations: in natural populations, whether from adult or from juvenile individuals, oaks display two well defined phenotypes with a low number of intermediate individuals. This does not preclude whether there are hybrid individuals within the tested populations; it merely means that the majority of individuals may be assigned to one or the other species based on their phenotype, and that this is true for pure as well as for mixed populations.

Figure 4 : Distribution of the saplings along the main axis of a factorial analysis of their leaf morphology. Left mode of the distribution: *Q robur*, with individuals from the mixed populations showing a slightly extended distribution with respect to those from the pure populations. Right: *Q petraea*, and the same remark applies. Individuals from the mixed populations overlap more than those from the pure populations. But the difference remains small



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A second important result from this study was to evidence a clear difference in leaf mass-to-area ration among the two species. The difference remains rather small but was significant. The population within each species did not differ significantly (lack of population effect). *Q*

robur always displayed a smaller LMA than *Q petraea*. This difference was never evidenced before; it was detected in this case due to the large number of individuals tested.

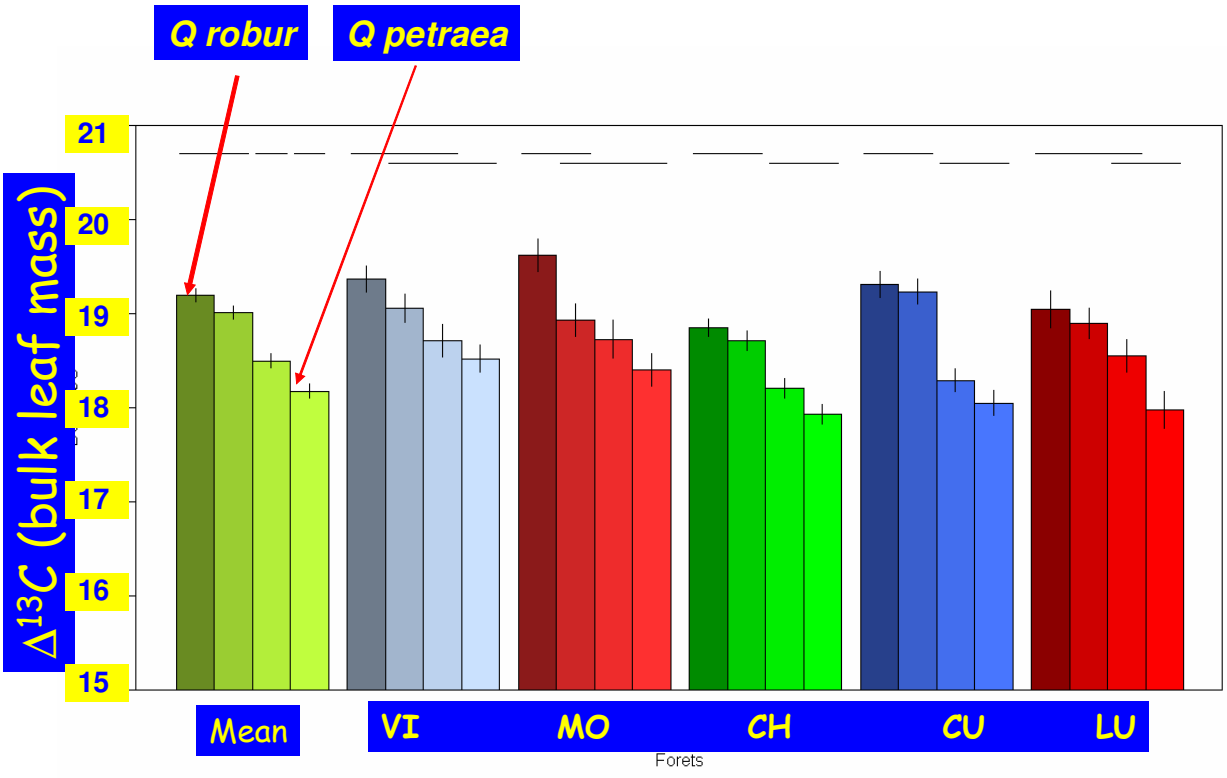
This study also confirmed on a large statistical basis that the two species differed in $\Delta^{13}C$, with a mean difference of about 0.9 ‰. The statistical analysis confirmed that this species effect was visible in all 5 tested forests, and was of a similar extent. This is therefore a very demonstrative confirmation of the result shown by Ponton et al, (2001, 2002): *Q petraea* systematically displays a smaller discrimination that *Q robur*, i.e., a higher water use efficiency. An additional and important result was that populations within species differed slightly but significantly. In *Q robur*, the population from the contact zone differed from that from bottomland with a smaller $\Delta^{13}C$ (i.e., a larger water-use efficiency); in *Q petraea*, the population of the contact zone similarly differed from that of top of the hills, with a smaller Wi. (figure 5). This was the first demonstration of the occurrence of a spatial structuration of oak populations with respect to the genetic component of Wi. It opens a series of questions:

1. is the difference between marginal populations and central populations due to the occurrence of hybrids in the marginal populations?

2. was the difference due to few outlier individuals or to a whole population shift?

To answer these two questions, a study was undertaken in order to assign the individuals from mixed stands to one or the other species or to hybrids using a range of microsatellites. The species assignment is currently done at INRA Bordeaux.

Figure 5 : Values of $\Delta^{13}C$ recorded in the leaves of 3 years old seedlings originating from pure populations of *Q robur* (extreme left) or *Q. petraea* (extreme right), or from mixed populations (centre, with *robur* on the left and *petraea* on the right). N= 150 ndividuals per treatment, means and SE (Faivre-Vuillin et al, in prep). The first series of bars represents the mean. VI, MO, CH , CU and LU indicate 5 forests along a North South gradient in Lorraine.



3.4 Conclusion and perspectives.

1. We confirmed that markers for interspecific differences among the two oak species are probably functional markers rather than neutral ones. Several elements support this conclusion: the fact that SNPs displayed larger values of genetic differentiation index (Gst) than microsatellites and AFLPs is one of them.

2. The functional markers involved in species differentiation are not grouped on a small fraction of the genome, but spread over the whole genome. This was confirmed by the mapping of Gst values for different markers (no hot spot with high Gst values but a few outlier markers, with associated neutral markers that displayed a linkage disequilibrium (at a distance below 2 cM). It was also very clearly confirmed by QTL analysis of traits that usually differ among species. All groups of traits resulted in significant QTLs that were spread ever the genome. For instance, QTLs were found for different leaf shape parameters, with a high heritability and a high clonal effect; their QTLs were present on 6 different linkage groups. Water use efficiency that was confirmed as displaying a persistent difference among the two species, was controlled by 2 main hot spots on two linkage groups in addition to several less significant QTLs.

3. The study allowed substantial progress in the understanding of the genetic control and the ecological significance of water use efficiency in oaks. A few QTLs with a large effect were consistently detected in the same genomic region of the tested mapping progeny. These QTLs were associated with QTLs for traits like stomatal conductance: this opens an avenue for dissecting the genetic control and the physiological background of the diversity in $\Delta^{13}C$ and intrinsic water use efficiency (W_i) in oaks. The systematic difference among the two species found in natural populations (around 1‰) corresponds to approximately a 15% difference in W_i., and such a difference could be of ecological significance. Moreover, within each species, genetic differences were detected among populations depending on the position along topographic clines. This was probably one of the first case of non-neutral distribution of W_i genotypes in natural ecosystems.

4. Our studies also allowed to address the question of the physiological and genetic control of the tolerance to water-logging. A genetic control was evidenced for some traits related to the tolerance to water-logging in the *Q robur* full-sib progeny. Unfortunately, only few QTLs with a small effect were detected for some of the traits, but not all of them. There is still a

need to improve our knowledge about the impact of this complex traits, given its ecological importance.

5. Ecological fitness of hybrids: no really strong conclusion reached until now, the main problem being the detection of hybrids, and the identification of a sufficient number of hybrids to allow statistical comparisons with parent species.

4 Impact of gene flow on management of oak stands

4.1 Introduction

A synoptic view on the project results was taken for the development of practical guidelines and recommendations for managing different types of oak stands with the objective to transfer the scientific results of the project to practical forestry and silviculture. Results described above in chapters 2 and 3 were used to address practical questions concerning the management of common oak stands for wood production, to recommend appropriate management techniques for oak stands used for seed collection or for the conservation of genetic resources, and to develop guidelines for both natural and artificial regeneration of oak stands.

Stakeholders were invited to all project meetings and directed the project towards their needs and most important practical problems. Oak forests and their management differ widely among countries and regions in Europe. Thus, priority problems of stakeholders were intensely and occasionally controversially discussed during all project meetings and in particular at the final meeting in Odense, DK.

Outcomes of the project are summarized for the two main topics 'Natural regeneration in common oak stands' and 'Design and management of seed and conservation stands', taking into account the comments and expressions of interest of stakeholders.

4.2 Natural regeneration in common oak stands

The size of ISPs ranged from approximately 5 to almost 50 ha. Thus, it corresponded well to the average size of typical management units (stands; forest compartments) in Europe, and results obtained in the ISPs can be transferred to the majority of oak stands in Europe.

4.2.1 Maintenance of genetic diversity in regeneration

The project confirmed previous findings that oak stands harbour considerable genetic variation. Gene flow through pollen is the main evolutionary factor responsible for the maintenance of high diversity within populations and low differentiation among populations. A high percentage of the pollen parents of investigated progenies were located out of the ISPs and hence were not identified in all locations (Table 5). On average, approximately 60% of successful pollen parents were not located within ISPs. The high level of gene flow through pollen is an efficient natural mechanism ensuring the maintenance of large effective

population sizes and, hence, the maintenance of genetic diversity in the progeny generation. Surprisingly high levels of gene flow were also estimated for seed dispersal by an investigation of natural regeneration in seven ISPs (section 2.2.2; Tables 11 and 14). Seed migration from outside the ISP was estimated for almost 40% of the investigated seedlings.

The project confirmed that long-distance gene flow through pollen and seed is effective in all studied ISPs even though the proportion of estimated external pollen varied considerable among populations from approximately 21% to almost 88%.

Even within stands, a high number of pollen parents contributed to the produced progenies (Table 6). Exemptions were only those ISPs (P 4, Spain, and P 12, Hungary) which were characterized by very high levels of external pollen flow (>80%). Thus, a large number of pollen parents contribute to the produced progenies even in a single seed year.

In summary, the maintenance of 'neutral' genetic diversity is safeguarded by the efficiency of gene flow through pollen and seed in oak populations. Even years with a moderate to low seed crop can be used for the establishment of the next generation in silvicultural systems based on natural regeneration without excessive losses of genetic diversity, as long as the number of produced acorns and germinating seedlings are regarded as sufficient from the silvicultural point of view. However, the extension of the initiation of natural regeneration to several periods of flowering and fruiting is likely to even further promote diversity in the regeneration due to the genetic differentiation among seed trees and their respective effective pollen clouds in different years.

4.2.2 Impacts of small-scale spatial structures and inbreeding

Extensive gene flow through pollen does not preclude the existence of family structures in oak stands. Since the majority of oaks germinate under their respective seed trees due to the weight of acorns, natural regeneration is expected to induce small-scale spatial structures since trees with the same seed parent have a greater probability of growing in neighbourhood to each other in comparison to unrelated trees.

A detailed study on small-scale spatial structures was conduced in the Swiss ISP (P11). A spatially explicit analysis revealed that the small-scale distribution of genetic variation coincided with leaf morphological variation differentiating the taxa *Q. robur* and *Q. petraea* (Gugerli et al. 2007). The small-scale spatial distribution of trees within stands in groups

assigned to a single species based on genetic and morphological traits is possibly contribution to the maintenance of species identity even in mixed stands.

Spatial genetic structures need to be considered as a possible cause of inbreeding with the consequence of reduced viability and vigour due to inbreeding depression if mating predominantly occurs within neighbourhoods, i.e. if pollen dispersal within stands is limited. A weak, but significant tendency for preferred mating among neighbouring trees was observed in all stands apart from P12 (Hungary) (Table 8). However, the low frequency of selfing observed at the stage of seeds at the population level in all populations, and the high efficiency of gene flow through pollen (see section 4.2.1) strongly suggest that inbreeding is no main threat to the viability of the progeny generation regardless of the applied management system as long as a reasonable number of seed parents is maintained. However, comparatively high selfing rates were observed for a few adult oaks in several stands, suggesting a genetic control of the ability to produce seeds originating from selfing.

4.2.3 Silvicultural management during regeneration

Oaks are light-demanding species. Hence, felling operations are needed to initiate natural regeneration or to promote the development of newly established regeneration. The possibilities to regenerate oak stands naturally differ widely across Europe. For example, natural regeneration is favoured in most areas in France but rarely successful in Germany or Denmark.

Changes of genetic structures due to dysgenic selection are possible if trees with large diameters and good stem form are preferentially harvested before the initiation of regeneration. In this case, only ‘inferior’ trees, i.e. trees with less preferred phenotypes, have the chance to contribute their genes to the next generation. Target-diameter felling and similar silvicultural practices to initiate or to promote natural regeneration have potentially detrimental consequences for oaks as for any other tree species (Finkeldey and Ziehe 2004).

Traits possibly negatively affected by dysgenic selection are difficult to monitor in long-living organisms and were not the main focus of this project. However, strong evidence was obtained pointing towards the genetic control of important adaptive traits such as water use efficiency and water-logging tolerance at least during early life stages. Thus, there is no reason to assume that other adaptive traits and traits of economic significance are not partially under genetic control and, hence, possibly negatively affected by dysgenic

selection. Furthermore, the results of the studies on pollen dispersal point towards the importance to consider spatial aspects for an assessment of the effects of different silvicultural selection methods during regeneration. Early removal of the qualitatively best seed trees might at least be partially compensated by extensive pollen flow from surrounding forests, if target-diameter felling or other silvicultural systems based on an early cutting of the 'best' trees are restricted to comparatively small areas of a few hectares in size. However, the simultaneous regeneration of large forests must not be initiated by the removal of qualitatively superior trees prior to the initiation of the regeneration on the complete area.

4.2.4 Impact of hybridization on oak stands

A main conclusion of the project is that hybridization among oaks is a comparatively common and natural process throughout Europe, which cannot be completely avoided. The long overlap of flowering periods and the importance of long-distance dispersal strongly suggest the occurrence of species hybrids even in 'pure' oak forests, i.e. in forests with a single species of adults only. However, results also point towards strong differences concerning the frequency of hybrids, which were estimated at less than 2% in the ISPs of P11 (Switzerland) and P12 (Hungary), but around 30% or even higher in the ISPs in Holland (P3) and Great Britain (P5) (Table 7).

The important question concerning the fitness of hybrids under controlled conditions and in the ISPs is not yet fully resolved. Preliminary evidence obtained in some ISPs points towards reduced viability of hybrids at least under special environmental conditions (Gugerli et al. 2007). A presumed selective disadvantage of species hybrids possibly contributes to the maintenance of the two separate taxa in Central Europe (*Q. petraea* and *Q. robur*), and keeps the botanical species differentiated from each other also in more species-rich oak communities (Curtu et al. 2007).

In conclusion, the occurrence of two (or even more) oak species in a forest stand certainly contributes to the evolutionary adaptive potential by increasing genetic diversity (Finkeldey 2000a) and providing a basis for selection due to the differentiation of the species at adaptive traits such as water use efficiency and water-logging resistance (section 3.2.3). The role of hybridization for the adaptation to changing environmental condition, and the recognition of particular environments offering selective advantages for hybrids (if any) deserve to be studied in more detail.

4.3 Design and management of seed and conservation stands

Different rules concerning the harvest of forest reproductive material are described in the relevant EU Council Directive 1999/105/EC of 22 December 1999, which was translated into national laws by most EU member countries, and by the OECD scheme on forest reproductive material (Nanson 2001). Most countries developed national strategies to conserve genetic resources within the more general context of forest protection in Europe (Mayer and Buck 2005). Results of OAKFLOW have implications concerning the production and use of reproductive material of oaks and concerning the selection and management of stands for the conservation of genetic resources of oaks.

4.3.1 Selection and management of seed production areas

A main result of the project is the uniform high rate of external pollen contributing to the production of seeds in all ISPs (Table 5). A high proportion of external pollen was successful in all ISPs regardless of their isolation, spatial structure, topography of the area, and density of oak trees. Even though the origin of external pollen is still unknown, long distance pollen transport of several kilometres seems to be very common. Thus, reproductive isolation of seed production areas from non-selected stands of usually inferior phenotypic appearance cannot be achieved. This results calls for the selection of large oak forests with uniform good phenotypic characters, and the collection of seeds preferably only in the central compartments of those large oak forests.

The harvest of acorns in mixed oak forests for the production of forest reproductive material is a matter of controversial discussions (Finkeldey 2001b). Currently, it is not advisable to give general recommendations concerning seed harvest in mixed stands since the long-term phenotypic performance and the adaptability of hybrids need further studies. Moreover, the project revealed large differences with regard to the proportion of hybrids produced in mixed oak forests with a frequency of less than 2% in the ISPs in Switzerland (P11) and Hungary (P12), but more than 20 times higher estimates in the ISPs in the Netherlands (P 3; seed crop from year 2002), Great Britain (P 5), and Denmark (P 8) (Table 7). The reasons for these large differences are not clear. Stand structure and proportion of different species among the adult stand are not responsible alone for the different hybrid proportions produced as revealed by the repeated analysis of seeds from different years in the ISP in the Netherlands (P 3). The estimated for the production of species hybrids is

almost twice as high from the seed crop in 1998 (>33 %) in comparison to the year 2002 (<18%) (Table 7).

Disregarding the considerable differences with regard to the proportion of hybrids produced in mixed forests, results confirm the existence of two clearly separated groups of trees which can be assigned to the taxa *Q. robur* and *Q. petraea* not only in adult populations in mixed stands, but also in progenies from those mixed populations (section 3.3.8). Furthermore, results revealed the genetic basis for differences among the two species *Q. robur* and *Q. petraea* at important ecophysiological traits such as leaf mass to area ratio and water use efficiency (section 3.3.8). These results suggest the use of reproductive material from mixed oak forests at least for planting sites with high uncertainty concerning future environmental conditions. In particular, reproductive material from mixed forests might be used at sites suitable for *Q. robur* in the past, which are forecasted to become drier and warmer in the future due to climate change. The different adaptive potential of both species is expected to result in selection favouring one species. Thus, more seed harvest from mixed oak stands needs to be considered as a means to mitigate the effects of global change on oak forests.

The regulation of the species composition is an important aspect for the management of seed production areas. This applies both to thinning operation aimed at removing trees other than oaks from the forest to promote gene flow among remaining oak trees, and to the regulation of the species composition in stands with more than a single oak species. Results from OAKFLOW revealed a high effective number of pollen parents, low selfing rates, and considerable long-distance gene dispersal for oaks throughout Europe irrespective of stand structures and density of oaks. Thus, other trees appear to be no obstacle for wind pollination in oaks, and thinning operations favouring oaks are only recommended if they are recommended from a silvicultural point of view and in order to promote flowering and seed production, but not to improve gene flow through pollen. Reproductive material from mixed oak forests might be used more frequently than before in response to global change (see above). Thus, it is not recommended to decrease the species diversity of oaks in mixed seed production areas.

4.3.2 Seed harvesting operations

All investigated ISPs were characterized by high rates of successful external pollen, a large effective number of pollen parents, large average dispersal distances of pollen within ISPs,

even though a slight tendency towards preferential mating among neighbouring trees was observed, and low selfing rates at the population level. Thus, high genetic diversity was realized even in the progenies from single seed trees due to diverse pollen contributions.

Acorns of oaks for the production of seedlings in nurseries are typically collected directly from the ground. Thus, a large number of seed parents, and an even much larger number of pollen parents, typically contribute to the production of collected progenies.

The general considerations mentioned above and the results of the analysis of pollen transport suggest that a high genetic diversity is usually realized in progenies after commercial seed harvesting irrespective of the chosen sampling strategy. However, sampling from very limited areas should be avoided even if only a small amount of seeds need to be collected.

Seed harvesting in mixed oak forests might become an important issue in the future (see section 4.3.1). If it is aimed to increase diversity and adaptive potential of progenies, it will be important to identify (sub)plots dominated by a single species only or with an intense mixture of two ore more species, since seed must not be harvested in a plot dominated by a single species only in this case.

4.3.3 Conservation stands

Most considerations described above (section 4.3.1) with regard to the selection of seed stands also apply to the identification of stands for the conservation of genetic resources of oaks. The efficiency and high rate of long-distance pollen dispersal needs to be considered for all types of *in situ* conservation of oak genetic resources of common species in Europe. *In situ* conservation will be very dynamic, i.e. changes of genetic structures are expected during the regeneration of oak forests. These changes due to high rates of gene exchange and migration are potentially contributing to the adaptive potential of stands, but may result in the loss of particular local adaptations.

Autochthonous stands are preferred targets for the conservation of forest genetic resources. Results from previous projects, in particular FAIROAK, proved the usefulness of variation of cpDNA to identify human seed transfer and, hence, also to conclude on autochthony vs. allochthony of stands (Petit et al. 2002a; Petit et al. 2002b). These results were further refined and used to differentiate autochthonous stands from transferred material at a regional level (e.g. Gailing et al. 2007). The spatially explicit analysis of small-scale structures of adults at highly variable SSRs within stands conducted within OAKFLOW can

be used as a complementary tool to distinguish stands originating from natural regeneration and planted stands, since spatial structures (family structures) are only expected, if stands were naturally regenerated. However, the observation of significant spatial structures does not prove autochthony, since a family structure may result from a single generation of natural regeneration.

The selection of conservation stands will greatly profit from the identification of genes of adaptive significance, and the observation of variation patterns at those genes. Initial steps towards the identification of those genes were made (see sections 3.3.1 to sections 3.3.3; section 3.3.6). These efforts form an important basis for ongoing related research in later project such as EVOLTREE (NoE; 6th FP). However, the results of this work can not yet be immediately applied to guide the selection of priority stands for the conservation of oak genetic resources.

5 Overall conclusion to the scientific results

In regards to the three main objectives of the OAKFLOW project, the following main conclusions can be drawn:

(7) An overall assessment of pollen and seed flow was obtained from the different intensive study plots (ISP) that were installed across Europe under various ecological conditions. Within the size of the plots varying between 4.5 to 50 ha, the pollen dispersal curve followed a negative power exponential function, with mean dispersal distances varying between 47 to 870 meters (and standard deviation from 40 to 2400 meters). However, in more than 60% of the pollination events (from 39% to 88% depending on the ISP), the male parents could not be assigned, suggesting that most of the pollen originates from outside the Intensive Study Plots. These results support the general view that pollen flow is the result of two processes: local dispersion (that has been addressed in OAKFLOW) and long-distance transport, the ratio between both processes amounting to approximately 40/60. Seed dispersal distances varied between 10 to 155 meters, and seed dispersal from outside the ISP was much lower than for pollen (on average 39 % , varying between 15% and 48%.

(8) Hybridisation occurred in all ISP, regardless of the density or the census numbers of the different species. The average proportion of hybrids over all ISPs amounted to 17 % (from 1% to 41%). This is however a lower limit of hybridization rates, as it accounted only

for those mating events where both parents could be assigned by parentage analysis. These figures do not take into account pollen donors from outside the study plots, as they could not be identified. In contrast to previous papers, there was no preferential direction of hybridisation between *Q. robur* and *Q. petraea.*

(9) In several case studies distributed across Europe, past artificial seed transfer could be identified by chloroplast DNA fingerprints and their spatial structure in the studied stands. These results suggest that the method could be used for certification purposes, if autochthony of the stand is to be used as a certification criteria

(10) Genetic maps for *Q. robur* were constructed in two full sib pedigrees, that were further used for QTL detection. Strong colinearity was found with the map of *Castanea sativa*, suggesting that the different genera of Fagaceae may share the same genetic system. These results open new perspectives for genetic and genomic research in the Fagaceae

(11) Quantitative Traits Loci (QTLs) were detected for traits showing interspecific adaptive differentiation between *Quercus petreae* and *Quercus robur* : leaf morphology and pubescence, water use efficiency, response to waterlogging. The percentage of phenotypic variation explained by the QTLs remains moderate to low (from 0 % to 12% in general). Interestingly for water use efficiency (as assessed by carbon isotope discrimination ($\delta^{13}C$)), the proportion of phenotypic variance explained by one QTL amounted to 20 - 25% (on linkage group 11)

(12) Differentially molecular screening methods were used to identify genes that exhibit different expression profiling between the two species under stressed conditions: hypoxia and osmotic stress. The corresponding genes were sequenced and mapped on the *Quercus robur* map.

(7) Results obtained in the project were shared an discussed with end-users (forest managers, conservation agencies). Stakeholders were invited to all project meetings and directed the project towards their needs and most important practical problems. Outcomes of the project , mainly of the gene flow studies, were discussed in regards to two main topics ‘Natural regeneration in common oak stands’ and ‘Design and management of seed

and conservation stands', taking into account the comments and expressions of interest of stakeholders

6. EXPLOITATION AND DISSEMINATION OF RESULTS

6.1 Exploitation of technical and scientific results in operational forestry and basic research

Among the deliverables produced by the project, some can lead to large scale applications and could further be implemented in operational plans for management of gene diversity. Others lead to important achievements in basic genetics, that can be used in future genomic research of the Fagaceae.

- A user friendly computer package for assessing gene flow comprising the calculation of likelihoods for parentage analysis, the spatial distribution of the adult and juvenile cohorts, the retrospective construction of pollen dispersal and various statistics (parental mating success, hybridisation rates, population size) was developed within the OAKFLOW. The publication describing the software Famoz (Gerber et al. 2003) was cited more than 30 times and the software was used for different purposes in several papers dealing with tree species, both temperate and tropical, and annual plants as well. Animal researchers are also attracted by the software: it was used in published papers to study different fish populations, but also bird species, a rodent and even an insect.
- Molecular methods for DNA extraction and microsatellite scoring. A set of 5 to 10 microsatellites is now available for assessing routinely intra specific and interspecific gene flow. This set is already implemented in the form of a high throughput genotyping assay (multiplexing) to allow large scale analysis.
- Chloroplast DNA fingerprints which allow to identify allochtonous origins of oak stands were confirmed in case studies conducted in France, Netherlands and Germany. These fingerprints can be used by forest managers to check the genetic origins when forest management plans are established
- Oak genetic maps were created and scans for genomic regions of interest (either genes or QTLs). A major result of this project was the colinearity of the genetic maps between *Quercus* and *Castanea.* These findings open new prospects in genomic research in oak, chestnut and beech.

6.2 Dissemination of scientific results

Scientific results obtained in this project were published in international journals and made available to the scientific and professional community through websites of the different partners. The website of the project can further be opened (http://www.pierroton.inra.fr/Oakflow/). A total of 25 peer reviewed papers were published at this stage in international journals

6.3 Dissemination to the end-users

For each of the eleven countries participating to the project, end-users from various origins were associated to the project as subcontractors: Forest services (Office National des Forêts in France, Forestry Commission in Great Britain, National Board of Forestry in Sweden, Forestry Research Institute in Hungary, Forest Service in Germany), Conservation Agencies (Information and Knowledge Centre for Nature in The Netherlands, Spanish General Directorate for Nature Conservation in Spain, Danish National Forest and Nature Agency in Denmark, Swiss Agency for the Environment, Forest and Landscape), Land Use Agencies (Communita Montana in Italy, Austrian Ministry of Agriculture in Austria). Most of the end users were laso owner of the ISPs were gene flow was monitored during the project. The end users were invited to each of the yearly meetings (Vitoria, Firenze, Sopron and Odense) to follow the achievements and results obtained by the project.

During the last meeting workshops were organized between scientists and end-users. Three separate workshops were organised where issues of the different subtasks (WP5.1: Natural regeneration in common oak stands; WP 5.2: Design and management of seed and conservation stands; WP5.3 Even and uneven aged management regimes) were discussed between the scientists and the end-users. The following specific topics were discussed.

(1) main project results (based on presentations of the previous days) regarding the issue

(2) original expectations regarding project results

(3) current understanding of applicable results

(4) "grading" of results with regard to applicability

(5) urgent scientific problems left to be addressed

(6) ways of transferring knowledge into praxis (with and without support from OAKFLOW)

The partners were subdivided in three different groups as follows:

- Natural regeneration in oak stands

- Design and management of seed and conservation stands
- Even and uneven aged management regimes

Minutes of these workshops are available on the website of the project http://www.pierroton.inra.fr/Oakflow/ (under page Minutes of the final meeting Odense), and the main conclusions are presented in part 4 of the final report.

6.4 Dissemination through networks

Under the aegis of the former IPGRI, a permanent European programme (EUFORGEN) was created to coordinate the communities conservation activities. In 1997, EUFORGEN initiated a conservation program for oaks in which member countries had to identify conservation stands through a network called “Social hardwoods”, that was further enlarged and called “Stand forming broadleaves”. Several contractors and subcontractors of the project are also members of the this EUFORGEN network. EUFORGEN develops joint products that complement and promote the implementation of scientific knowledge into practice. The EUFORGEN community has a track record of ensuring integration of relevant technical advice on forest genetic resources into national and European policy processes, policies and regulations. Scientists of OAKFLOW participated to the regular meetings of the EUFORGEN network "Stand forming broadleaves". Joint members of the OAKFLOW and of the EUFORGEN network contributed to the writing of the Technical guidelines for forest genetic resources are one of the main products of the Euforgen (Ducousso and Bordacs, 2004)

7 Policy related benefits

7.1 Contribution to EU policies for the certification of forest reproductive material

Trade and use of forest reproductive material is regulated and supervised in all developed countries. The necessity arises from the need to credibly certify the origin and genetic quality of forest reproductive material. In addition to national legislation, the guidelines of the OECD C(74)29 (OECD Forest tree and plant scheme, Paris June 2007) and AGR/CA (96)25/REV2 as well as the directive 105/99/E of the European Union provide the basis for regulation and certification. The principles underlying the mentioned regulations have been elaborated in recent decades on the basis of available (mostly quantitative) genetic information from field tests, in most cases the only way of documenting of genetic quality was the certification of the place of collection/production.

For example, among the six requirements for certification of "selected stands" under the OECD scheme, two will directly benefit from results obtained in OAKFLOW

Requirement b) "it must be stated whether the basic material is autochthonous/indigenous, non-autochthonous/ non-indigenous or the origin is unknown and for non-autochthonous/non-indigenous basic material the origin must be stated if known"

Requirement d) "the basic material will be sufficiently isolated (by approval of the Designated Authority) from trees which would potentially dilute or compromise the genetic composition of the reproductive material. In particular, basic material in close proximity to trees/stands of non-autochthonous/non-indigenous origin, unknown origin, or derived from plant breeding techniques should not be approved".

The findings of OAKFLOW in terms of pollination distance, but also in chloroplast DNA fingerprinting, can lead to operational implementation permitting to check for these requirements.

7.2 Contribution to EU policies for conservation and management of genetic resources

The European ministers of Environment commit themselves to take actions to halt the loss of biological diversity at all levels by the year 2010. Within the 8 resolutions adopted, three are directly addressed by the activities conducted within OAKFLOW (R1: Forestry and Biodiversity, R2: Agriculture and Biodiversity; R3: Pan-European Ecological networking; R8: Biodiversity monitoring and indicators). PEBLDS recognizes that forest trees and

woodlands are essential elements of the European biodiversity and recommend strong action to be implemented in wooded landscapes through MCPFE (Ministerial Conferences on the Protection of Forests in Europe). MCPFE conferences are the successively held in Strasbourg (1990), Helsinki (1993), Lisbon (1998), Vienna (2003), and Warsaw (2007). They provide the basis for implementing forestry strategies for conservation to slow down the loss of biodiversity and to enhance its contribution to a further development and sustained use. The MCPFE is a high level political initiative involving 44 European countries, European Community and cooperates with other countries, as well as international organizations that participate as observers. The MCPFE is a major part of the European Forest Strategy (EFS) and part of the Common Agricultural Policy (CAP) of the EC. The MCPFE recommended several resolutions for the future protection of forests in Europe, which are directly concerned with the conservation of biological diversity in general and genetic, within species, diversity in particular: resolution S2 (Conservation of Forest Genetic Resources), H1 (General Guidelines for the Sustainable Management of Forest in Europe), H2 (General Guidelines for the Conservation of Biodiversity in European Forests), and H4 (Strategies for a Process of Long-Term Adaptation of Forest in Europe to Climate Change).

The results obtained in OAKFLOW contributed to conservation measures taken in order to fulfil these resolutions. As detailed in paragraph 6.4, scientists of OAKFLOW take part to the EUFORGEN network “Stand forming broadleaves” and contributed to the “Technical guidelines for genetic conservation and use for pedunculate and sessile oak”.

7.3 Contribution to European research infrastructures

In the fifth framework programme (Quality of life and management of living resources), the EU has expressed needs for installing infrastructures of widespread significance for enhancing provision in biological information resources (Support of Research Infrastructures). OAKFLOW has installed a unique network of infrastructures (ISPs) that can monitor gene flow on a European scale in various ecological conditions. This network may be helpful for other ecological surveys.

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