

# Distribution of genomic regions differentiating oak species assessed by QTL detection

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Pedunculate oak and sessile oak are two sympatric interfertile species that exhibit leaf morphological differences. We aimed to detect quantitative trait loci (QTLs) of these traits in order to locate genomic regions involved in species differentiation. A total of 15 leaf morphological traits were assessed in a mixed forest stand composed of *Quercus petraea* and *Q. robur* and in a full-sib pedigree of *Q. robur*. The progeny of the full-sib family were vegetatively propagated in two successive experiments comprising 174 and 216 sibs, and assessments were made on two leaves collected on each of the 1080 and 1530 cuttings corresponding to the two experiments. Traits that exhibited strong species differences in the mixed stand tended also to have higher repeatability values in the mapping population, thus indicating higher genetic control. A genetic map was

constructed for QTL detection. Composite interval mapping with the one QTL model was used for QTL detection. From one to three QTLs were detected for 13 traits. In-depth analysis of the QTLs, controlling the five morphological traits that exhibited the highest interspecific differences in the mixed stand, indicated that they were distributed on six linkage groups, with two clusters comprising QTLs of at least two discriminant traits. These results were reinforced when error 1 for QTL detection was set at 5% at the chromosome level, as up to nine clusters could be identified. In conclusion, traits involved in interspecific differentiation of oaks are under polygenic control and widespread in clusters across the genome.

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## Introduction

Pedunculate oak (*Quercus robur* L.) and Sessile oak (*Q. petraea* (Matt.) Liebl.) are closely related species with wide sympatric distributions in Europe, although their taxonomic distinctiveness is not clearly defined and has raised conflicting opinions between botanists. Since sessile and pedunculate oak are interfertile in natural conditions (Rushton, 1977), the biological species concept (Mayr, 1963) has been challenged by some authors (Kleinschmit and Kleinschmit, 2000). Even if the two species are sympatric, they occupy different ecological niches within the same stands. Indeed, *Q. robur* preferentially grows in rich, wetter, and more alkaline habitats, whereas *Q. petraea* occurs on drier and more acidic soils (Becker and Levy, 1990). Nevertheless, in spite of their ecological differences, the two species coexist in mixed stands and intermediate forms appear under these circumstances (Olsson, 1975; Rushton, 1979; Dupouey, 1983; Bacilieri *et al.*, 1996).

Interspecific variation in mixed oak stands has raised a general interest in Europe since decades among scientists and foresters. The two species exhibit extremely low genetic differentiation, because of their interfertility and their sympatry. Species differentiation has been tested using various genetic markers, for example, isozymes

(Zanetto *et al.*, 1994; Gömöry, 2000), ribosomal DNA (Petit and Kremer, 1993; Muir *et al.*, 2001), chloroplast DNA (Petit and Kremer, 1993), proteins (Barreneche *et al.*, 1996), and anonymous DNA fragments (Moreau *et al.*, 1994; Bodénès *et al.*, 1997; Cervera *et al.*, 2000; Zoldos *et al.*, 2001; Mariette *et al.*, 2002). These investigations showed the absence of species-specific markers, and that the two species displayed only allele frequency differences. These results differed markedly from phenotypic observations made in mixed stands when the two species coexist, where they show clear ecological preferences and differences in leaf morphological characters. Earlier regional investigations of leaf morphological variation indicated that the two species exhibit quantitative differences when multivariate methods are used (Aas, 1993; Dupouey and Badeau, 1993). A recent study, using the same techniques, over a wider sample of populations confirmed these observations (Kremer *et al.*, 2002) that leaf morphological traits show continuous but bimodal distribution in mixed stands. The discrepancy between genetic and phenotypic differentiation is well illustrated by the genetic survey of Bodénès *et al.* (1997) where only 2% of the 2800 random amplified polymorphic DNA (RAPD) fragments exhibited significant frequency differences between the two species. These results suggested that genomic regions involved in species differentiation are rare and explain why earlier molecular investigations, using a low number of loci, proved to be inefficient.

In this contribution, we attempt to investigate the distribution of these informative (species-discriminant) genomic regions within the oak genome. The strategy

adopted is a downstream approach from the informative and discriminant phenotypic traits to their underlying loci. As quantitative differences in phenotypes between individuals are due to the segregation of alleles at multiple quantitative trait loci (QTLs), we intend to map the QTLs that control species-discriminant leaf morphological traits. With the recent and widespread availability of polymorphic molecular markers, QTL mapping has been used to locate the genomic regions involved in interspecific differentiation in various species, for example *Drosophila* (Zeng *et al*, 2000), tomato (Grandillo and Tanksley, 1996), monkeyflower (Bradshaw *et al*, 1998), rice (Brondani *et al*, 2002), maize (Westerbergh and Doebley, 2002), millet (Poncet *et al*, 2002), and sunflowers (Kim and Rieseberg, 1999). In the present study, we aimed to locate QTLs of 15 leaf morphological characters that are traditionally used for oak species identification. The QTL detection was performed in a cloned full-sib family of *Q. robur* comprising 278 progeny, while interspecific variation in morphological characters was first estimated on phenotypic values recorded in a natural mixed stand.

## Material and methods

### Natural population

Leaf morphological traits were assessed in a mixed stand of *Q. petraea* and *Q. robur* located in the northwest of France (Bacilieri *et al*, 1995; Kremer *et al*, 2002). The study area is square (250 m × 250 m) and comprises 426 adult trees (older than 100 years), approximately evenly distributed between the two species (199 *Q. petraea*, 215 *Q. robur*, and 12 so-called intermediate). The data of the intermediate trees were not used. The sampling was exhaustive and five leaves were sampled within the upper crown of each tree. The morphological data, previously reported (Bacilieri *et al*, 1995; Kremer *et al*, 2002), will be used here only for the estimation of the variance components of the traits.

### Mapping population

An intraspecific hybrid family of *Q. robur* was obtained by crossing two adult trees. The female parent (accession 3P) was located on the Forestry Research Station of Pierroton (latitude 44.44 N, longitude 0.46 W), and the male parent (accession A4) originated from Arcachon (latitude 44.40 N, longitude 1.11 W). More than 400 acorns were obtained from the controlled cross, from which 278 seedlings were produced. A subset of 94 individuals were previously used for the construction of parental genetic maps (Barreneche *et al*, 1998) and 184 additional full sibs were genotyped for QTL mapping in the present study. The 278 seedlings were raised in a seedbed in the nursery until age 3, and they were then transplanted in a clonal bank as stool-beds. The spacing between the stool-beds was 1.5 m × 1.5 m. After 2 years following the transplantation, when the seedlings were 5 years old, each stool-bed was hedged at the level of the ground in late-February. The stool-beds stump sprouted in late April, and when the sprouts were fully elongated, cuttings were prepared for vegetative propagation. The cuttings consisted of stem segments about 10–12 cm long, comprising at least two internodes. They were dipped in a rooting hormone (Rhizozone AA 0.5) and transplanted

in 80 cm<sup>3</sup> containers with a 60/40 mixture of peat/perlite. The cuttings were then transferred into a greenhouse that was watered by a fog system. The first rooting occurred after 5 weeks, and more than 60% of the cuttings rooted within 2 months. The rooted cuttings were further transplanted into 4 l containers (with a mixture of 2/1/2: peat/sand/pine bark) in September. They over-wintered in a greenhouse and grew outdoors in the nursery for an additional year. The procedure for vegetative propagation was used recurrently over 4 successive years. The QTL detection was carried out in two experiments.

**Experiment 1:** In total, 174 full sibs were successively propagated in summer 1997 and transplanted from the nursery to a field test located in Bourran (latitude 44.20 N, longitude 0.24 W, along the Garonne River) in southwest France. The experiment design comprised 36 incomplete blocks each containing single vegetative replicates of 30 full sibs. The whole experiment comprised 1080 cuttings (on average six vegetative replicates per full sib). The cuttings were planted in the spring 1999, and two fully elongated leaves were collected from each cutting in summer 1999. Leaf morphological data were adjusted to block effects prior to the detection of QTLs.

**Experiment 2:** This experiment comprised 216 full sibs of the same mapping pedigree. Vegetative propagation took place in spring 1998. Two leaves were collected in summer 1999 in the nursery from each of 1530 cuttings (on average seven vegetative replicates per full sib). The experimental design was considered as fully randomised.

### Assessment of leaf morphological traits

In all, 15 morphological traits were assessed on all the leaves collected in the natural populations and in the two experiments. The measurements were made following the procedure indicated by Kremer *et al* (2002): lamina length (LL), petiole length (PL), lobe width (LW), sinus width (SW), length of lamina at widest point (WP), number of lobes (NL), number of intercalary veins (NV), basal shape of the lamina (BS), lamina shape (OB = WP/LL), petiole ratio (PR = (PL/(PL + LL))), lobe-depth ratio (LDR = (LW - SW)/LW), percentage venation (PV = NV/NL), lobe-width ratio (LWR = LW/LL). In addition to the morphological assessments, observation of pubescence (PU) was made at two different spots on the abaxial surface of each leaf: in the central part of the lamina (PUI) and along the veins (PUV). The measurements of pubescence along the veins were only made on the cuttings and not on the adult leaves. Observations of pubescence were made following Kissling's grading system (Kissling, 1980), ranging from 1 (no pubescence) to 6 (dense pubescence); pubescence was assessed using a stereo microscope.

### Statistical analysis

**Estimation of variance components in the natural population:** The value of each morphological trait ( $X_{ijk}$ ) was decomposed according to the following two-way analysis of variance (ANOVA) model, using the

OPEP Software (Baradat and Labbé, 1995):

$$X_{ijk} = \mu + S_i + T_{ij} + L_{ijk} \quad (1)$$

where  $S_i$  stands for the effect of species  $i$ ,  $T_{ij}$  for the effect of tree  $j$  within species  $i$ , and  $L_{ijk}$  for the effect of leaf  $k$  within tree  $j$  and within species  $i$ .

In a second step, the subdivision was made separately within each species. Model (2) was successively used for *Q. petraea* and *Q. robur*

$$Y_{jk} = \mu + T_j + L_{jk} \quad (2)$$

where  $Y_{jk}$  is the phenotypic value of a leaf morphological trait,  $T_j$  the effect of tree  $j$ , and  $L_{jk}$  is the effect of tree  $j$  within tree  $k$ .

All sources of variation were considered as random. Consequently, the following ratios were calculated to account for the proportion of the total variation explained by different sources of variation.

$S = \sigma_S^2 / \sigma_X^2$  derived from model (1) is an estimate of the variation explained by species differentiation, whereas  $T = \sigma_T^2 / \sigma_Y^2$  derived from model (2) represents the proportion of variation within a species that is due to tree variation within that species.

**Estimation of variance components in the full-sibs pedigree:** All data were analysed using the ANOVA with the OPEP Software (Baradat and Labbé, 1995). Since vegetative propagation was used to propagate the full sibs of the mapping pedigree, the subdivision of the phenotypic value ( $Z_{ij}$ ) of a cutting could be made with the following model in the two experiments:

$$Z_{ij} = \mu + C_j + Cu_{ij} \quad (3)$$

where  $C_i$  is the effect of clone  $i$  and  $Cu_{ij}$  is the effect of cutting  $j$  within clone  $i$ .

Based on the variance components obtained by the ANOVA (model 2), the repeatability could be calculated as

$$R = \frac{\sigma_C^2}{\sigma_C^2 + \frac{\sigma_{Cu}}{n}}$$

where  $n$  is the number of cuttings for each clone.

### Construction of the linkage map

The mapping strategy used in this study was the so-called double-pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994). Based on the framework map that was obtained on the same pedigree but only on 94 full sibs (Barreneche *et al*, 1998), we extended the mapping to 278 individuals and mapped additional anchor markers on the total set of individuals. The additional mapping consisted in 38 microsatellites, six SCARs (Bodénès *et al*, 1996), and 13 AFLP primer-enzymes combinations (eight *Eco*/*Mse* and five *Pst*/*Mse*). Amplification fragment length polymorphism (AFLP) genotyping was performed as described by Vos *et al* (1995). AFLP markers were named according to the restriction enzyme used, the three selective nucleotides used on the amplification primers, the size of the band, and the quality of the scoring (1 (difficult to read) to 5 (very easy)). Microsatellites genotyping was performed using the primers developed by Kamper *et al* (1998) and Steinkellner *et al*

(1997). AFLP fragments and microsatellites were separated by electrophoresis on an LI-COR (model 4000) DNA sequencer. Primers were labelled with the fluorescent infrared dye IRD800 (LI-COR, Lincoln, Neb.) and fragment analysis recorded by a laser system.

Mapmaker 2.0 (Lander *et al*, 1987) was used to infer linkage relationships among markers. To allow the detection of linkage of markers in repulsion phase, the data set was duplicated and recoded. First, markers were divided into linkage groups with the group command (parameters LOD=7;  $\theta=0.25$ ). Second, markers were ordered within the linkage group with the first-order command (LOD=3;  $\theta=0.40$ ). The ordered marker sequences were confirmed using the ripple and LOD table commands. Kosambi's (1944) mapping function was then used to transform the recombination frequency between linked loci into centimorgan (cM) distances. The graphical presentation of linkage maps and QTL was obtained using the MapChart software version 2.0 (Voorrips, 2001).

### QTL detection

The analytical method used to identify putative QTLs and to estimate their phenotypic effect was composite interval mapping (CIM; Jansen and Stam, 1994; Zeng, 1994), which is an extension of interval mapping (IM; Lander and Botstein, 1989). IM calculates the likelihood score for a putative QTL located at any position within an interval flanked by two adjacent markers. CIM extends this method by fitting the most significant markers outside the interval into the model, allowing more accuracy and efficiency of QTL mapping (Zeng, 1994). The MultiQtl software (Britvin *et al*, 2001; <http://esti.haifa.ac.il/~poptheor>) was used with no extended parameters (no LOD normalization and no missing data restoration). The SD for each QTL position was estimated by bootstrap (Visscher *et al*, 1996) with 1000 samplings. Empirical statistical significance thresholds for declaring the presence of a QTL were determined by permutations of the data set (Churchill and Doerge, 1994). In all, 1000 different samplings were generated from the actual data by shuffling the trait values with respect to the marker genotypes. Each individual genotyped was randomly assigned to one of the trait values from the sample. In the single QTL model, permutations permitted the null hypothesis of no QTL contributing to the trait to be tested.

Since the permutations tests were calculated at the chromosome level, we further computed the corresponding Type I error rate at the whole genome level. The relationship between Type I error rate at the genome level ( $\alpha_g$ ) and Type I error rate at the chromosome level ( $\alpha_{chr}$ ) is as follows:

$$\alpha_{chr} = 1 - \left\{ 1 - [1 - (1 - \alpha_g)^{1/M}] \right\}^m$$

where  $M$  is the total number of markers used for the QTL detection on each map and  $m$  the number of markers in the linkage group.

The QTL detection was carried out separately for each parental map in both the experiments.

For each combination of morphological trait and marker, the following ANOVA model was used to subdivide the variance components of the clonal means

in the QTL detection:

$$\bar{Z}_{ij} = \mu + M_i + C_{ij} \quad (4)$$

where  $\bar{Z}_{ij}$  is the clonal mean as derived from model (3),  $M_i$  the marker effect, and  $C_{ij}$  the effect of clone  $j$  (within the marker class).

If  $\sigma_Z^2$  is the variance of the clonal means ( $\sigma_Z^2 = \sigma_C^2 + \sigma_{Cu}^2/n$ ), and if  $P$  is the proportion of  $\sigma_Z^2$  explained by the QTL, then the proportion  $Q$  of the clonal variance explained by the QTL is  $Q$  with  $Q = P/R$ .

## Results

### Subdivision of the phenotypic variation in the natural population

All sources of variation contributed significantly to the diversity of leaf morphology as shown by the different ANOVAs computed. However, the contributions of the different sources were quite different according to the traits considered (Table 1). For size-related traits, such as lamina length (LL) and lobe width (LW), species differences represent only a minor part of the total variation. Conversely, more than 80% of the total variation was due to species subdivision for petiole length (PL) and petiole ratio (PR). To a lesser degree, venation (NV, 67% and PV, 70%), pubescence (PUI, 77%), sinus width (SW, 59%), and basal shape of lamina (BS, 72%) exhibited important species variation. However, despite the important species variation, the range of variation within a species significantly overlapped the range of variation for the other species. As an example, *Q. petraea* exhibited a PL varying between 3 and 33 mm, whereas PL varied between 0 and 12 mm in *Q. robur*. For pubescence, *Q. robur* is often reported as glabrous and indeed 84% of the leaves did not exhibit any pubescence,

but 16% of the leaves did exhibit hairs on the abaxial surface of the lamina. Similarly, leaves of *Q. petraea* have usually only a few intercalary veins. Indeed, only 75% of the leaves did not show any intercalary veins in *Q. petraea* and this percentage amounted to 5% in *Q. robur*. The subdivision of variation within a species indicated that the traits that showed high species differentiation also exhibited an important within-species tree-to-tree variation (Table 1).

### Subdivision of phenotypic variation in the mapping pedigree

In experiment 1, data were first adjusted to block effects prior to using ANOVA model (3), whereas in experiment 2, data were directly analysed using ANOVA model (3). All phenotypic values, except PUV, were normally distributed as tested by the Box and Cox method (Box and Cox, 1964). The clonal effect was significant in all ANOVAs, except for OB and LWR in experiment 1 (data not shown).

Repeatability values were slightly higher in experiment 2, as the result of higher environmental variance due to the heterogeneity of environmental conditions in the field test (experiment 1, Table 2). Pubescence (either PUI or PUV) had the highest repeatability, and OB or LWR exhibited the lowest genetic control in both experiments. Overall, morphological traits reported to be strongly involved in species differences (PL, SW, NV, BS, PU, and their derived variables PR, LDR, PV; see Table 1) were also among those that had the largest repeatability values, especially in experiment 2.

### Correlations between leaf morphological traits

Correlations between tree values derived from ANOVA model (2) in the natural population (Table 3a), or clone

**Table 1** Partitioning of leaf morphology variation in the natural population

Trait	<i>Q. petraea</i>		<i>Q. robur</i>		
	Mean	T	Mean	T	S
LL (mm)	91.4	0.59	77.5	0.64	0.23
PL (mm)	16	0.70	4.4	0.52	0.81
LW (mm)	28.2	0.61	24.5	0.63	0.14
SW (mm)	20.5	0.62	13.4	0.50	0.59
WP (mm)	49.7	0.42	44.4	0.50	0.08
NL	11.2	0.52	8.7	0.50	0.51
NV	0.35	0.32	3.1	0.49	0.67
BS	3.9	0.47	6.8	0.48	0.72
PUI	3.4	0.58	1.2	0.74	0.77
OB (%)	54.1	0.16	57.3	0.17	0.07
PR (%)	14.8	0.68	5.4	0.50	0.84
LDR (%)	26.7	0.47	44.5	0.44	0.67
PV (%)	3.32	0.34	36.0	0.48	0.70
LWR (%)	30.8	0.51	31.6	0.40	0.02

$S = \sigma_Z^2/\sigma_X^2$  (between species variance/total variance, see model (1) in the text),  $T = \sigma_T^2/\sigma_Y^2$  (between tree variance/total variance, see model (2) in the text), LL=lamina length, PL=petiole length, LW=lobe width, SW=sinus width, WP=length of lamina at widest point, NL=number of lobes, NV=number of intercalary veins, BS=basal shape of the lamina, OB=lamina shape, PR=petiole ratio, LDR=lobe-depth ratio, PV=percentage venation, LWR=lobe-width ratio, PUI pubescence on the central part of the lamina.

**Table 2** Mean values and repeatability of leaf morphology traits in the mapping pedigree

Trait	Experiment 1		Experiment 2	
	Mean ± SD	R	Mean ± SD	R
LL (mm)	58.5 ± 15.2	0.56	69.8 ± 20.4	0.66
PL (mm)	2.6 ± 0.86	0.3	3.2 ± 1.0	0.74
LW (mm)	17.8 ± 5.19	0.54	20.7 ± 7.23	0.65
SW (mm)	6.4 ± 2.5	0.62	8.4 ± 3.8	0.73
WP (mm)	34.6 ± 11.9	0.49	41.9 ± 14.8	0.65
NL	7.7 ± 1.7	0.58	8.5 ± 1.9	0.75
NV	5.8 ± 2.3	0.49	4.2 ± 1.9	0.84
BS	5.1 ± 1.4	0.53	4.6 ± 1.7	0.68
Puv	1.5 ± 0.74	0.8	1.7 ± 0.93	0.90
PUI	2.8 ± 0.80	0.74	2.2 ± 0.92	0.84
OB (%)	59.6 ± 18.8	0	61.1 ± 28.5	0.44
PR (%)	4.4 ± 1.7	0.38	4.5 ± 1.6	0.63
LDR (%)	62.9 ± 13.0	0.51	58.9 ± 13.1	0.70
PV (%)	76.7 ± 37.5	0.17	52.9 ± 37.2	0.73
LWR (%)	31.0 ± 9.87	0.07	30.0 ± 11.4	0.43

SD=standard deviation, R=repeatability of the clonal mean, LL=lamina length, PL=petiole length, LW=lobe width, SW=sinus width, WP=length of lamina at widest point, NL=number of lobes, NV=number of intercalary veins, BS=basal shape of the lamina, OB=lamina shape, PR=petiole ratio, LDR=lobe-depth ratio, PV=percentage venation, LWR=lobe-width ratio, PUI pubescence on the central part of the lamina, PUV pubescence along the veins.

**Table 3** Matrix of correlation coefficients between leaf morphological traits calculated

	PL	SW	NV	BS	Pul
<i>(a) Natural population<sup>a</sup></i>					
PL		<b>0.58</b>	-0.11	-0.20	<u>-0.21</u>
SW	<b>0.23</b>		-0.05	-0.05	<u>-0.27</u>
NV	-0.01	-0.04		0.09	<u>-0.23</u>
BS	-0.04	0.01	-0.09		-0.001
PUI	-0.05	-0.06	0.07	-0.03	
<i>(b) Two experiments of the mapping pedigree of Q. robur<sup>b</sup></i>					
PL	<b>0.17</b>	<b>0.26</b>	<b>0.18</b>	-0.20	-0.03
SW	<b>0.43</b>	<b>0.24</b>	<u>-0.46</u>	0.06	-0.05
NV	0.13	-0.05	0.09	<b>0.21</b>	0.01
BS	<u>-0.24</u>	0.11	0.13	0.11	-0.01
PUI	-0.13	-0.13	<u>-0.32</u>	0.11	<b>0.20</b>

NL = number of lobes, PL = petiole length, NV = number of intercalary veins, SW = sinus width, PUI = pubescence in the central part of the lamina. Underlined values correspond to correlations that exhibit opposite signs at the inter- and intraspecific level (see text), values in bold are significant at  $P \leq 5\%$ .

<sup>a</sup>The correlations are based on the tree effect (model 2). Above the diagonal, values correspond to *Q. petraea*, Below the diagonal, values correspond to *Quercus robur*.

<sup>b</sup>Correlations are based on the clone effect (model 3). Above the diagonal, genetic correlation for experiment 1. Below the diagonal, genetic correlation for experiment 2. Diagonal, genetic correlation between experiments 1 and 2.

values derived from ANOVA model (3) in the mapping pedigree (Table 3b) were calculated. Size-related leaf characters (LL, SW, LW, WP) were significantly correlated, mostly as a result of the common size effect (data not shown). We focused our attention here only on those traits that exhibited a high between-species variation according to Table 1 (eg, PL, SW, NV, BS, PU). The values of these five characters were all significantly different between *Q. petraea* and *Q. robur*. *Q. petraea* has larger petioles (PL), fewer intercalary veins (NV), less pronounced sinuses (SW), sharper basal leaf lamina (BS), and more pubescence (PU) than *Q. robur* (Table 1). One can therefore consider these five characters to be correlated at the interspecific level.

Correlations among these characters are less pronounced at the intraspecific level. In the natural populations, only five correlations among the 10 pairwise associations were significant in *Q. petraea*, and only one in *Q. robur* (Table 3a). In the mapping pedigree, where the computed correlations represent mostly genetic correlations, five of the 10 correlations were significant in experiment 1 and three in experiment 2 (Table 3b). A more intriguing feature of the significant correlations is that in some cases, the sign of the correlation is different between the inter- and intraspecific levels. Among the 14 significant correlations (Table 3a and b), three changed sign from the inter- to the intraspecific level. This is illustrated by the correlation between PL and NV. The leaves of *Q. robur* have smaller petioles (PL) and more intercalary veins (NV, Table 1). However, within the mapping pedigree in *Q. robur*, clones that had smaller petioles tended to have fewer intercalary veins (Table 3b). Correlations of opposite sign between the inter- and intraspecific levels also occurred in *Q. petraea* (Table 3a).

### Linkage map for QTL detection

The 13 AFLP primer-enzyme combinations provided 201 markers segregating in a 1:1 ratio. Among them, 4% showed a significant deviation ( $P < 1\%$ ) from Mendelian segregation and were discarded. The genotyping of the offspring also included 38 microsatellites and six SCARs. Nine additional RAPD markers were selected from the reference map (Barreneche *et al*, 1998) and were included to fill gaps in some linkage groups. Among the 254 markers obtained, a subset of 128 markers (84 AFLP, 34 microsatellites, one SCAR, nine RAPD), evenly distributed across the genome, were finally selected to construct the QTL map. The two maps shared 19 microsatellites. The female framework map was composed of 75 markers over a total distance of 890 cM, covering 75% of the genome (estimated total length is 1192 cM (Barreneche *et al*, 1998)) with an average spacing between markers of 14 cM. The male framework map comprised 72 markers over a total distance of 929 cM, covering 75% of the genome (estimated total length is 1235 cM) with an average marker spacing of 15.5 cM.

### QTL mapping

No QTL was detected for two traits (LW, OB) among the 15 leaf morphological traits assessed when the probability threshold was set at 5% at the genome level. Genetic variation for LW and OB was low as shown by their repeatability values (Table 2).

For the remaining 13 traits, between one and two significant QTLs were detected, which on average explained from 10.7 to 13.8% of the clonal mean variance (Table 4). Overall, the proportion of phenotypic variance explained by the QTLs detected in this pedigree remains moderate, despite the high repeatability values of the traits, especially in experiment 2. There was no obvious relationship between the level of the repeatability and the number of QTLs, or the proportion of phenotypic variance explained by the QTLs. For example, in the case of pubescence (PU<sub>v</sub> or PUI), which exhibited the highest repeatability (Table 2), two QTLs were detected only in experiment 1, with each QTL explaining between 12.9 and 17.2% of the phenotypic variance (Table 4). For six traits (PL, SW, NV, NV, LDR, PV), QTLs were detected in both experiments. Interestingly, these traits are strongly involved in species differentiation. When considering only the five traits that exhibited the highest species differentiation (eg the same traits presented in Table 3: PL, SW, NV, BS, PU), their QTLs were located on six different linkage groups (LG1, LG3, LG4, LG5, LG11, LG12) when bulking the information over both experiments (Table 4, Figure 1). Some of these QTLs were detected on the same linkage group in both experiments (for PL on LG1, SW on LG 3). Colocalisation of QTLs of at least two different traits involved in species differentiation can be seen on LG1 (PL, PU) and LG3 (NV, SW). A clustering of QTLs controlling raw or derived traits involved in species differentiation can clearly be seen on LG3 (PV, NV, LDR, SW).

When the Type I error rate for the QTL detection was relaxed from 5% at the genome level to 5% at the chromosome level, then the former clusters of QTLs involved in species differentiation were reinforced by the

**Table 4** QTL detection summary for the leaf morphological traits

Traits	LG	N	Lod	$\alpha_{chr}$	P1	P2	CI	D	P
<i>Experiment 1</i>									
PL	1f	124	3.9	0.004	55.4	46.7	20.2	0.32	13.6
PR	10f	134	3.2	0.005	63.1	60.9	18.5	0.48	9.7
LWR	10f	131	2.8	0.005	68.9	65.5	19	2.26	9.9
SW	3f	126	3.7	0.002	22.3	22.1	6.9	1.00	15.5
SW	3m	137	3.6	0.003	19.3	17.2	9.2	0.81	10.9
LDR	3f	126	3.7	0.002	20.5	18.2	16.3	-4.99	14.5
LDR	3m	137	3.0	0.003	19.1	18.5	7.9	-3.84	9.9
PUI	12f	110	4.8	0.003	14.9	17.6	11.9	0.37	17.2
PUI	1m	108	3.0	0.003	29.3	30.8	12.9	-0.33	12.9
NL	2f	89	3.9	0.005	67	50.7	39.7	0.94	22.9
NV	3f	126	4.7	0.002	20.2	15.8	15.7	-0.91	16.4
PV	3f	126	5.7	0.002	20.4	18.3	12.8	-12.19	21.7
Mean values									13.8
<i>Experiment 2</i>									
LL	11f	171	3.2	0.004	2.7	7.3	27.8	6.64	8.8
LL	6m	184	2.5	0.003	26	25.3	10.3	-5.70	6
PL	1f	156	4.2	0.004	39	44.2	17.1	0.41	11.2
PL	11f	171	3.4	0.004	17.7	19.4	23.3	0.36	7.9
PR	1f	165	3.6	0.004	9	12.2	25.5	0.66	11.8
SW	3f	162	3.1	0.002	17.9	18.3	5.4	1.51	8.4
SW	3m	160	2.5	0.003	43.2	27.6	33.1	1.61	9.7
LDR	3f	162	7.1	0.002	17.9	17.7	3.0	-6.14	19.1
LDR	3m	160	4.5	0.003	41.1	34.2	21	-6.41	8.2
BS	4m	131	2.6	0.004	86.3	74.4	33.7	-0.54	8.4
WP	11f	171	3.0	0.004	5.2	9.6	30.4	4.69	8.4
NL	8m	166	3.1	0.005	11.6	16	34.1	-0.71	8.4
NV	5f	168	7.4	0.005	31.4	32.2	14.1	1.00	17.6
PV	5f	168	6.2	0.005	31.4	30.7	6.1	15.53	15.7
Mean values									10.7

LG = linkage group (m: male map; f: female map), N = number of individuals genotyped for the flanking markers, Lod = highest LOD in the interval,  $\alpha_{chr}$  = theoretical P-value at the chromosome level corresponding to the P-value of 5% at the genome level, P1 = position of the highest LOD score from the composite Interval mapping, P2 = position of the mean value for maximum LOD score after bootstrap analysis (1000 bootstrap samples), CI = QTL confidence interval (95%) based on the 1000 bootstrap samples, D = size of effect, P = percentage of the clonal mean variance explained by the QTL.

addition of suggestive QTLs for other informative morphological traits (Figure 2):

- on LG1F (c. 30 cM) : PUI1, PL1, PL2, BS2, and PUI1 on LG1M;
- on LG3F (c. 20 cM): NV1, NV2, PL2, SW1, SW2, and BS2, SW1, SW2 on LG3M;
- on LG4M (c.10 cM): PL1 and PUv2;
- on LG6M (c. 40 cM): PUI2, SW1, and PUI1 on LG6F;
- on LG9F (c. 10 cM): PUv1, PUv2, SW1, and BS2 on LG9M;
- on LG12F (c. 30 cM): PUI1 and NV2.

Two additional ones were also identified (data not shown):

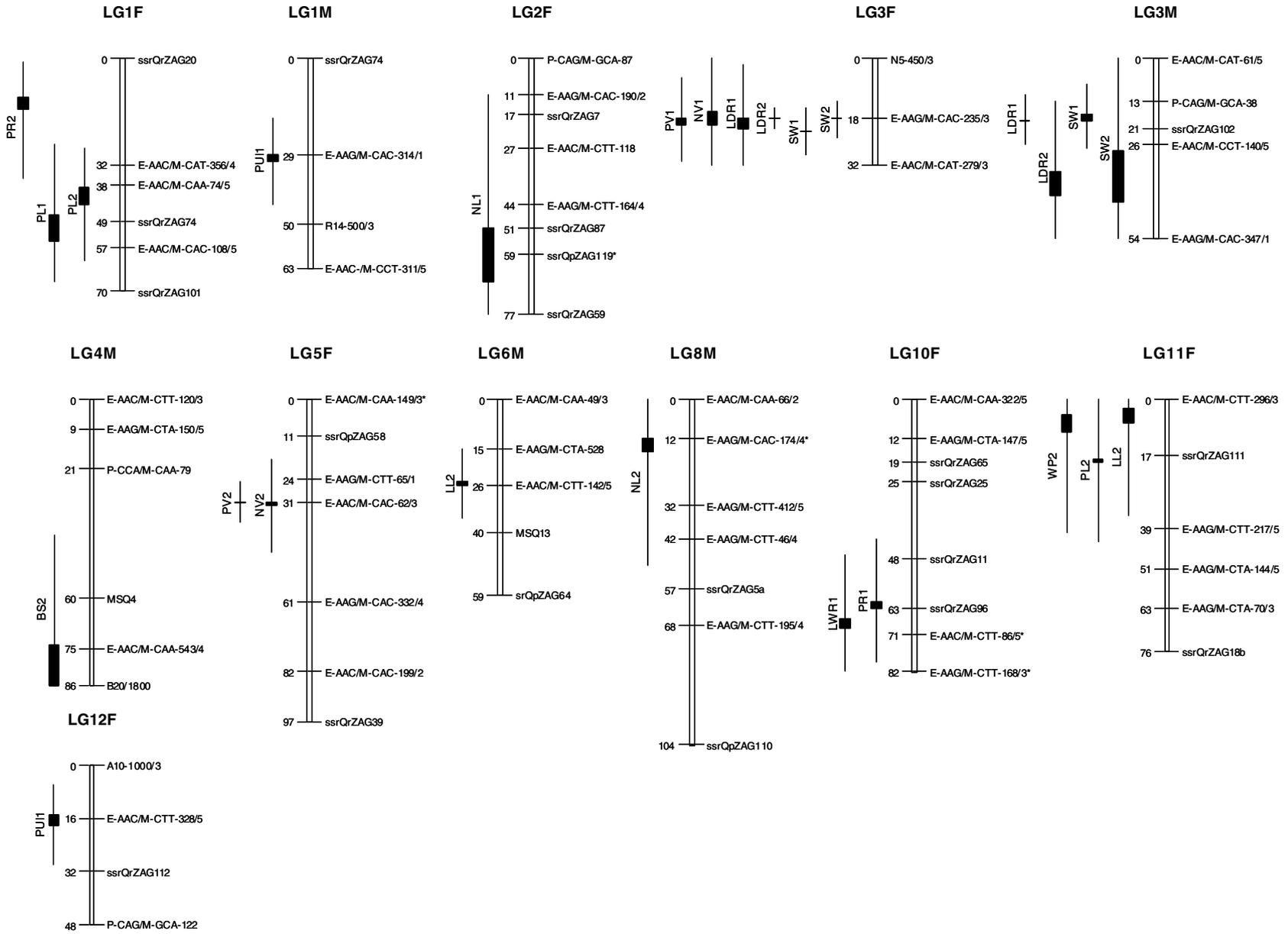
- on LG5F (c. 40 cM): NV2 and SW1 on LG5M;
- on LG7F (c. 30 cM): PUv1, PUv2, and PUI2 on LG7M.

## Discussion

QTL mapping as a method for detecting genomic regions involved in oak species differentiation

As leaf morphology traits are of low economic importance, they have seldom been studied for estimating genetic variances or QTL detection. Most of the 15 traits

assessed exhibited high repeatability values. Leaf pubescence was the trait with highest repeatability, and in general there was a trend towards larger repeatabilities for those traits that show higher species differentiation. QTLs were detected for 13 traits among the 15 studied. The QTLs explained on average 11.1–13.8% of the clonal mean variance. Assuming dominance is zero, these values can be translated in the proportion of additive variance explained by the QTLs, varying between 9 and 66% in experiment 1, and 5 and 14% in experiment 2. These values can be compared to other reported studies on leaf morphological traits providing estimates of the proportion of the additive variance explained by the QTLs: from 2 to 37% in *Brassica oleracea* (Lan and Paterson, 2001) and from 5 to 52% in *Gossypium hirsutum* and *G. barbadense* (Jiang *et al*, 2000). The major objective of this research was to map genomic regions involved in species differentiation, by locating QTLs involved in phenotypic differences between *Q. petraea* and *Q. robur*. We chose leaf morphology traits as they have been explored in many studies for species identification. Indeed, we reanalysed the variation of 15 of these traits and showed that five of them exhibit important variation among the two species. The QTL strategy should not, however, be considered as an exhaustive way of identifying informative regions. First



of all, our mapping pedigree was an intraspecific full-sib family, which may have reduced the chances of detecting informative QTLs. Some of these loci may be fixed within a species for different alleles and exhibit complete interspecific differentiation. Hence, they would not segregate in a full-sib intraspecific family. Ideally, an interspecific mapping pedigree (either F2 or backcross) would be necessary to detect these QTLs, for example, *Drosophila* (Zeng *et al*, 2000) and *Mimulus* (Lin and Ritland, 1997). However, due to experimental constraints related to the length of generations, F2 or backcrosses are not available in oaks. Second, some of the loci may be missed by chance just because they are not polymorphic between the two parents of the mapping pedigree. Finally, if QTLs controlling morphological traits are numerous and if each one contributes only a small part of the phenotypic variation of the trait, then it can also be missed because the family size of our mapping pedigree was too low (the so-called Baevis effect; Beavis, 1995), despite the vegetative propagation used to increase the power for QTL (Bradshaw and Foster, 1992). Neither of these hypotheses can be completely excluded. However, the existing data of genetic diversity within the complex of the two species (Kremer and Petit, 1993; Mariette *et al*, 2002) suggest that they are highly polymorphic, and that fixation within species has never been found for nuclear markers that were polymorphic within the complex. Even when the genomes of the two species were screened for numerous markers (RAPDs, Bodénès *et al*, 1996 or AFLPs, Mariette *et al*, 2002), polymorphism was always shared among the two species. Hence, we expected that polymorphism for QTLs involved in species differentiation is likely to be shared as well. High levels of polymorphism in oaks increase the chances of detecting QTLs in a single full-sib family, but additional experiments in other pedigrees need to be implemented to complete the detection. Finally, larger family sizes would be needed to increase statistical power of QTL detection of morphological traits, as our results indicated that most QTLs contributed individually only a minor part of the phenotypic variation of the trait.

**Distribution of genomic regions differentiating oak species**  
When the analysis was restricted to the five morphological characters involved in species differentiation, we found that their QTLs were distributed on six linkage groups when the Type I error rate was set at 5% for the whole genome, and on nine linkage groups when it was set at 5% for the chromosome level. Recalling that our method provides an incomplete number of informative QTL, we may conclude that species differentiation occurs at multiple sites within the genome and is not confined to a few spots with limited recombination. Our results in oaks contribute to the heterogeneity of results in the same area that have recently been reviewed by Orr

(2001). In a synthesis of QTLs detections oriented towards species differentiation, Orr reported that the number of informative QTLs varied between one and 20. Examples of the heterogeneity of results are given in *Drosophilae* (*Drosophila simulans* and *D. mauritiana*; Zeng *et al*, 2000) or *Mimulus* (Lin and Ritland, 1997). In both cases, depending on the traits measured, there was a large variation in the number of QTLs. Part of the inconsistency could be explained by experimental constraints, as QTL detection is highly dependent on sample sizes in mapping pedigrees (Wilcox *et al*, 1997), as is illustrated in this study by the differences observed between the two experiments. Given that sample sizes of mapping pedigrees in the few hundred usually tend to inflate the contribution of the detected QTLs, we may conclude that a larger number of informative genomic regions for species differentiation exists within the genome. In addition, our results, based on an underestimate of these regions, clearly indicated that species differentiation is spread throughout the genome. However, QTLs involved in different discriminant morphological characters tend to be distributed in clusters. No conclusion can be drawn as to whether these clusters correspond to a single locus with pleiotropic effects or to linked loci contributing to different morphological traits. The occurrence of the clusters is also a direct expression of the correlations among traits, suggesting that they may contribute to variation of the morphological trait due to linkage disequilibrium. A further question is whether interactions among these clusters (epistatic effects) or interlocus disequilibria may also contribute to the overall differentiation among the two species.

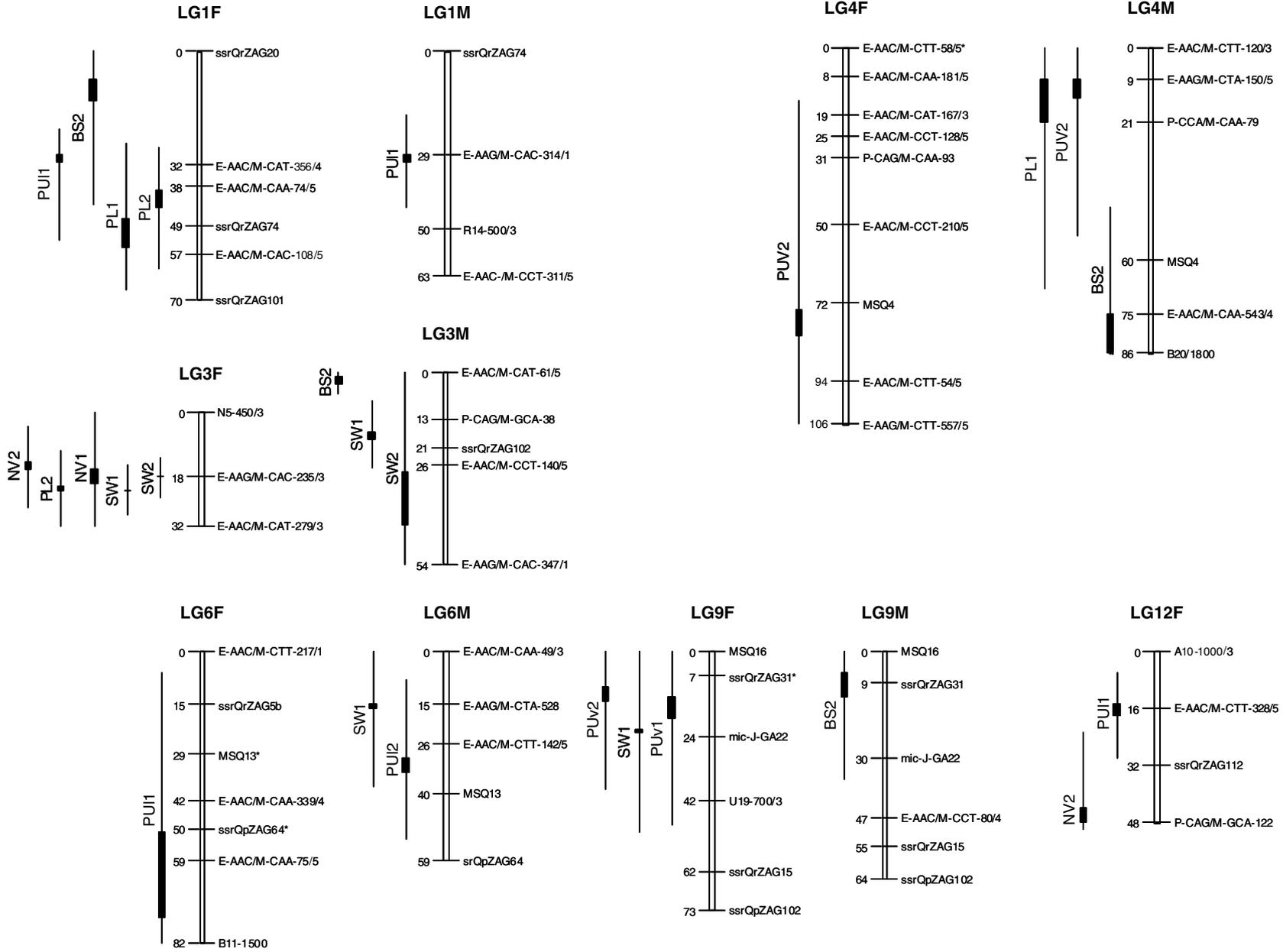
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**Figure 1** Distribution of QTLs for leaf morphology traits with a threshold fixed to 5% at the genome level: lamina length (LL), number of lobes (NL), petiole length (PL), number of intercalary veins (NV), sinus width (SW), petiole ratio (PR), lobe-depth ratio (LDR), percentage venation (PV), length of lamina at widest point (WP), pubescence in the central part of the lamina (PUI), lobe-width ratio (LWR). Index 1 corresponds to QTLs detected in experiment 1, index 2 corresponds to QTLs detected in experiment 2. Each QTL is delineated by the position of the highest LOD score and the bootstrap mean value of the highest LOD score. Confidence intervals of their position based on 1000 bootstrap samples are indicated as lines.



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**Figure 2** Distribution of QTLs for the most discriminant leaf morphology traits ( $P < 5\%$  at the chromosome level): petiole length (PL), number of intercalary veins (NV), sinus width (SW), pubescence in the central part of the lamina (PU1), pubescence along the vein (PUv), basal shape lamina (BS). Index 1 corresponds to QTLs detected in experiment 1, index 2 corresponds to QTLs detected in experiment 2. Each QTL is delineated by the position of the highest LOD score and the bootstrap mean value of the highest LOD score. Confidence intervals of their position based on 1000 bootstrap samples are indicated as lines.

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