Theor Appl Genet (2002) 105:431–439
DOI 10.1007/s00122-002-0920-6

E. Coart · V. Lamote · M. De Loose
E. Van Bockstaele · P. Lootens · I. Roldán-Ruiz

AFLP markers demonstrate local genetic differentiation between two indigenous oak species [*Quercus robur L.* and *Quercus petraea (Matt.) Liebl.*] in Flemish populations

Received: 8 June 2001 / Accepted: 26 November 2001 / Published online: 31 May 2002

Abstract The nuclear genetic variation within and between four sessile (*Q. petraea*) and six pedunculate (*Q. robur*) autochthonous Flemish oak populations was investigated with AFLP markers. One sessile and one pedunculate oak population were additionally screened for detailed leaf characteristics using an image analysis system. Principal coordinate analysis on the AFLP data classified the oaks in two main groups, according to their taxonomic status. No species-specific AFLP markers were found using four primer combinations, but marker frequency differences up to 71% were recorded between both species. Analysis of the genetic structure showed that the divergence between species, as observed by ordination, was significant. Both species revealed similar diversity levels. A smaller though significant differentiation was also revealed for both species among populations within species. Molecular and morphology based approaches showed a high degree of consistency. Screening of 60 AFLP primer combinations using a bulking strategy did not allow identifying species-specific markers, which supports the conclusions reached in previous studies. The distribution of genetic variability at the species and at the population level is discussed.

Communicated by C. Möllers

E. Coart (✉) · V. Lamote · M. De Loose · E. Van Bockstaele
I. Roldán-Ruiz
Department for Plant Genetics and Breeding,
Agricultural Research Centre,
Caritasstraat 21, 9090 Melle, Belgium
e-mail: e.coart@clo.fgov.be
Tel.: +32-9-2722900, Fax: +32-9-2722901

V. Lamote · E. Van Bockstaele
University of Ghent,
Faculty of Agricultural and Applied Biological sciences,
Coupure Links 653, 9000 Gent, Belgium

P. Lootens
Department of Crop Husbandry and Ecophysiology,
Agricultural Research Centre, Burg. Van Gansberghelaan 109,
9820 Merelbeke, Belgium

Keywords AFLP · Morphology · Genetic variability · Differentiation · *Quercus robur* · *Quercus petraea*

Introduction

The oak species *Quercus robur* L. (pedunculate oak) and *Quercus petraea* (Matt.) Liebl. (sessile oak) are the only indigenous oak species in the Flemish region (Belgium). Both white oak species are largely sympatric, widespread in Europe from Spain to Russia and from Scotland to Turkey, and generally occupy different but proximal ecological niches. The presence of morphological intermediates between both species at first led taxonomists and foresters to believe they were freely interfertile (Wigston 1974). The authors that support the widespread occurrence of hybridisation suggested a revision of their taxonomic status to the status of subspecies (e.g. Olsson 1975; Van Valen 1976). However, crossing experiments (Rushton 1977; Aas 1990; Steinhoff 1993; Kleinschmit and Kleinschmit 1996) showed that the number of successful interspecific crosses was low and that the fertilisation of *Q. robur* with *Q. petraea* pollen was much more successful than the reciprocal crosses. This evidence of fertility barriers led to much speculation about the origin and taxonomic status of the morphological intermediate forms. Several authors postulated that the variation within the two species had been underestimated (e.g. Jones 1959; Gathy 1969; Dupouey 1983; Aas 1993; Dupouey and Badeau 1993) and stated that, if hybridisation had been at all common, the close relationship between the two species and their sympatric distribution would by now have led to the complete loss of their separate identities.

Up to now no complete differentiation between the two species nor a diagnostic character were detected in any morphological study, although in surveys based on multivariate approaches for data analysis (e.g. Aas 1993; Dupouey and Badeau 1993; Bacilieri et al. 1995) the number of morphological intermediates was low (less than 5%). The phenotypic studies published to-date were

carried out in various regions of the geographical range of the oaks. Because for these studies a range of measured characters were taken into different statistical approaches, it is very difficult to attribute the differences found to methodological and/or geographical effects, or to state whether the morphological character's variation detected is either the result of modification or of genetically fixed variability (Kleinschmit et al. 1995).

The development of molecular markers provided new tools for further investigation of the genetic relationship and interspecific variability of the sessile and pedunculate oaks. However, the first studies were discouraging because molecular markers appeared to be less discriminating than observations of the phenotype. Isozyme-analyses could not reveal any species-specific marker (Zanetto and Kremer 1995; Bacilieri et al. 1995), but confirmed directional hybridisation under natural conditions (Bacilieri et al. 1996) as previously demonstrated in artificial crosses (Steinhoff 1993). RAPD analyses supported the hypothesis that differentiation between both white oak species is likely to be controlled by only a few loci (Bodénès et al. 1997a). The overall nucleotide divergence detected between the species was very low (0.5%); however, a few 'hot spots' for differentiation with an increased divergence (3%) were observed. Furthermore, these 'hot spots' are located in highly polymorphic genomic regions (Bodénès et al. 1997b).

Additionally, chloroplast markers were found to be present with similar frequencies in both oak species over the European continent (e.g. Petit et al. 1993; Dumolin-Lapègue et al. 1999). According to Muir et al. (2000), microsatellites could be the marker of choice for discrimination between related oak species at the population level. Microsatellite analysis enabled us to group several European oak populations per species, with highly significant values, suggesting that sessile and pedunculate oaks represent clearly separate taxonomic units. Furthermore, a recently analysed isozyme locus provided preliminary results on its possible use for differentiation between the two oak species (Gömöry 2000).

As far as we know, no study on the genetic variability within and between natural populations of white oak species has been published in which the AFLP (amplified fragment length polymorphism, Vos et al. 1995) fingerprinting technique was used. De Greef et al. (1998) employed AFLPs to support the breeding and selection of *Q. petraea*. Gerber et al. (2000) applied AFLPs to *Q. macrocarpa* samples to study the power of AFLPs for parentage analysis. AFLPs usually yield several DNA polymorphisms amplified by one primer combination. High data output per reaction and good reproducibility make this technique a reliable marker system for characterising natural populations and breeding gene pools (e.g. De Riek et al. 1999; Roldán-Ruiz et al. 2000).

On behalf of the Flemish Forest and Green Areas Division, a study was started with the aim of characterising the genetic diversity and differentiation present within and between populations of both indigenous oak species (*Q. petraea* and *Q. robur*). This paper reports on the results obtained using AFLP markers and morphological characterisation.

Materials and methods

Plant material

Ten oak populations were sampled in the eastern part of Flanders (Table 1). These stands were chosen according to a survey for autochthonous tree populations (Maes and Rövenkamp 1999). In each stand, 30 trees were sampled at random across the complete surface of the stand. Minimum distances between trees varied from circa 50 up to circa 100 m. In the small relict populations QR5 and QR6, all trees were sampled, respectively 8 and 14 individuals. Oaks were sampled regardless of their morphologic characters and presumed taxonomic status. The populations are named after the dominant oak species (QR for *Q. robur* populations and QP for *Q. petraea* populations), the presence of a (minor) mixture of the other oak species is given in Table 1.

Table 1 Description of the populations analysed. QP = population dominated by *Q. petraea* trees; QR = population dominated by *Q. robur*. The last column indicates the number of samples retained in the statistical analysis after elimination of those samples for which it was impossible to isolate DNA of appropriate quality, and samples for which not all primer combinations resulted in scoreable AFLP fingerprints. *Populations that were eliminated from the calculations of AFLP marker frequencies per population due to small population sizes

Code	Location name	Estimated area (ha)	Other oak species or intermediate forms present	Longitude/latitude	Number of samples collected	Number of samples in AFLP analysis (4PC)
QP1	Klaverberg-As	11	Yes	5°32′51″/51°1′18″	30	30
QP2	Gemeentebos Hechtel-Eksel	18	Yes	5°20′43″/51°6′51″	30	22
QP3	Windelsteen-Maasmechelen	1.8	Not detected	5°38′33″/50°59′53″	30	21
QP4	Opgrimbie	6	Yes	5°37′39″/50°56′39″	30	15
QR1	Kruiskensberg-Gruitrode	24	Yes	5°34′17″/51°03′43″	30	20
QR2	Gemeentebos Hechtel-Eksel	1.1	Not detected	5°20′43″/51°6′51″	30	18
QR3	Gemeentebos Hechtel-Eksel	8	Not detected	5°20′43″/51°6′51″	30	24
QR4	Speelbos-Wetteren	5	Not detected	3°54′10″/51°0′34″	30	22
QR5*	Heuvelken-Tessenderlo	0.6	Not detected	5°1′12″/51°1′24″	8	6
QR6*	Paddenhoek-Tessenderlo	0.5	Not detected	5°1′52″/51°4′10″	14	10

Table 2 List of directly measured and derived morphological characters. *Character evaluated by eye

Measured variables		
Abbreviation	Character	Measure
LL	Lamina length	Pixel (1/3 mm)
PL	Petiole length	Pixel (1/3 mm)
LW	Lobe width: width of the deepest lobe from the mid rib to the top	Pixel (1/3 mm)
SW	Sinus width: lamina width from the mid rib to the base of the sinus, at the widest part of the lamina	Pixel (1/3 mm)
WP	Widest part: length of lamina from the lamina base to the widest part of the lamina	Pixel (1/3 mm)
NL	Number of lobes	Count
NV*	Number of intercalary veins*	Count
BW_0, BW_{10}, BW_{20}, BW_{-10}, BW_{-20}	Basal width of the lamina measured at the lamina base and 20 and 10 pixels up and down	Pixel (1/3 mm)
PUB*	Abaxial lamina pubescence*	Categorical, from 1 to 6
Derived characters		
Abbreviation	Character	Calculation formula
LS	Shape of lamina	LL/WP
PR	Petiole ratio	PL/(LL+PL)
LDR	Lobe depth ratio	LW/(LW-SW)
PV	Percentage venation	NV*100/NL
BS	Basal shape	$(BW_{-20}+BW_{-10}+BW_0+BW_{10}+BW_{20})/5$

DNA extraction

Five mature oak leaves were harvested on each tree, immediately frozen in liquid nitrogen during sampling and lyophilised for 48 h. The dried material was further stored under vacuum conditions until DNA extraction. Forty milligrams of the dried material were ground and a DNA-isolation was carried out according to the CTAB-extraction procedure of Lefort and Douglas (1999). This method yielded up to 25 μg of genomic DNA per extraction. DNA concentrations were determined, relative to uncut lambda DNA, on 1.5% agarose gels.

AFLP reactions and PAGE

AFLP was performed according to Vos et al. (1995) with available products and kits (Preamp primer mix and AFLP Core reagent kits, Gibco-BRL). Two hundred and fifty nanograms of genomic DNA was digested for 2 h at 37 °C in a final volume of 50 μl containing 10 mM of MgOAc, 50 mM of KOAc, 10 mM Tris-HCl pH 7.5, 2.5 U of *Eco*RI (Gibco-BRL) and 2.5 U of *Mse*I (Gibco-BRL). Two adaptors, one for the *Eco*RI ends and one for the *Mse*I ends, designed to avoid the reconstruction of the restriction sites, were ligated to the restriction fragments by adding 24 μl of a mix containing 5 pmol of *Eco*RI adaptor, 50 pmol of *Mse*I adaptor, 10 mM of ATP, 10 mM of Tris-HCL, 10 mM of MgOAc, 50 mM of KOAc and 1 U of T4 DNA ligase (Gibco-BRL). The ligation mixture was incubated for 2 h at 37 °C.

A pre-amplification step was performed with primers complementary to the *Eco*RI and *Mse*I adaptors with an additional selective 3′ nucleotide. The PCR reactions were performed in a 50 μl volume of 10 mM Tris-HCL pH 8.3, 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.2 mM of each dNTP, 25 ng of each primer (Gibco-BRL), 1 U of *Taq* DNA polymerase (Applied Biosystems) and 5 μl of the fragments. The PCR amplifications were carried out in a Hybaid Omni Gene cycler using 20 cycles, each cycle consisting of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. For the selective amplification, primers with six selective bases were used. The *Eco*RI primer was labelled with a fluorescent near-infrared group (IRD-700 or IRD-800). The PCR amplification mixture was composed of 3 μl of diluted pre-amplification product (1/10 of their initial concentration), 1 μl of *Mse*I primer at 5 μM, 1 μl of *Eco*RI primer at 1 μM, 1 U of *Taq* DNA polymerase (Applied Biosystems), 15 μl of 10 × PCR Buffer (Applied Biosystems) and 1 μl of dNTPs (20-mM each). The selective amplification was carried out in a Perkin Elmer Geneamp PCR system 9600 with the following parameters: 1 cycle of 2 min at 94 °C, 30 s at 65 °C, 2 min at 72 °C, followed by nine cycles in which the annealing temperature decreases 1 °C per cycle, followed by 23 cycles of 1 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C. After amplification, the samples were denatured by adding 1 μl of formamide buffer (LI-COR, Inc.) to 2 μl of the sample and heating for 3 min at 90 °C. AFLP fragments were separated by PAGE on a LI-COR 4200 DNA Analyzer Sequencer on 25-cm gels using 6.5% denaturing polyacrylamide gels (KB-plus solution, LI-COR, Incorporated). Near-infrared labelled size standards (LI-COR, Incorporated) were loaded on each gel at regular intervals for sizing of the AFLP fragments.

Data analysis

Gene ImagIR software 3.55 (LI-COR, Incorporated) was used to score the fragment size and to define marker bins. Each marker was coded as present (1) or absent (0) for each plant, thus creating a binary data matrix.

Spearman correlations between marker presences over the plants were calculated to estimate the redundancy in the data set. In addition, a principal coordinate analysis (PCO) was performed to calculate principal co-ordinates from pairwise Euclidian distance estimates between individual genotypes (MVSP, http://www.kovcomp.com). The first two axes were plotted graphically (SPSS, http://www.SPSS.com). Furthermore, marker frequencies per population were used to calculate squared Euclidean distances and for subsequent UPGMA clustering. The reproducibility of the ordination was tested by random re-sampling (1,000 bootstrapped datasets) using Phylip (Felsenstein 1993). All cluster analyses were performed with SPSS.

Genetic diversity and differentiation statistics were calculated using the program Aflpsurv V.1.0. (Vekemans 2001). Allelic frequencies at AFLP loci were computed from the observed frequencies of fragments using the Bayesian approach proposed by Zhivotovsky (1999) for diploid species, assuming some deviation

Table 3 Comparison of the results obtained in the preliminary study using ten primer combinations. Reproducibility values are given as percentage of bands that were identically scored in three independent repeats, starting from different DNA extractions.
*The four primer combinations selected for the diversity screening

Primer combination	Number of markers scored (in 40 samples)	Number of polymorphic markers (in 40 samples)	Reproducibility value
E-ACA + M-CAA	58	32	98.6%
E-ACA + M-CAC	42	29	98.8%
E-ACA + M-CTC*	49	38	99.1%
E-ACA + M-CTG	40	32	99.0%
E-ACC + M-CAT*	45	36	98.6%
E-ACC + M-CTA	Not scoreable	/	/
E-ACG + M-CTC*	44	37	98.8%
E-ACT + M-CAT	43	36	98.8%
E-AGC + M-CTT	Not scoreable	/	/
E-AGG + M-CAA*	53	35	98.8%

from Hardy Weinberg genotypic proportions. Deviation from Hardy Weinberg genotypic proportions were accounted for through the use of a hypothesised value of the inbreeding coefficient (F_{is} = 0.15, see discussion). A non-uniform prior distribution of allelic frequencies was assumed with its parameters derived from the observed distribution of fragment frequencies among loci (see Note 4 in Zhivotovsky 1999). These allelic frequencies were used as input for the analysis of genetic diversity within and between populations following the method described in Lynch and Milligan (1994). The significance of the genetic differentiation between species or populations was tested by comparison of the observed F_{ST} with a distribution of F_{ST} under the hypothesis of no genetic structure, obtained by means of 5,000 random permutations of individuals among species or populations.

Morphological analysis

In one *Q. robur* (QR4) and one *Q. petraea* (QP1) population, leaf material was collected on the same trees sampled for the DNA analysis (30 trees per population). Ten mature leaves were collected from the upper part of the crown of each sampled tree. Leaves were dried and stored in a herbarium. Five intact leaves were chosen for further analysis. The image-analysis system used for the morphological characterisation of the leaves consisted of a camera (KY-F55B, JVC, Japan), a frame grabber card (Flashbus, Integral Technologies, USA), an IBM-compatible PC and image analysis software (Wit version 5.31, Logical Vision, Canada). A programme was developed to measure the leaf characteristics automatically. Additional estimations of lamina pubescence were made with a binocular microscope (magnification 40 ×) and the number of intercalary veins was counted by eye. Table 2 summarises the measured parameters and the derived characters used for further computations. A Mann-Whitney U test was performed to estimate the power of the morphological parameters for species discrimination. In addition, a PCO analysis was performed (using MVSP and SPSS).

Results

AFLP analysis

Selection of primer combinations

Twenty eight primer combinations were tested on five samples from different populations. The generated fingerprints were evaluated for overall clearness of the banding pattern and the number of polymorphic markers present was recorded (data not shown). Ten primer combinations (Table 3) were chosen for further screening on 40 oak samples. The number of markers scored and the degree of polymorphism are shown in Table 3. To evaluate the reproducibility of the fingerprints, three completely independent AFLP fingerprints were generated for ten samples, starting from different DNA extractions. Mean reproducibility values (calculated as the percentage of markers that were identical in the three repeats for the same plant) were very high and ranged from 98.6 to 99.1% for the different primer combinations (Table 3). Two primer combinations resulted in not-scoreable fingerprints due to the amplification of too many and/or faint bands. Four primer combinations were finally chosen for the diversity screening: *Eco*RI-ACA/*Mse*I-CTC, *Eco*RI-ACC/*Mse*I-CAT, *Eco*RI-ACG/*Mse*I-CTC and *Eco*RI-AGG/*Mse*I-CAA.

Diversity screening

The application of the four primer combinations on 188 oak genotypes resulted in 202 scoreable markers, of which 170 were polymorphic. All trees were characterised by a unique banding pattern. Only oaks, for which all four fingerprints were successfully produced, were taken into account for further analysis. For the stands QR5 and QR6, only six and ten samples were successfully analysed (Table 1) and were not included in the calculations based on marker frequencies per population.

After elimination of the monomorphic markers, Spearman correlations were calculated between all pairs of markers. The low average-pairwise correlation value of 0.074 suggests the presence of only a limited amount of redundant information in the data set. This can be attributed to the high degree of polymorphism detected within and between the populations analysed.

A PCO analysis (Fig. 1) clearly separated sessile and pedunculate oaks into two differentiated groups, leaving only ten (out of 188) oaks to be apparently ‘misclassified’. These atypical samples most-likely represent minor mixtures in a stand dominated by the opposite oak species, as will confirmed by their morphologic evaluation (see below). The two plotted axes accounted for respectively 7.0 and 6.5% of the variation present at the molecular level. The vast majority of individuals with a

Table 4 Results of the analysis of genetic diversity based on 170 AFLP markers scored in 88 *Q. petraea* and 84 *Q. robur* individuals. All calculations are performed using the program AFLPsurv 1.0. (Vekemans 2001). Names of populations refer to Table 1. *n* number of populations; *Ht* total diversity; *Hw* average diversity within populations; *Hb* average diversity between populations; F_{ST} differentiation between populations; Low 99% F_{ST}-Upper 99% F_{ST} critical values at the 99% level of the randomisation distribution of F_{ST} assuming no population structure, based on 5,000 random permutations

Populations	*n*	*Ht*	*Hw*	SE (*Hw*)	*Hb*	SE (*Hb*)	F_{ST}	Low 99% F_{ST}	Upper 99% F_{ST}	*P*-value
QP1–4; QR1–4	2	0.3021	0.2800	0.000120	0.0222	0.000000	0.0733	0	0.0053	< 0.0001
QP1, 2, 3, 4	4	0.2949	0.2887	0.010312	0.0061	0.000000	0.0208	0	0.0076	< 0.0001
QR1, 2, 3, 4	4	0.2901	0.2840	0.010366	0.0062	0.001911	0.0213	0	0.0112	0.0012

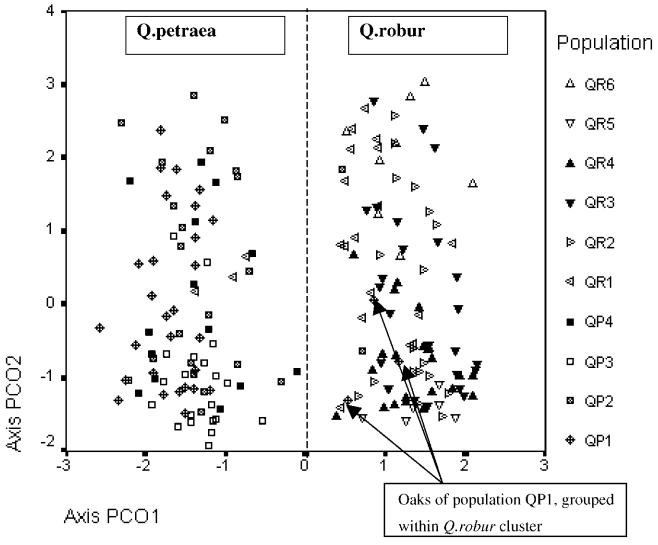

Fig. 1 Plot of the first two principal coordinates calculated from the Euclidean distance of presence/absence data for 170 polymorphic AFLP markers. Samples that do not cluster within their population and for which phenotypic results are available are indicated with *arrows*. Individuals are coded according to the stand. Names of stands refer to Table 1

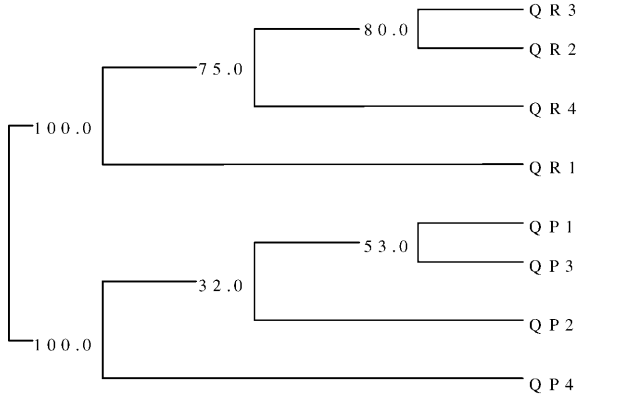

Fig. 2 Dendrogram (UPGMA) calculated from the squared Euclidian distance of AFLP marker frequencies per population. Names of populations refer to Table 1. *Numbers* at forks are bootstrap support values, based on 1,000 bootstrapped datasets

negative score for the first principal coordinate belong to the *Q. petraea* species-group; individuals with a positive score belong to the *Q. robur* group.

The reproducibility of the grouping was further tested by performing a hierarchical cluster analysis (UPGMA), based on the frequency data of AFLP markers per population, and the stability of the resulting dendrogram was tested by bootstrapping procedures (Fig. 2). All oak populations grouped together with populations from the same species. The separation between both species has very strong bootstrap support (100%). The relationships at the population (within-species) level were less stable (bootstrap values between 32% and 80%).

After exclusion of the ten atypical samples (Fig. 1), an analysis of the population genetic structure indicated a significant differentiation between the two species (F_{ST} = 0.0733, P < 0.0001, Table 4). The differentiation among populations within species was lower though also significant for both species [F_{ST} = 0.0208 (P < 0.0001) for *Q. petraea*; F_{ST} = 0.0213 (P = 0.0012) for *Q. robur*]. The vast majority of the diversity present was attributed to the within-population level for both species. The levels of diversity within both species, either at the population (*Hw*) or the whole-sample level (*Ht*), were strikingly similar.

Identification of diagnostic markers for species assignment

No species-specific markers were detected by the four AFLP primer combinations used in the diversity study, and the significant differentiation found at species level was due to AFLP marker-frequency differences. The average difference in marker frequency between species was 12%, but one marker displayed a difference in frequency up to 71%.

A genotypic difference of 71% will correspond with an allele frequency difference of 71% if the vast majority of plants in which the marker is present are homozygous for this marker locus. In case of heterozygosity for this locus, allele frequency differences will be lower than 71%. Therefore, the observed 71% of genotypic difference is most-likely an overestimate of the true allele frequency difference. The allele frequency difference at this specific marker locus is estimated as 62% (using the Bayesian approach from Zhivotovsky, see data analysis).

Table 5 Descriptive statistics and significance values for the phenotypic characters. Five leaves of 30 sessile (QP1) and 30 pedunculate (QR4) oaks were analysed. Mean values per character were used for data analysis

Parameter	Mean	Std. deviation	Minimum	Maximum	Mann-Whitney U	Significance (2-tailed)
PUB	2.63	1.40	1.00	5.00	40.00	0.000
BS	22.71	8.63	8.32	55.16	269.50	0.002
LDR	3.03	0.60	2.13	4.71	118.00	0.000
LS	1.68	0.10	1.45	1.90	474.00	0.773
PR	0.0826	0.0439	0.01	0.18	27.00	0.000
PV	27.92	24.06	0.00	89.69	57.00	0.000

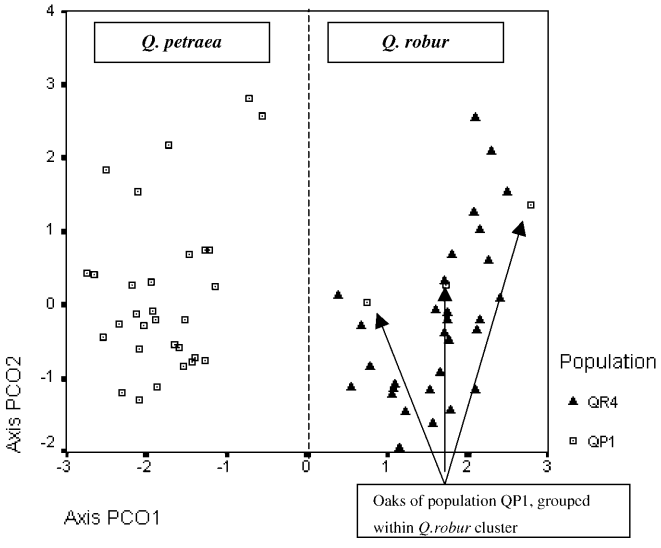

Fig. 3 Plot of the first two principal coordinates calculated from Euclidean distances based on five morphological characters (Table 2) of 60 trees (populations QR4 and QP1). Individuals are marked according to their population. Names of stands refer to Table 1. The same oaks of QP1 are clustered within the *Q. robur* group (indicated with *arrows*) as on the PCO plot based on AFLP markers (Fig. 1)

Phenotypic analysis

Secondary characters were calculated, based on the measured leaf parameters (Table 2) for 30 trees from one sessile oak (QP1) and one pedunculate oak (QR4) population. The discriminatory power of these parameters was computed (Table 5). All but one character (LS, lamina shape) were significantly different between sessile and pedunculate oaks. PCO analysis (Fig. 3) divided the samples into two completely separated groups, without any intermediate positions. The first axis, representing the species divergence, explains 55.0% of the variation and axis 2 stands for 20.1% of the variation present in the morphology dataset. As expected, the three oaks of stand QP1 that were identified as *Q. robur* based on AFLP genotyping were here again positioned within the *Q. robur* group.

Discussion

Use of AFLP markers for population genetic analysis of Flemish oak populations

One of the major drawbacks of using dominant markers such as AFLPs for population genetics is that they only provide information on genotypic frequencies and not on the underlying allelic frequencies. On the other hand, the power of AFLP, generating a huge amount of markers in comparison to other molecular marker systems, makes it an important tool for population studies.

In order to estimate population genetic parameters one can either assume Hardy Weinberg equilibrium (for an outcrossing species) or use estimates of the inbreeding coefficient (F_{is}) from other studies performed with codominant markers to calculate allelic frequencies (Vekemans et al. 2002). In this case it is assumed that the F_{is} values estimated in different populations and using different marker systems are the same. Alternatively, an AMOVA approach (Excoffier et al. 1992), assuming the input of co-dominant data, could be applied in order to avoid the inaccuracies of estimating F_{is} values. This method can, however, give significantly biased estimates of population genetic parameters when dominant marker data are used, especially for outcrossing species such as oaks. No information on allelic frequencies has been published yet for Flemish oak populations. It was therefore decided to use an average value for F_{is} (F_{is} = 0.15), computed in other natural oak populations distributed over the geographic range of the oak species (Bacilieri et al. 1994; Streiff et al. 1999). Analysis was repeated with F_{is} values of 0.1 and 0.2 which resulted in comparable parameters and identical conclusions.

Interspecific variation and species differentiation

The present dataset revealed a clear differentiation of the Flemish oak gene pool in sessile and pedunculate oaks. The clustering based on the frequency data of AFLP markers, grouped the populations of the same species together, with a bootstrap value of 100%. Similar results were obtained in a microsatellite survey of European oak populations (Muir et al. 2000). Therefore, the power of AFLP markers to discriminate the sessile and pedunculate oaks is comparable to the power obtained by Muir et al. (2000) using 20 microsatellite markers. Further-

more, PCO analysis on the AFLP data was able to assign the individual trees to the 'right' species. At first sight, only ten trees were atypical. These ten samples displayed an AFLP fingerprint that did not fit in the stand where they were sampled. Most of these oaks had also been designated as the species other than the dominant one in the stand where they are located during the collection of the material in the field. For the three atypical oaks that were also characterised morphologically, the results of both genotypic and phenotypic classification were identical at the individual tree level. The combination of the AFLP results, the morphological study and additional field information show clearly that these apparently atypical oaks were in fact classified correctly based on their AFLP fingerprints, and that they represent minor mixtures in a stand dominated by the other oak species.

The differences between species as detected by PCO analysis proved to be highly significant (F_{ST} = 0.0733, $P < 0.0001$). This differentiation value is lower than often found between taxonomic closely related species (e.g. Lowe et al. 2000), but the differentiation detected in the present study was stronger than formerly observed in these oak species using other molecular-marker techniques (with isozymes: e.g. Zanetto et al. 1994, with RAPDs; Moreau et al. 1994). AFLP, generating many polymorphic markers, was successfully applied for identifying the two major taxonomic units present in the Flemish oak gene pool. This supports the hypothesis that the formerly, often observed, discrepancy between the discriminatory power of phenotypic (high) and genetic (low) differentiation between both species can be due to a sampling effect: too few loci were investigated with molecular markers to reveal the same divergence at the molecular level.

PCO plots were produced for each AFLP primer combination separately and for all possible combinations of two primer combinations (data not shown). None of the (sets of) primer combinations had enough discriminatory power to make the same clear differentiation between species as found using the whole dataset (four primer combinations). However, if only the ten most species-specific markers out of the complete dataset (ten markers showing the highest difference in frequency between both species, marker frequency differences range from 71% to 39% in this group of ten markers) were used, similar results were obtained as when the complete data set of 170 polymorphic markers was used. Most of the AFLP markers are assumed to be neutral markers (Vos et al. 1995) and are therefore not likely to be linked to the plant's phenotype. Nevertheless, when selecting species-diagnostic AFLP markers, a selection is made towards markers associated with genomic regions of species-divergence. Hence, these markers are more-likely to be associated with the phenotype, if the observed morphological differences are truly diagnostic characters and not the result of phenotypic plasticity or modifications. The presence of these species-diagnostic markers will be further analysed in oak trees originating from a wider geographic range to estimate their true diagnostic value for the species assignment of sessile and pedunculate oaks.

Although many AFLP markers were generated using four AFLP primer combinations, only a relatively small portion of the genome was sampled. For rapidly screening more regions of the genome, 60 different AFLP primer combinations were tested on DNA bulks. Six primer combinations identified a total of eight putative species-specific markers. None of the eight markers selected in the bulks was confirmed as species-specific when individual trees were analysed. The markers were present in maximum eight out of the ten trees from the same species and were often too faint to score on individual plants (data not shown).

These results confirm the previous studies on the divergence of sessile and pedunculate oaks. Up to now, no single marker has been identified which allowed differentiating the two species at the individual tree level (Moreau et al. 1994, using RAPDs; Zanetto and Kremer 1995, using isozymes; Bodénès et al. 1997a, using 2,800 PCR-amplification products; Muir et al. 2000, using SSR markers and Muir et al. 2001, using rDNA). As stated, the overall nucleotide divergence is limited (Bodénès et al. 1997a) and past and recent hybridisation events are likely to have occurred, which renders the chance of finding species-specific markers with a random PCR-technique low.

Intraspecific variation and population differentiation

Oaks exhibit a level of diversity that is amongst the highest of all woody species (Kremer and Petit 1993), despite their relatively small genome (Zoldos et al. 1998). Bacilieri et al. (1996) demonstrated that the maintenance of the genetic diversity in these species is associated with almost complete outcrossing. It has been shown that outcrossing woody species typically display greater genetic variation within populations than among populations (e.g. Hamrick and Godt 1996; Heaton et al. 1999). The current study, however, found that AFLP variation was not distributed entirely homogeneously across the sampled populations. A small, though significant, proportion of the total variation was assigned to differences among populations [F_{ST} = 0.0208 ($P < 0.0001$) for *Q. petraea*, F_{ST} = 0.0213 (P = 0.0012) for *Q. robur*]. The vast majority of variation present in the dataset was attributed to the within-population level (ratio H_w/H_t = 0.979 for both species). These values are comparable with other oak studies based on isozyme analysis (Kremer et al. 1991: 91% of the variation was attributed to within-populations differences). In other studies of oaks over a large European range, codominant and dominant markers revealed the presence of population differentiation in both sessile and pedunculate oaks (Bodénès et al. 1997b; Le Corre et al. 1997). However, in contrast with previous studies based on isozymes and microsatellites (Zanetto et al. 1994 and Streiff et al. 1999) where a higher genetic diversity was detected within *Q. petraea*, similar levels of diversity were found for both species. The present analyses confirm that the studied Flemish oak stands, located within a distance of 150 kilo-

metres, cannot be considered as one panmictic unit. This is rather unexpected when considering the long-distance transport of oak pollen (Lahtinen et al. 1996; Streiff et al. 1999). A hypothesis on the origin of the detected differentiation can be found in the history of many Flemish forests. As has been revealed by recent molecular analysis, historical events such as habitat fragmentation and population bottlenecks can have an important influence on the present patterns of genetic differentiation (Newton et al. 1999). The autochthonous Flemish oak stands are old coppiced populations and are assumed to be relicts of populations, established during the re-migration of the oaks after the last glacial period. During history, the surfaces of (ancient) woods have often declined significantly due to a high anthropogenic pressure. When pressure diminished, populations could be re-established out of the preserved remnants. Hence, this enforced bottleneck and subsequent genetic drift may account for the currently detected differentiation at the population level. The presence of some of the oldest Flemish oak stools on top of land dunes (e.g. populations QR4, QR3, QP1) further support this hypothesis: all land except for the dry and unfertile land dunes might have been de-forested, leaving the old coppice stools as seed sources for the future re-establishment of the oak populations.

Taxonomic status and hybridisation

Many experimental results support the hypothesis of the existence of interspecific gene flow and introgression between *Q. petraea* and *Q. robur*, the most important being: (1) the possibility of interspecific crossings (Steinhoff 1993), (2) the occurrence of natural hybrids and the so called 'regeneration' of *Q. petraea* from successive unidirectional hybridisation with *Q. robur* (Bacilieri et al. 1996; Petit et al. 1997), and (3) the similar geographic structure of chloroplast haplotypes (Dumolin-Lapègue et al. 1999). Moreover, analyses of chloroplast and mitochondrial DNA variation revealed that hybridisations took place during the stay of the oaks in glacial refugia, but that also recent hybridisation and introgression events are most-likely to occur (Petit et al. 1997; Dumolin-Lapègue et al. 1999).

On the other hand, nuclear markers have shown that both species are differentiated throughout the geographical range of their natural distribution (e.g. Zanetto et al. 1994). AFLP markers reflect the divergence of the Flemish oaks into two main groups, according to their taxonomic species, suggesting that the gene flow between both groups is limited. Even in populations were both species coexist (QR1, QP1, QP2, QP4), only very few putative hybrid forms were detected. Assuming the occurrence of past and recent interspecific gene flow, two hypotheses can be put forward to explain the absence of genetic intermediate individuals in a stand where both species are present. First of all, and as suggested by Bacilieri et al. (1995), if hybridisation rates are low, the number of samples in the present survey may be too small to detect the rare hybrid forms. Secondly, directional mating and subsequent asymmetrical backcrosses to the *Q. petraea* parent will reduce the occurrence of intermediate forms. When analysing individuals resulting from several backcrosses with a *Q. petraea* parent, the generated AFLP fingerprints will be regarded as part of the genetic variability present within the *Q. petraea* gene pool. In addition, after repeated backcrosses, the morphology of the tree will probably be more similar to *Q. petraea* than to *Q. robur*. A case study on one oak population, using the same AFLP markers will be carried out in order to further investigate the first hypothesis. Assuming that most acorns germinate in the vicinity of the mother tree (Petit et al. 1997) and the preferential directionality in interspecific crosses with *Q. petraea* as pollinator (Bacilieri et al. 1996), most putative hybrids will be located in the vicinity of *Q. robur* trees in a stand dominated with *Q. petraea*. An AFLP analysis of the saplings and seedlings surrounding these pedunculate oaks will be included when studying a population into more detail.

Acknowledgements The authors thank Nancy Mergan and Katrien Liebaut for excellent technical assistance, Paul Vandecasteele for the development of the software for the automatic measurement of morphological parameters, Jan De Riek for help with software programs, fruitful discussions and comments on this manuscript, Rémy Petit, Jos van Slycken, An Vanden Broeck and Kristine Vander Mijnsbrugge for useful comments on the manuscript. We are very grateful to Xavier Vekemans for supplying the software and discussions on data analysis. Also thanks to two anonymous referees for useful comments on an earlier draft of the manuscript. This work was supported by the Ministry of the Flemish Community (AMINAL, Forests and Green Areas Division and Nature Division).

References

Aas G (1990) Kreuzbarkeit und Unterscheidung von Stiel- und Traubeneiche. Allg Forst-u J-Ztg 9–10:219–221

Aas G (1993) Taxonomical impact of morphological variation in *Quercus robur* and *Quercus petraea*: a contribution to the hybrid controversy. Ann Sci For 50(suppl 1):107–113

Bacilieri R, Labbe T, Kremer A (1994) Intraspecific genetic structure in a mixed population of *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. Heredity 73:130–141

Bacilieri R, Ducousso A, Kremer A (1995) Genetic, morphological, ecological and phenological differentiation between *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. in a mixed stand of Northwest of France. Sylvae Genet 44:1–10

Bacilieri R, Ducousso A, Petit RJ, Kremer A (1996) Mating system and asymmetric hybridization in a mixed stand of European oaks. Evolution 50:900–908

Bodénès C, Joandet S, Laigret F, Kremer A (1997a) Detection of genomic regions differentiating two closely related oak species *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. Heredity 78:433–441

Bodénès C, LabbéT, Pradère S, Kremer A (1997b) General vs local differentiation between two closely related white oak species. Mol Ecol 6:713–724

De Greef B, Triest L, De Cuyper B and Van Slycken J (1998) Assessment of intraspecific variation in half-sibs of *Quercus petraea* (Matt.) Liebl. 'plus' trees. Heredity 81:284–290

De Riek J, Dendauw J, Mertens M, De Loose M, Heursel J, Van Bockstaele E (1999) Validation of criteria for the selection of AFLP markers to assess the genetic variation of a breeders' collection of evergreen azaleas. Theor Appl Genet 99:1155–1165

Dumolin S, Demesure B, Petit RJ (1995) Inheritance of chloroplast and mitochondrial genomes in peduculate oak investigated with an efficient PCR method. Theor Appl Genet 91:1253–1256

Dumolin-Lapègue S, Demesure B, Fineschi S, Le Corre V, Petit RJ (1997) Phylogeographic structure of white oaks throughout the European continent. Genetics 146:1475–1487

Dumolin-Lapègue S, Kremer A, Petit RJ (1999) Are chloroplast and mitochondrial DNA variation species independent in oaks? Evolution 53:1406–1413

Dupouey JL (1983) Analyse multivariable de quelques charactères morphologiques de populations de chènes. Ann Sci For 40:265–282

Dupouey JL, Badeau V (1993) Morphological variability of oaks (*Quercus robur* L. *Quercus petraea* (Matt.) Liebl. *Quercus pubescens* (Willd.)) in northeastern France: preliminary results. Ann Sci For 50(suppl 1):35s–40s

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Felsenstein J (1993) PHYLIP, Phylogenetic inference package, version 3.5.7. A computer program distributed by the author, http://evolution.genetics.washington.edu/phylip.html. Department of genetics, University of Washington, Seattle, USA

Gathy P (1969) Contribution a l'étude de la génétique des chènes. FAO 2:979–987

Gerber S, Mariette S, Streiff R, Bodenes C, Kremer A (2000) Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. Mol Ecol 9:1037–1048

Gömöry D (2000) A gene coding for a non-specific NAD-dependent dehydrogenase shows a strong differentiation between *Quercus robur* and *Quercus petraea*. For Genet 7:167–170

Hamrick JL, Godt MJW (1996) Effect of life history traits on genetic diversity in plant species. Phil Trans Royal Soc Lond B 351:1291–1298

Heaton HJ, Whitkus R, Gómez-pompa A (1999) Extreme ecological and phenotypic differences in the tropical tree chicozapote (*Manilkara zapota* (L.) P. Royen) are not matched by genetic divergence: a random amplified polymorphic DNA (RAPD) analysis. Mol Ecol 8:627–632

Jones EW (1959) Biological flora of the British islands, *Quercus* L. J Ecol 17:169–222

Kleinschmit J, Bacilieri R, Kremer A, Roloff A (1995) Comparison of morphological and genetic traits of pedunculate oak (*Q. robur* L.) and sessile oak (*Q. petraea* (Matt.) Liebl.). Silvae Genet 5–6:256–267

Kleinschmit JRG, Kleinschmit J (1996) Artificial hybridization between *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. In: Inter- and intra-specific variation in European oaks: evolutionary implications and practical consequences Kremer A, Mühs H (eds) European commission, EUR 1617EN, Luxembourg, pp 69–87

Kremer A, Petit RJ (1993) Gene diversity in natural populations of oak species. Ann Sci For 50 (suppl 1):186s–202s

Kremer A, Petit RJ, Zanetto A, Fougère V, Ducousso A, Wagner D, Chauvin C (1991) Nuclear and organelle gene diversity in *Quercus robur* and *Quercus petraea*. In: Genetic variation in European populations of forest trees Müller-Starck G, Ziehe M (eds) Sauerländers Verlag, Frankfurt am Main, pp 141–166

Kremer A, Zanetto A, Ducousso A (1997) Multilocus and multitrait measures of differentiation for gene markers and phenotypic traits. Genetics 145:1229–1241

Lahtinen MJ, Pulkkinen P, Helander ML (1996) Potential gene flow by pollen between English oak (*Quercus robur* L.) stands in Finland. For Studies 28:47–50

Lexer C, Heinze B, Gerber S, et al. (2000) Microsatellite analysis of maternal half-sib families of *Quercus robur*, pedunculate oak. II. Inferring the number of pollen donors from the offspring. Theor Appl Genet 100:858–865

Le Corre V, Dumolin-Lapegue S, Kremer A (1997) Genetic variation at allozyme and RAPD loci in sessile oak (*Quercus petraea)*: the role of history and geography. Mol Ecol 6:519–529

Lefort F, Douglas GC (1999) An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species *Acer*, *Fraxinus*, *Prunus* and *Quercus*. Ann Sci For 56:259–263

Lowe AJ, Gillies ACM, Wilson J, Dawson IK (2000) Conservation genetics of bush mango from central/west Africa: implications from a random amplified polymorphic DNA analysis. Mol Ecol 9:831–841

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. Mol Ecol 3:91–99

Maes N, Rövenkamp C (1999) Onderzoek naar autochtone genenbronnen in de houtvesterijen Hechtel en Bree. Vertrouwelijke rapporten, AMINAL, Forest and Green Area Division, Brussels

Moreau F, Kleinschmit J, Kremer A (1994) Molecular differentiation between *Quercus petraea* and *Quercus robur* assessed by random amplified DNA fragments. For Genet 1:51–64

Muir G, Fleming CC, Schlötterer C (2000) Species status of hybridizing oaks. Nature 405:1016

Muir G, Fleming CC, Schlötterer C (2001) Three divergent rDNA clusters predate the species divergence in *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. Mol Biol Evol 18:112–119

Newton AC, Allnut TR, Gillies ACM, Lowe AJ, Ennos RA (1999) Molecular phylogeography, intraspecific variation and the conservation of tree species. Trends Ecol Evol 14:140–145

Olsson V (1975) Morphological analysis of phenotype in populations of *Quercus* species in Sweden. Bot Notiser 128:55–69

Petit RJ, Kremer A, Wagner DB (1993) Geographical structure of chloroplast DNA polymorphisms in European oaks. Theor Appl Genet 87:122–128

Petit RJ, Pineau E, Demesure B, Bacilieri R, Ducousso A, Kremer A (1997) Chloroplast DNA footprints of postglacial recolonisation by oaks. Proc Natl Acad Sci USA 94:9996–10,001

Roldán-Ruiz I, Dendauw J, Van Bockstaele E, Depicker A, De Loose M (2000) AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). Mol Breed 6:125–134

Rushton BS (1977) Artificial hybridization between *Q. robur* and *Q. petraea*. Watsonia 11:229–236

Steinhoff S (1993) Results of species hybridization with *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. Ann Sci For 53:173–180

Streiff R, Ducousso A, Lexer C, Steinkellner H, Gloessl J, Kremer A (1999) Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. Mol Ecol 8:831–841

Van Valen L (1976) Ecological species, multispecies and oaks. Taxon 25:233–239

Vekemans X (2001) AFLPsurv V.1.0. a software for genetic diversity analysis with AFLP population data, distributed by the author, xvekema@ulb.ac.be, Université Libre de Bruxelles

Vekemans X, Beauwens T, Lemaire M, Roldán-Ruiz I (2002) Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Mol Ecol 11:139–151

Vos P, Hogers R, Bleeker M et al. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wigston DL (1974) Cytology and genetics of oaks. In: Morris MG, Perring FH (eds) The British oak London

Zanetto A, Kremer A (1995) Geographical structure of gene diversity in *Quercus petraea* monolocus patterns of variation. Heredity 75:506–517

Zanetto A, Roussel G, Kremer A (1994) Geographic variation of inter-specific differentiation between *Quercus robur* and *Quercus petraea*. For Genet 1:111–123

Zhivotovsky LA (1999) Estimating population structure in diploids with multilocus dominant DNA markers. Mol Ecol 8:907–913

Zoldos V, Papes D, Brown SC, Panaud O, Siljak-Yakovlev S (1998) Genome size and base composition of seven *Quercus* species: inter- and intra-population variation. Genome 41:162–168