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SHORT COMMUNICATION

Genetic mapping of EST-derived simple sequence repeats (EST-SSRs) to identify QTL for leaf morphological characters in a *Quercus robur* full-sib family

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Abstract The availability of genomic resources such as expressed sequence tag-derived simple sequence repeat (EST-SSR) markers in adaptive genes with high transferability across related species allows the construction of genetic maps and the comparison of genome structure and quantitative trait loci (QTL) positions. In the present study, genetic linkage maps were constructed for both parents of a *Quercus robur* × *Q. robur* ssp. *slavonica* full-sib pedigree. A total of 182 markers (61 AFLPs, 23 nuclear SSRs, 98 EST-SSRs) and 172 markers (49 AFLPs, 21 nSSRs, 101 EST-SSRs, 1 isozyme) were mapped on the female and male linkage maps, respectively. The total map length and average marker spacing were 1,038 and 5.7 cM for the female map and 998.5 and 5.8 cM for the male map. A total of 68 nuclear SSRs and EST-SSRs segregating in both parents allowed to define homologous linkage groups (LG) between both parental maps. QTL for leaf morphological traits were mapped on all 12 LG at a chromosome-wide level and on 6 LG at a genome-wide level. The phenotypic effects explained by each single QTL ranged from 4.0 % for leaf area to 15.8 % for the number of intercalary veins. QTL clusters for leaf characters that discriminate between *Q. robur* and *Quercus petraea* were mapped reproducibly on three LG, and some putative candidate genes among potentially many others were identified on LG3 and LG5. Genetic linkage maps based on EST-SSRs can be valuable tools for the identification of genes involved in adaptive trait variation and for comparative mapping.

Keywords Oaks · Linkage maps · Adaptive traits · Comparative mapping

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Introduction

Genetic linkage maps are essential tools to localize chromosomal regions, quantitative trait loci (QTL), that are associated with quantitative trait variation (e.g., reviewed in Bergelson and Roux 2010; Neale and Kremer 2011). Simple sequence repeat (SSR) markers are the markers of choice for comparative mapping since they show multiple alleles and can thus be mapped in different pedigrees (Varshney et al. 2005; Bodénès et al. 2012). The abundance of SSR markers in expressed genes (EST-SSRs) with generally high transferability and polymorphism across species within genera (Ellis and Burke 2007) allows comparative mapping in phylogenetically related species to reveal structural rearrangements of the genome (e.g., Burke et al. 2004) and to identify the genetic architecture of adaptive traits in different genetic backgrounds (e.g., Chagne et al. 2003; Casasoli et al. 2006; Kremer et al. 2007).

Quercus species are economically and ecologically important keystone taxa with more than 400 species in the Northern hemisphere and have been a focus of genetic, evolutionary, and phylogeographic studies over the last decades (Aldrich and Cavender-Bares 2011; Kremer et al. 2007). With the availability of large amounts of EST sequences (Ueno et al. 2010) especially in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl., more than 570 EST-SSR markers were developed for genetic mapping purposes (Durand et al. 2010; Bodénès et al. 2012). These EST-SSRs showed high transferability and polymorphism across oak species (Bodénès et al. 2012; Sullivan et al. 2013) and even across genera within the Fagaceae, making them especially useful for comparative mapping (Bodénès et al. 2012). Thus, comparisons of earlier *Q. robur* and *Castanea sativa* genetic linkage maps using orthologous nuclear SSRs (nSSRs) and EST-derived markers showed a strong correspondence in the map location of QTL for vegetative bud burst (Casasoli et al. 2006). The availability of EST-SSR-based linkage maps will allow aligning linkage maps with anchor markers that segregate in multiple pedigrees as shown in oaks by Bodénès et al. (2012) in order to study the architecture of adaptive traits in different genetic backgrounds. The localization of EST-SSRs and SNP markers on high density genetic linkage maps will facilitate the identification of functional and positional candidate genes underlying major QTL for validation in natural populations using association mapping approaches.

In oaks, QTL for characters differentiating between the co-occuring interfertile species *Q. robur* and *Q. petraea* were characterized in intraspecific *Q. robur* pedigrees (Kremer et al. 2007) since appropriate interspecific families were not available. Likewise, QTL for leaf morphological characters that differentiate between species were mapped based on *Q. robur* mapping pedigrees suggesting within-species variation for species discriminating characters (Saintagne et al. 2004; Gailing 2008). Co-localization of QTL on LG3 and LG5 was detected for species discriminating characters such as number of intercalary veins (NV) and percentage venation (PV) (Gailing 2008; Saintagne et al. 2004). The availability of annotated EST-SSR markers located in expressed genes (Durand et al. 2010; Bodénès et al. 2012) will now allow for the identification of functional candidate genes underlying these QTL. A subset of the mapping pedigree used in the present study and the male EST-SSR map (112 markers) was included in comparative mapping analyses in *Q. robur* and *Q. petraea* and in the construction of a consensus genetic map (Bodénès et al. 2012). Here, we present male and female EST-SSR/nSSR/AFLP genetic linkage maps based on about twice the number of segregating progeny and markers (286 markers) and QTL mapping of leaf morphological characters.

The aims of the present study were threefold: (1) establish genetic linkage maps from a *Q. robur*-intraspecific cross using AFLP, nuclear SSR, and EST-SSR markers; (2) map QTL for species discriminating leaf morphological traits in multiple years and compare with QTL that have already been identified in the same pedigree on an earlier AFLP/nSSR linkage map under greenhouse conditions (Gailing 2008); and (3) compare and identify underlying putative candidate genes for major QTL.

Material and methods

Mapping population

The mapping pedigree (HSL03) consisted of 192 full-sibs derived from an intraspecific cross of *Q. robur* (EF03) × *Q. robur* subsp. *slavonica* (SL03) (Gailing 2008). Pronounced differences in leaf morphological characters between crossing parents were observed with the male parent (*Q. robur* subsp. *slavonica*) showing larger leaves and less intercalary veins (Gailing 2008). Seedlings were grown in a greenhouse at the Institute of Forest Genetics and Forest Tree Breeding at Göttingen University from 2004 to 2006 and transferred to a fenced field site adjacent to the Institute in spring 2007. Seedlings were planted at a distance of 1.5 m from each other.

Morphological measurements

Two or three largest and fully expanded leaves were collected from the first flush of the year in the upper part of the main shoot in the middle of May from 2005 to 2008. Leaf morphological characters were measured and mean values were calculated as described in Kremer et al. (2002) and Gailing (2008) (Supplementary Table 1). Additionally, leaf area (LA) and leaf perimeter (LP) were determined on digital images of scanned leaves with the program WINFOLIA (Regnet Instruments Inc., Quebec, Canada). A leaf shape index (LS) was calculated as LA/LP. The number of epidermis cells (EP) was counted from imprints taken from the lower leaf surface in the intercostal zone between two third-order veins of a central lobe (see Gailing et al. 2008).

Marker analysis

Out of 22 AFLP enzyme primer combinations tested, a total of eight *Eco*RI/*Mse*I and seven *Pst*I/*Mse*I combinations were used for map construction (Gailing 2008; Gailing et al. 2008). Additionally, 46 nuclear fluorescent-labeled microsatellites developed for *Q. robur* (Kampfer et al. 1998), *Q. petraea* (Steinkellner et al. 1997), and *Q.*

macrocarpa (Dow and Ashley 1996) and the isozyme maker isocitrate dehydrogenase (1.1.1.42, IDH-B) were tested for polymorphisms in the mapping parents. Finally, a total of 332 EST-SSRs earlier described in Durand et al. (2010) were tested in the mapping parents and polymorphic markers were used to genotype the progeny. For a more cost-effective primer screening, a M13 tail (5′-TGT AAA ACG ACG GCC AGT-3′) was added to the 5′-end of the forward primer (Schuelke 2000) in order to amplify the fragments using a complementary adapter with a 6-FAM dye at its 5′-end. PCR reactions were carried out as described in Durand et al. (2010). Fragments were separated on an ABI 3100 capillary sequencer and data were analyzed with Genescan 3.7 and Genotyper 3.7 (Applied Biosystems). Functional annotation of EST-SSRs was performed by applying a homology search of reassembled ESTs (Durand et al. 2010; Bodénès et al. 2012) against the non-redundant (nr) NCBI database using the BLASTx algorithm (Altschul et al. 1997) in the Blast2GO software (Conesa et al. 2005).

Map construction

The genetic linkage maps were constructed based on segregation data obtained with 192 offspring using the two-way pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994). To compare marker order and QTL positions, polymorphic markers segregating approximately in the 1:1 or 1:1:1:1 ratios were used to calculate separate male and female linkage maps, including markers with segregation distortion. Maps were calculated with JoinMap 4 (Van Ooijen 2006) using regression mapping with the suggested default settings of a maximum recombination frequency of 0.40 and a minimum LOD score of 1.0. Haldane's mapping function was applied to convert recombination frequencies into centiMorgan (cM). Male and female linkage groups were aligned using heterozygous markers in both parents. Segregation distortion was assessed for individual markers using chi-square tests, and distorted markers ($p<0.1$) were plotted on male and female linkage maps using JoinMap.

QTL detection

QTL detection was performed in MapQTL 5 (Van Ooijen 2004). Interval mapping was applied to calculate the likelihood of a QTL at any position of the map (Lander and Botstein 1989) using 1 cM interval size and to estimate the phenotypic variance explained (PVE) by each QTL. In order to increase the power and precision of QTL detection and to distinguish between one or more QTL per linkage group, the MQM mapping method using automatic cofactor selection was used to identify cofactors as background control (Jansen and Stam 1994). Permutation tests with 10,000 iterations were applied to calculate chromosome and genome-wide significance thresholds of the LOD score at $p<0.05$.

Results

Leaf morphological characters

Considerable variation was found for leaf morphological characters within and among years (Supplementary Table 2). Reproducibility among years as measured by Pearson's correlation coefficient was generally low indicating a strong ontogenic and/or environmental effect on leaf development. Significant correlations ($p<0.05$) between years were especially found for NV and PV in four out of six comparisons ($r=0.157$ to 0.248), and for LWR in five out of six comparisons ($r=0.173$ to 0.380).

Construction of linkage maps

The coverage of the new maps has increased considerably as compared to an earlier AFLP/nSSR linkage map that contained 74 markers on the female map and 69 markers on the male map (Gailing 2008). A total of 182 markers (61 AFLPs, 23 nSSRs, and 98 EST-SSRs) segregated in the testcross configuration in the female parent and were assigned to the 12 linkage groups (i.e., LG1f to LG12f). The total length of the female map was 1,038 cM with an average marker spacing of 5.7 cM. The total number of markers mapped on the male map was 172 (49 AFLPs, 21 nSSRs, 101 EST-SSRs, 1 isozyme IDH) resulting in a map length of 998.5 cM and an average marker spacing of 5.8 cM. The number of markers per linkage groups ranged from 10 (LG12f) to 25 (LG2f) on the female map and from 7 (LG7m) to 24 (LG2m) on the male map. Number of markers, the length of linkage groups, and the average marker spacing were significantly correlated between male and female map with the lowest correlation found for the length of the linkage groups ($r=0.80$, $p=0.0009$; $r=0.57$, $p=0.03$; $r=0.80$, $p=0.0009$). A total of 68 shared nSSR and EST-SSR markers were used to align male and female maps. The marker order was the same on both maps for most chromosomal regions, and inverted marker orders were found in dense marker regions especially on LG2 (Supplementary Fig. 1). The number of markers per linkage groups and the length of linkage groups were significantly correlated ($r=0.49$, $p=0.007$).

Segregation distortion was found for single markers and groups of markers (22.7 % of all markers) on most linkage groups with the exception of LG8f and LG9. Chromosomal regions showing segregation distortion were only partly overlapping between male (28 distorted markers at $p<0.1$) and female (38 distorted markers at $p<0.1$) linkage groups

(Supplementary Figs. 1 and 2). Only EST-SSR marker POR025 (unknown protein) showed segregation distortion ($p<0.05$) on LG7f and LG7m. Especially on LG7f and LG11f, larger regions showed significant segregation distortion.

QTL mapping

For all characters, a total of 104 QTL and 21 QTL were significant at the chromosome (Supplementary Table 3) and genome levels (Table 1), respectively. The phenotypic effect explained by individual QTL ranged from 4.0 % for LA2 to 15.8 % for NV2. Chromosome-wide QTL and genome-wide QTL were detected on 12 and 7 linkage groups, respectively. QTL detected in more than 1 year in clusters on either male or female linkage groups were mapped for LWR on LG2 and LG5m, for LP and SW on LG3f, for NV on LG5f, for SW and LDR on LG6, and for OB on LG9 (Supplementary Fig. 2). Major QTL clusters that showed significant marker phenotype associations with several leaf morphological traits across more than 2 years were found on LG1f, LG2, LG3f, LG5, and LG6m. QTL clusters including species discriminating traits PV, NV, LDR, SW, and NL were found on LG3f, LG5f, and LG6m.

Putative candidate genes for leaf size and shape underlying QTL regions were identified on LG3f (PIE242, pectate lyase-like gene), LG3m (GOT021, putative histidine kinase 4-like), and LG5f (PIE246, farnesylpyrophosphate synthase; POR030, alpha n-terminal protein methyltransferase 1-like) (Supplementary Table 4). PIE242 (pectate lyase 5, putative) and PORO30 (alpha n-terminal protein methyltransferase 1-like) showed significant but minor segregation distortion in the male and female parent, respectively.

Discussion

EST-SSR markers for the construction of genetic linkage maps

About the same percentage of the tested EST-SSR markers were polymorphic and in testcross configuration in the male (30.4 %) and in the female parent (29.5 %). Each of the linkage groups contained at least two markers that segregated in both parents with an average of 5.5 markers per linkage group allowing the alignment of both maps and the comparison of QTL positions on male and female maps. The relatively large number of EST-SSRs (98 on the female

Table 1 Genome level quantitative trait loci (QTL) for leaf morphological characters

	Linkage group	LOD score	Allelic effect	LDD max (cM)	Range (cM)	PVE (%)	GIC
EP3	5f	2.80	19.7	57.1	44.7 to 59.1	8.5	0.954
LA1	5f	2.77	8.4	56.1	48.7 to 57.1	9.1	0.924
LA2	6m	3.06	9.7	57.0	51.4 to 59.0	7.6	0.790
LDR2	3m	2.99	6.9	75.6	70.6 to 80.6	7.4	0.747
LP2	5f	3.52	64	30.7	27.6 to 39.7	8.7	0.919
LS1	5f	3.00	1.14	56.1	48.7 to 59.1	9.8	0.924
LS2	5m	3.29	0.9	97.7	94.7 to 101.0	8.2	0.959
LW4	5m	4.22	3.8	62.7	57.6 to 68.7	12.2	0.923
LW4	1f	3.17	3.0	30.9	28.7 to 32.2	9.3	0.839
LWR2	1f	3.13	1.7	69.8	64.5 to 71.6	7.7	0.927
LWR2	2m	2.65	1.6	48.1	42.0 to 52.0	7.7	0.971
LWR3	2m	3.59	2.9	36.7	32.7 to 42.7	10.9	0.929
LWR4	2f	3.38	2.6	37.7	34.7 to 40.0	9.9	0.858
NL1	6m	3.98	2.4	31.2	22.0 to 36.2	12.1	0.754
NV2	5f	6.70	1.8	30.6	24.6 to 43.6	15.8	0.919
OB3	9m	3.14	5.0	68.4	65.0 to 68.4	10.4	0.703
PV2	5f	4.05	10.4	30.6	24.6 to 38.6	9.8	0.919
PV2	8f	3.96	11.2	27.1	15.6 to 37.1	9.6	0.766
SW2	6m	4.94	0.37	59.6	57.0 to 63.3	11.9	0.750
WP2	5f	3.65	1.03	30.7	26.6 to 37.6	9.0	0.919
WP2	6m	3.60	1.13	57.0	50.4 to 59.0	8.8	0.747

LDD: position (centiMorgan) of the maximum LOD score, allelic effect: absolute difference between phenotypic means of QTL alleles, range: range of LOD scores above the 5 % chromosome-wide threshold using 10,000 permutations, PVE: phenotypic variance explained by QTL, GIC: genomic information content ranging from 0 (no marker information) to 1 (maximum marker information)

map and 101 on the male map) has also made it possible to integrate these maps with EST-SSR maps derived from other full-sib families (Bodénès et al. 2012).

Markers showing segregation distortion at the 0.1 and 0.001 levels, respectively, were found on 11 and 7 out of the 12 linkage groups. A large number of markers with segregation distortion were found on the male LG2 (5 out of 24 markers at $p<0.1$) and LG10 (6 out of 16 markers at $p<0.1$), while a clustering of distorted markers was observed on the female map for LG7 (7 out of 12 markers at $p<0.05$) and LG11 (9 out of 13 markers at $p<0.05$). Moderate segregation distortion for candidate gene markers co-localizing with leaf QTL, PIE242 (pectate lyase 5, putative), PORO030 (alpha n-terminal protein methyltransferase 1-like) on LG5f, and FIR027 (u-box domain-containing protein 4-like) on LG6m, might be due to selection acting on genes located in these chromosomal segments (Yin et al. 2004).

QTL analysis

QTL for leaf morphological characters that were mapped in the same mapping pedigree on earlier AFLP/nSSR linkage maps under greenhouse conditions (Gailing 2008) were confirmed in the present study based on higher density linkage maps and data obtained in two more years under field conditions. The association of chromosome segments with a larger number of traits across several years as observed on LG2m, LG3f, LG5, and LG6m indicates either a major QTL with pleiotropic effects on correlated traits or closely linked QTL. While QTL for individual traits were observed in at least 1 year on each linkage group, marker–phenotype associations for several traits and across years were only detected on the four linkage groups listed above, therefore presenting interesting targets for map-based cloning. QTL for leaf morphological traits that showed a high differentiation between the closely related species *Q. robur* and *Q. petraea* were detected in more than 1 year on LG3f (SW), LG5f (NV), and LG6 (LDR, SW). The presence of QTL clusters in intraspecific crosses for traits showing high interspecific differentiation indicates considerable genetic variation for species discriminating leaf characters within a single species. Accordingly, QTL for the same and additional species discriminating leaf morphological characters were mapped on 8 out of 12 linkage groups in another intraspecific *Q. robur* cross (Saintagne et al. 2004). The large number of QTL that segregate in intraspecific crosses of *Q. robur* and across-year variability in QTL expression suggests that high leaf morphological variation within *Q. robur* is due to genetic variation for leaf morphological characters and to plastic trait expression in different environments.

For leaf morphological characters that were analyzed in both *Q. robur* pedigrees (Saintagne et al. 2004 and present study), QTL clusters for species discriminating traits PV, NV, LDR, and SW were mapped on LG3 and LG5. QTL on LG3 explained a maximum of 21.47 % for PV and of 19.1 % for LDR of the clonal mean variance (Saintagne et al. 2004). In the present study, a QTL for LDR2 with genome-wide significance explaining 7.4 % of the total phenotypic variance (PVE) was found on LG3m, and a QTL cluster associated with NV, SW, and other leaf size characteristics was mapped on another part of LG3f. Annotation of the ESTs containing the QTL-linked EST-SSR marker PIE242 on LG3f identified a pectate lyase as putative functional and positional candidate gene (e-value$<e^{-50}$). Interestingly, mutations in the pectate lyase-like gene *PMR6* in *Arabidopsis* resulted in altered leaf morphology and decreased leaf size that was explained by a decrease in cell expansion consistent with the putative role of pectins in cell wall loosening (Vogel et al. 2002). Also GOT021 (putative Histidine kinase 4-like) could be associated with leaf shape character LDR on LG3m. Triple mutants of *Arabidopsis* Histidine kinase genes resulted in altered leaf shape and size and vasculature (Nishimura et al. 2004). In addition, GOT021 was identified as outlier locus in four neighboring *Quercus ellipsoidalis* and *Q. rubra* populations showing largely reduced genetic variation in populations of *Q. ellipsoidalis* (Sullivan et al. 2013; Lind and Gailing 2013) with morphological (e.g., deeply dissected leaves) and physiological adaptations (high turgor maintenance capacity) to drought (Abrams 1990). On LG5, major QTL for PV and NV were mapped in one out of two experiments of vegetatively replicated full-sibs explaining 17.6 % of the clonal mean variance for NV and 15.7 % for PV in the other intraspecific *Q. robur* pedigree (Saintagne et al. 2004). While QTL for PV and NV were mapped on the same linkage group in the present study on LG5f (PVE=15.8 % for NV, PVE=9.8 % for PV), their position relative to the anchor marker ssrQpZAG58 differed. The major leaf QTL cluster on LG5f co-located with PIE246 (farnesyl pyrophosphate synthase) and PORO30 (alpha n-terminal protein methyltransferase 1-like). Overexpression of farnesyl pyrophosphate synthase genes affected leaf size and shape in *Artemisia annua* (Banyai et al. 2010). While a small number of putative candidate genes among potentially many others were found to underlie QTL for leaf morphology, the resolution of QTL mapping is yet too low to narrow down the chromosomal region to a small number of potential genes (Neale and Kremer 2011). For example, in poplar, an average QTL interval of 31 cM contained approximately 426 genes, nearly half of them with unknown function (Novaes et al. 2009). The availability of a genome sequence in *Q. robur* and *Q. petraea* in the near future and the use of larger full-sib families for QTL mapping will allow for the identification of all candidate genes that underlie these QTL (Neale and Kremer 2011).

While there is evidence that a few QTL on homologous linkage groups are involved in the control of leaf

morphological traits in different genetic backgrounds, a generally low correspondence of QTL positions among years and, taking earlier QTL studies into account, in different genetic backgrounds was confirmed in the present study (see also Gailing 2008; Saintagne et al. 2004). Different numbers and positions of QTL can be explained by the relatively small sample size of the mapping pedigree (192 full-sibs) that limits the detection of minor QTL and leads to an overestimation of the phenotypic effect of significant QTL (Beavis 1998; Tanksley 1993). Also the detection of QTL is limited by the number of genes underlying leaf morphological variation that segregate in the mapping pedigree (Neale and Kremer 2011). Thus, the number of QTL for interspecific differences could have been underestimated in intraspecific crosses as used in the present and earlier studies (Saintagne et al. 2004; Gailing 2008), since some species-specific loci might be fixed or nearly fixed in one species and segregation of species-specific alleles could only be observed in F_2 backcrosses of a F_1 hybrid plant between *Q. robur* and *Q. petraea*.

Conclusions

QTL clusters with effects on several leaf morphological characters and across more than 1 year were identified on linkage groups 2, 3, 5, and 6. While candidate genes with a putative role in leaf development were identified in chromosomal segments on LG3 and LG5, due to limited resolution of QTL mapping and without the availability of a reference genome, the number of underlying candidate genes cannot be determined. The observation of genetic variation for species discriminative leaf morphological characters within *Q. robur*-intraspecific crosses across years suggests that there is considerable within-species genetic variation for leaf morphological characters that are used to differentiate between the closely related species *Q. robur* and *Q. petraea*.

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Data Archiving Statement Data used in this manuscript have been made publicly available through the Quercus Portal (https://w3.pierroton.inra.fr/QuercusPortal/index.php) and will be submitted to the TreeGenes Database.

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