High-quality SNPs from genic regions highlight introgression patterns among European white oaks (*Quercus petraea* and *Q. robur*).

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Keywords: SNPs, functional candidate genes, *Quercus robur*, *Q. petraea*, Sanger amplicon resequencing, introgression, species differentiation

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Running title: High-quality SNPs for *Quercus* species

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Abstract

In the post-genomics era, non-model species like most *Fagaceae* still lack operational diversity resources for population genomics studies. S~~anger s~~equence data~~s~~ were produced from over 800 gene fragments covering ~530 kb across the genic partition of European oaks, in a discovery panel~~range wide sampling~~ of 25 individuals from western and central Europe (11 *Quercus petraea*, 13 *Q. robur*, one *Q. ilex* as an outgroup). Regions targeted represented broad functional categories potentially involved in species ecological preferences, and a random set of genes. Using a high-quality dedicated pipeline, we provide a detailed characterization of these genic regions, which included over 14500 polymorphisms, with ~12500 SNPs -218 being triallelic-, over 1500 insertion-deletions, and ~200 novel di- and tri-nucleotide SSR loci. This catalog also provides various summary statistics within and among species, gene ontology information, and standard formats to assist loci choice for genotyping projects. The distribution of nucleotide diversity ($\theta\pi$) and differentiation (F_{ST}) across genic regions are also described for the first time in those species, with a ~~(~~ mean $\theta\pi$ close to ~0.0049 in *Q. petraea* and to ~0.0045 in *Q. robur* across random regions, and a mean F_{ST} ~0.13 across SNPs. The magnitude of diversity across genes is~~)~~ within the range estimated for long-term perennial outcrossers, and can be considered relatively high in the plant kingdom, with an estimate across the genome of 41 to 51 million SNPs expected in both species. Individuals with typical species morphology were more easily assigned to their corresponding genetic cluster for *Q. robur* than for *Q. petraea*, revealing higher or more recent introgression in *Q. petraea* and a stronger species integration in *Q. robur* in this particular discovery panel. We also observed robust patterns of a slightly but significantly higher diversity in *Q. petraea*, across a random gene set and in the abiotic stress functional category, and a heterogeneous landscape of both diversity and differentiation. To explain ~~T~~these patterns, we discuss an alternative and non-exclusive hypothesis of stronger selective constraints in *Q. robur*, the most pioneering species in oak forest stand dynamics, additionally to the recognized and ~~are discussed in the context of both species~~ documented introgression history in both species despite their strong reproductive barriers. The quality of the data provided here and their representativity in terms of species genomic diversity make them useful for possible applications in medium-~~-~~scale landscape and molecular ecology projects. Moreover, they can serve as reference resources for validation purposes in larger-scale resequencing projects. This type of project is preferentially recommended in oaks in contrast to SNP array

development, given the large nucleotide variation and the low levels of linkage disequilibrium revealed.

Introduction

High-throughput techniques of the next-generation sequencing (NGS) era and increased genome sequencing efforts in the last decade have greatly improved access to genomic resources in non-model forest tree species (Neale and Kremer 2011, Neale *et al*. 2013; Plomion *et al*. 2016). However, these have only been applied recently to large-scale ecological and population genomics research (Holliday *et al*. 2017). One notable exception are studies undertaken in the model genus *Populus* (e.g. Zhou *et al*. 2014, Geraldes *et al*. 2014, Christe *et al*. 2016b) that benefited from the first genome sequence completed in 2006 in *P. trichocarpa* (Tuskan *et al*. 2006). In *Fagaceae*, previous comparative mapping and "omics" technologies (reviewed in Kremer *et al*. 2012) with recent development of genomic resources (e.g. Faivre-Rampant *et al*. 2011; Tarkka *et al.* 2013; Lesur *et al*. 2015; Lepoittevin *et al*. 2015, Bodénès *et al*. 2016) set the path to very recent release of genome sequences to the research community (*Quercus lobata*, Sork *et al.* 2016; *Q. robur*, Plomion *et al*. 2016, 2018; *Q. suber*, Ramos *et al*. 2018; *Fagus sylvatica*, Mishra *et al*. 2018), and these provide great prospects for future evolutionary genomics studies (Petit *et al*. 2013; Parent *et al*. 2015; Cannon *et al*. 2018; Lesur *et al*. 2018).

Recently, building from the European oaks genomic resources (*Quercus Portal* at https://arachne.pierroton.inra.fr/QuercusPortal/ ~~https://quercusportal.pierroton.inra.fr/~~ and references therein), natural populations of 4 *Quercus* species (*Q. robur*, *Q. petraea*, *Q. pyrenaica*, *Q. pubescens*) were genotyped for ~4000 single-nucleotide polymorphisms (SNPs, from an initial 8K infinium array, Lepoittevin *et al*. 2015). The data were further analysed (Leroy *et al*. 2017), with results extending previous knowledge on their likely diversification during glacial periods, as well as their recolonization history across Europe and recent secondary contacts (SC) after the last glacial maximum (Hewitt 2000; Petit *et al*. 2002a; Brewer *et al*. 2002). Using recent model-based inference allowing for heterogeneity of migration rates (Roux *et al*. 2014; Tine *et al.* 2014), Leroy *et al*. (2017) showed that the most strongly supported demographic scenarios of species diversification, allowing for gene flow among any pair of ~~for all~~ the four~~4~~ species mentioned above~~,pairs,~~ included very recent SC, due to a much better fit for patterns of large heterogeneity of differentiation observed across SNP loci (confirmed by Leroy *et al*. 2019, using ~15 times more loci across the genome and the same inference strategy). These recent SC events have been documented in the last decade

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in many patchily distributed hybrid zones where current *in situ* hybridization can occur among European oak species (e.g. Curtu *et al*. 2007; Jensen *et al.* 2009; Lepais and Gerber 2011; Guichoux *et al*. 2013). The resulting low levels of differentiation among *Q. robur* and *Q. petraea* in particular is traditionally linked to a model of contrasted colonization dynamics, where the second-in-succession species (*Q. petraea*) is colonizing populations already occupied by the earlier pioneering *Q. robur* (Petit *et al.* 2003). This model predicts asymmetric introgression towards *Q. petraea* (see Currat *et al*. 2008), as often observed in interspecific gene exchanges (Abbott *et al*. 2003), and a greater diversity in *Q. petraea* was documented at SNP loci showing higher differentiation (Guichoux *et al.* 2013). The directionality of introgression in oaks was also shown to depend on species relative abundance during mating periods in particular stands (Lepais *et al*. 2009, 2011). Nethertheless, oaks like other hybridizing taxa are known for the integration of their species parental gene pools and strong reproductive isolation barriers (Muir *et al*. 2000; Muir and Schlötterer 2005; Abadie *et al.* 2012, Lepais *et al*. 2013; Ortiz-Barrientos and Baack 2014; Christe *et al*. 2016a), raising essential questions about the interacting roles of divergent (or other types of) selection, gene flow, and recombination rates variation in natural populations, and their imprints on genomic molecular patterns of variation (e.g. Zhang *et al*. 2016; Christe *et al*. 2016b; Payseur and Rieseberg 2016).

These issues will be better addressed with genome-wide sequence data in many samples (Buerkle *et al*. 2011), which will be facilitated in oaks by integrating the newly available genome sequence of *Quercus robur* to chosen HT resequencing methods (Jones and Good 2016; e.g. Zhou and Holliday 2012; Lesur *et al.* 2018 for the first target sequence capture study in oaks). However, obtaining high quality haplotype-based data required for nucleotide diversity estimation and more powerful population genetics inferences will likely require the development of complex bioinformatics pipelines dedicated to high heterozygosity genomes and solid validation methods for polymorphism detection (e.g. Geraldes *et al*. 2011; Christe *et al*. 2016b).

Therefore, the objectives of this work were first to provide a detailed characterization of sequence variation in *Quercus petraea* and *Quercus robur*. To that end, we validated previous unpublished ~~Sanger~~ sequence data from the classical Sanger' chain-terminating dideoxynucleotides method (Sanger *et al*. 1977). These sequences ~~for fragments of~~ targeted fragments of gene regions in a panel of individuals sampled across ~~a~~ the western and central European ~~large~~ part of both species geographic range. Both functional and expressional

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candidate genes potentially involved in species ecological preferences, phenology and host-pathogen interactions were targeted, as well as a reference set of fragments randomly chosen across the last oak unigene (Lesur *et al.* 2015). These data were obtained within the framework of the EVOLTREE network activities (http://www.evoltree.eu/). Second, we aimed at estimating the distributions of differentiation and nucleotide diversity across these targeted gene regions for the first time in those species, and further test the robustness of comparative diversity patterns observed in the context of both species contrasted dynamics and introgression asymmetry. We discuss the quality, representativity and usefulness of the resources provided for medium scale genotyping landscape ecology projects or as a reference resource for validation purposes in larger-scale resequencing projects.

Material and methods

Sample collection

The discovery panel (*DiP*) included 25 individuals from 11 widespread forest stands with 2 to 4 individuals per location (13 from *Q. robur*, 11 from *Q. petraea*, 1 from *Q. ilex* to serve as outgroup, in Table 1).

Table 1 Geographic location of 25 sampled individuals from *Quercus petraea*, *Q. robur* and *Q. ilex*.

Country	Sampling site	Latitude	Longitude	Morphological *Quercus* species	Original Identifier	European cpDNA lineages[#]	cpDNA haplotypes[**]
Spain	Arlaban	42.967	-2.55	*petraea*	Ar18	B	10, 11, 12
				robur	Ar22		12
France	Arcachon	44.663	-1.181	*robur*	A4*	B	11, 12
	Pierroton	44.737	-0.776	*ilex*	IL_C	Euro-Med	H12**
				robur	11P*	B	10, 12
				robur	3P*		
	Orléans	47.826	1.908	*petraea*	Qs21*	B	10, 11, 12
				petraea	Qs28*		
				petraea	Qs29*		
	Petite Charnie	48.083	-0.167	*petraea*	PC55	A	7
				robur	PC229		
				robur	PC233		
Switzerland	Büren	47.105	7.383	*petraea*	B3	C	1
				robur	B179		
Hungary	Sopron	47.717	16.642	*petraea*	S444	A	5, 7
				robur	S104		
The Netherlands	Meinweg	51.181	6.138	*petraea*	M51	A, C	1, 5
				robur	M7		
United Kingdom (UK)	Roudsea Wood	54.218	-3.018	*petraea*	RW108	B	10, 12
				robur	RW8		
				robur	RW11		
Germany	Rantzau	53.707	9.765	*petraea*	R100	A, C	7, 1
				petraea	R127		
				robur	R300		
				robur	R312		

Latitude and longitude are given in the WGS 84 coordinate system. Coordinates correspond either to a central point in the mixed forest stand, or the mean of individual trees coordinates. *: parents of controlled crosses used for genetic mapping. [#]: after Petit *et al.* (2002a), the putative glacial refugia for lineage B and C are located in the south of Spain, and for lineages A and C either in the south of Italy or in the Balkans or both~~(Petit *et al.* 2002)~~. **: cpDNA haplotypes are from trees previously sampled in Petit *et al.* (2002b), located within a 50 km radius of studied trees, based on the GD2 database (http://gd2.pierroton.inra.fr/). *Quercus* species were *a priori* assigned from morphological information by persons who sampled the trees, but see below for a comparison with genetic assignments and introgression analyses of each individual using the STRUCTURE bayesian inference method ("*Characterization of diversity*…" part).

These stands occur across a large part of both *Quercus* species natural distributions, spanning ~20° in longitude (~2200 km) and ~11° in latitude (~1250 km) in western and central Europe (Fig. S1, Supporting Information). They are also located in areas covering the three major cpDNA lineages A, B and C (among five) that indicate different historical glacial refugia

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(Petit *et al.* 2002a), and extend much further geographically towards northern, eastern and south-eastern European borders (Table 1, after Petit *et al.* 2002b). One stand (Sopron, in Hungary), also occurs within the large geographic distribution of the most Eastern lineage E, in a region where lineages A and C also occur. Individuals were chosen either on the basis of their differing leaf morphology among *Q. robur* and *Q. petraea* species (Kremer *et al.* 2002a), or as parents of mapping pedigrees (e.g. Bodénès *et al.* 2016, see Table 1).

Leaves were sampled, stored in silica gel and sent to INRA (Cestas, France) for DNA extraction following Guichoux *et al.* (2013). DNA quality and concentration were assessed with a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, 152 DE, USA) and by separating samples in 1% agarose gels stained with ethidium bromide. Extractions were repeated until we obtained at least 20 micrograms of genomic DNA per sample, which was needed for a few thousands individual PCRs.

Choice of genic regions for resequencing

Genic regions were chosen from over 103 000 Sanger sequences available in expressed sequence tags (EST) databases at the start of the project. These sequences corresponded to 14 cDNA libraries that were prepared with many individuals from both species. They ~~obtained from various tissues and developmental stages~~ were assembled before finally selecting 2000 fragments for resequencing (Appendix S1 and Fig. S2-A, Supporting information for more on methods producing the original working assembly (*orict*); see also Ueno *et al.* 2010). The targeted fragments were chosen from an extensive compilation of both expressional and functional candidate genes that would likely be involved in white oaks' divergent functions and/or local adaptation, using model and non-model species databases or published results (see Appendix S1 and Fig. S2-B, Supporting information for more details on the strategy followed, and Table S1 for designed primers).

~~bud, leaf, root and wood-forming tissues), and thus likely to target a large range of expressed genes. Overall, 146 individuals were sampled in 3 different French regions (South West, North East and North West). We performed the first working assembly for those sequences, with the main aim of avoiding paralog assembly while limiting split contigs with overlapping homolog sequences, the final assembly including 13477 contigs and 74 singletons (Appendices S1 and S2, Fig. S2-A, Supporting information). The libraries used in this assembly have since been named A, B, F to O, and S, and were included in larger transcriptome resources for Quercus species (Ueno et al. 2010).~~

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~~In parallel, expressional and functional candidate genes information was compiled for targeting those potentially involved in white oaks' divergence and/or local adaptation (Fig. S2-B and Table S1, Supporting information). Briefly, model species databases were searched for gene accessions by gene ontology (GO) and metabolic pathways keywords. Those sequences were first Blasted against our oak assembly (Altschul et al. 1990, 1997). Second, the sequences from their best hits were extracted (see filtering criteria in Fig. S2-B, Supporting information) and re-Blasted against the non-redundant protein (NR) database at NCBI. Third, their annotation was compared to those of the initial gene accessions, allowing 95% of hits from the oak assembly to be validated (step 2 in Fig. S2-B, Supporting information). Expressional candidate genes sequences from bud tissues or stress treatment libraries and a random set of ESTs were also directly sampled across the oak assembly generated above (see Table S1, column F, Supporting information). Primers were designed with the OSP software (Hillier and Green 1991) by setting up homogenous melting temperatures constraints and excluding low-complexity propositions. Predicted amplicons were Blasted against each other and onto our assembly to exclude those with potential amplification problems and multiband patterns. They were also checked for their depth and presence of polymorphisms in contigs alignment, yielding finally 2000 amplicons for resequencing (Fig. S2-B, Supporting information).~~

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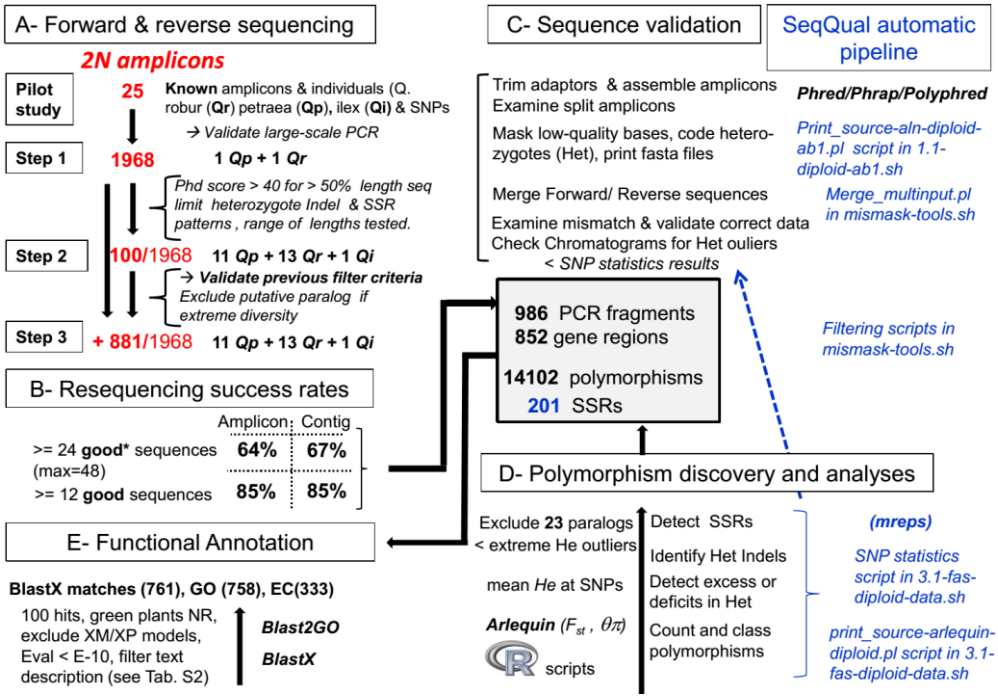
Data production and polymorphism discovery *in resequenced fragments*

All the sequencing work was performed by Beckman Coulter (Agencourt Bioscience Corporation, Beverly, MA, USA) on ABI3730 capillary sequencers (Applied Biosciences) after preparing DNA samples according to the company's guidelines. Various d~~D~~ata quality steps were followed for ~~designed throughout the process in order to~~ maximizinge the amount and quality of the sequences finally obtained (Fig. 1-A, and Appendix S1, Supporting information for further analyses across 2000 amplicons).

Figure 1 Bioinformatics strategy for sequence data production, amplicon assembly, functional annotation, and polymorphism discovery. Scripts used are in italics (see text for further details). GO: Gene Ontology, EC: Enzyme Commission ID. * A **good** sequence is defined as having a minimum of 50% of its nucleotides with a Phred score above 30.



Forward and reverse sequences were produced for 986 amplicons across 25 individuals (100+881 in steps 2 and 3, Fig. 1-A), and more than 85% of them yielded at least 12 high-quality sequences (Fig. 1-B and column L in Table S1, Supporting information). All amplicon assembly steps, merging, trimming, and filtering/masking based on quality were performed with ~~Bioperl scripts from~~ our *SeqQual* pipeline ~~(; available at~~ https://github.com/garniergere/SeqQual~~)~~, with examples of data and command files. This repository compiles and extends former work dealing with 454 data (Brousseau *et al*. 2014; El Mujtar *et al.*2014), providing Bioperl scripts used here that automatically deal with Sanger haploid or diploid DNA sequences and allow fasta files post-processing in batch (Fig. 1-C). Sequence variant~~Polymorphisms~~ discovery was finally performed ~~on nucleotide data with~~using an error rate below 0.001 (i.e. Phred score above 30, Ewing *et al*. 1998, and see Appendix S1, Supporting information for more details). Simple sequence repeat (SSR) patterns were further detected or confirmed from consensus sequences using the *mreps* software (Kolpakov *et al.* 2003~~; see~~, Fig. 1-D, and~~see for a R script parsing *mreps* output,~~ https://github.com/garniergere/Reference.Db.SNPs.Quercus/ for a R script parsing *mreps* output~~MREPS.parsing/ for a R script parsing *mreps* output~~). Various additional steps involving the treatment of insertion-deletion polymorphisms (indels) and heterozygote indels

(*HI*) in particular, allowed missing data from polymorphic diploid sequence to be minimized (see Appendix S1, Supporting information).

Functional annotation

Resequenced genic regions were annotated using the BlastN best hits offor their corresponding ~~our working assembly (*orict*) original~~ contigs and those of their ~~for the~~ expected amplicons (*orict-cut*) towere first retrieved using ~~Lesur *et al.* (2015)'~~ most recent oak assembly (*ocv4*, Lesur *et al.* (2015); see Table S2-C, Supporting information). Final c~~C~~onsensus sequences ~~o~~for these ~~candidate~~ regions originated from both *orict* and *ocv4* (396 and 368 respectively, see Table S2-A, S2-B, and Appendices~~x~~ S1 and S3, Supporting information), aiming at retrieving the longest ~~consensus~~ sequences~~ that included the resequenced gene regions~~, while avoiding to target those with possible chimeric sequences. Functional annotation was then performed via homology transfer using BlastX 2.6.0+ program at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with parameters to optimize speed, hits' annotation description and GO content (Fig. 1-E and Table-S2, Supporting information). Retrieval of GO terms were performed with Blast2GO (Conesa *et al.* 2005 free version at https://www.blast2go.com/blast2go-pro/b2g-register-basic) and validation of targeted annotations with Fisher Exact enrichment tests (details of Blast2GO analyses provided in Appendix S1, Supporting information).

Characterization of diversity and genetic clustering

Using the *SNP-stats* script for diploid data from *SeqQual*~~(see above)~~, simple statistics were computed across different types of polymorphisms (SNPs, indels, SSRs…) including minimum allele frequencies (*maf*) and heterozygote counts, Chi-square tests probability for Hardy-Weinberg proportions, G_{ST} (Nei 1987) and G_{ST}' standardized measure (Hedrick 2005). Complex polymorphisms (involving heterozygote indels (*HI*) and/or SSRs,) were also further characterized (see Appendix S1, Supporting information), and data formatted or analyzed using either Arlequin 3.5 (Excoffier and Lischer 2010), *SeqQual* (e.g. for Arlequin input file with phase unknown, Fig. 1-C), or R scripts. Nucleotide diversity $\theta\pi$ (Nei 1987), based on the average number of pairwise differences between sequences, and its evolutionary variance according to Tajima (1993), were also estimated and compared among species and across candidate genes grouped by broad functional categories (see column F in Table S1, Supporting information), and Weir and Cockerham (1984) F_{ST} estimates of differentiation

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were computed among species for SNP data along genic regions using analyses of molecular variance (Excoffier 2007).

The initial morphological species samples were compared to the genetic clusters obtained with the STRUCTURE v2.3.3 inference method (Falush *et al.* 2003) in order to test possible levels of introgression across individuals. We used the admixture model allowing for mixed ancestry and the correlated allele frequencies assumption for closely related populations as recommended defaults, and since they best represent previous knowledge on each~~both~~ species genetic divergence across their range (e.g. Guichoux *et al.* 2013). Preliminary replicate runs using the same sample of loci produced very low standard deviation across replicates of the data log likelihood given K (ln Pr(X/K), see Fig. S3-A, Supporting information). We thus resampled loci at random for each of 10 replicate datasets in 3 different manners to add genetic stochasticity: 1) one per region, 2) one per 100 bp block, and 3) one per 200 bp block along genes (see Appendix S1, Supporting information and https://github.com/garniergere/Reference.Db.SNPs.Quercus/tree/master/STRUCTURE.files for examples of STRUCTURE files as recommended by Gilbert *et al.* (2012), along with R scripts for outputs). Statistical independence among loci within each species was verified with Fisher's exact tests implemented in Genepop 4.4 (Rousset 2008).

Results

Polymorphisms typology and counts

Among the amplicons tested, 986 were successful, 13 did not produce any data and 23 were excluded because of paralog amplifications (Fig. 1-C and Table S1, Supporting information). Around 25% of the successful amplicons overlapped and were merged, consistently with their original design across contigs. Despite the presence of *HI* patterns due to SSR or indels, most amplicons were entirely recovered with forward and reverse sequencing. Several (5% of the total) were however kept separate, either because of functional annotation inconsistency, or because amplicon overlap was prevented by the presence of SSRs or putative large introns (see "Final gene region ID" column with -F/-R suffix in Table S1, Supporting information). We finally obtained 852 genic regions covering in total ~529 kilobases (kb), with an average size of 621 bp per region, ranging from 81 to 2009 bp (Table 2, and Appendix S4, Supporting information, for genomic consensus sequences).

Table 2 Typology of polymorphisms in successfully resequenced amplicons.

	Both species and introgressed individuals	*Q. petraea*	*Q. robur*	*Q. ilex*
Total length resequenced (bp)	529281	-	-	196676
Number (Nb) of amplicons	986	-	-	486
Nb of genic regions	852	-	-	394
Mean genic region size - N50 size (bp)	621-700	-	-	500-539
Minimum - Maximum genic region size (bp)	81-2009	-	-	198-1285
Estimated intron sequences (bp)	186827	-	-	-
Mean haploid sample size (total sequence)	34.71	13.35	18.28	-
Polymorphism in 852 genic regions				
Mean haploid sample size (variants)	32.16	12.57	13.85	-
Monomorphic genic regions	15 (1.76%)	18 (2.14%)	21 (2.52%)	-
Genes with at least one single base indel	591	345	379	-
" " " " one larger indel (>1 bp)	252	190	214	-
" " " " one SSR (>=di)	163	-	-	-
SNPs only (excluding 1 bp indels)	12478	7511	8078	-
Indels (1 bp)	1213	751	809	-
Indels (2-5 bp)	221	142	161	-
Indels (6-10 bp)	88	72	71	-
Indels (11-50 bp, excl. SSRs)	98	81	79	-
Indels (74,146,219,341 bp, excl. SSRs)	4	3	4	-
Total number of polymorphisms	**14102**	**8560**	**9202**	**676**
Triallelic SNPs	*218*	*141*	*165*	-
…Singletons (incl. 1 bp indels)	*4334*	*1990*	*2151*	-
…Variable SSRs (excl. homopolymers)	*111*	-	-	-
Total length with sequence variant positions	17594	10765	11451	-
Sequence length of indels and complex polymorphisms (Indels and SSRs)	5116	-	-	-

Counts for *Q. petraea* exclude the 2 most introgressed individuals (Qs28 and S444 in Table 1); SSR: simple sequence repeats; "N50 size" is the size for which the cumulative sum of gene amplicons' size equal or higher than this value corresponds to 50% of the total amplicons' size sum; The number of polymorphisms for *Q. ilex* equals the number of heterozygotes in the resequenced individual across amplicons; Numbers of monomorphic regions were computed for those with at least 10 gametes in both species; Some detected SSR patterns were not polymorphic in our samples (detailed in Tables S1 and S5, Supporting information).

Compared to the EST-based expected total fragment size of ~ 357 kb, around 187 kb of intron sequence was recovered across 460 of the resequenced regions (assuming intron presence if an amplicon size was above its expected size by 40 bp). Introns represented ~35% of genic regions in length and ~51% of those including introns.

We observed 14102 polymorphisms in both species across 852 gene regions, 15 of those regions (<2%) being monomorphic (Table 2). This corresponds to 1 polymorphism per ~38 bp, or 1 per ~30 bp when considering the total number of variant positions in both species (17594 bp, Table 2). Remarkably, variant positions involving larger indels, SSRs and mixed

complex polymorphism patterns represented ~30% of the total variant positions (Table 2, and see their exhaustive lists with various statistics in Table S3 and S4, Supporting information). We observed 12478 SNPs (88.5% of all polymorphisms), 1 SNP per 42 bp, and 218 triallelic SNPs (~1.75% of SNPs) were confirmed by visual examination of chromatograms.

Considering only one species, we observed on average 1 variant position per ~48 bp, 1 polymorphism per ~60 bp, and 1 SNP per ~68 bp. Among indels, 1213 (8.6% of all polymorphisms) were single base, 309 ranged from 2 to 10 bp, and 102 had sizes above 10 bp which were mostly shared among species (Table 2). In this range-wide sample, there were 4334 singletons among all single base polymorphisms, 506 of them being indels. Overall, indels were present in 69% of gene regions and non-single base ones across ~30% of them. Excluding homopolymers (see Appendix S1, Supporting information), we detected 201 SSRs occurring on 163 gene regions by considering a minimum repeat numbers of 4 and a mismatch rate among repeats below 10% (Table 2, Table S1 and Table S5, Supporting information), and 55% (111) were polymorphic in our sample of individuals (Table 2). Among them, 89 (44%) had dinucleotide repeats and 65 (32%) trinucleotide repeats. The SSRs with the lowest number of repeats (<5) had a majority (59%) of repeat sizes between 4 and 7, the rest being trinucleotides (Table S5, Supporting information).

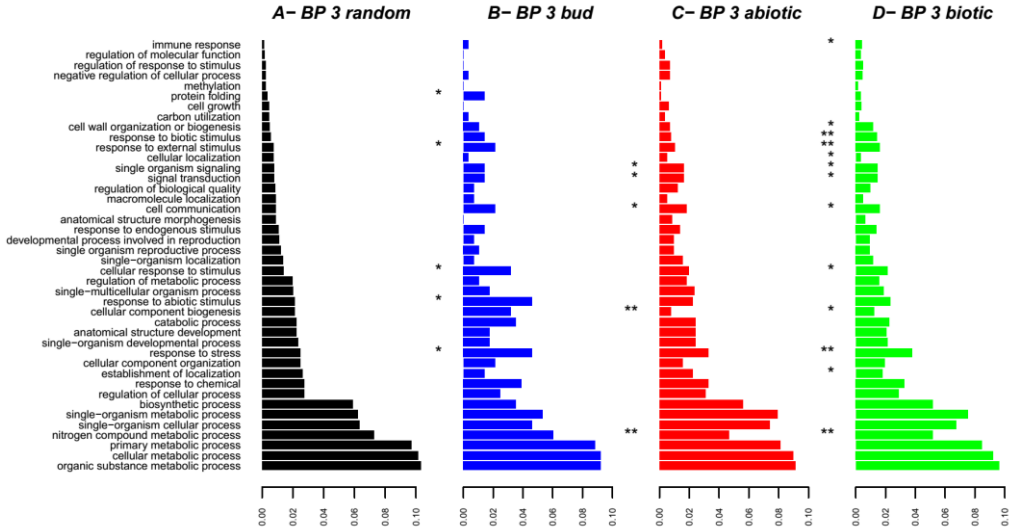
Using the same PCR conditions, homologous sequence data were obtained for one individual of the outgroup *Quercus ilex* across 37% of the gene regions (~197 kb, 397 sequences, 676 heterozygous sites in Table 2), which illustrates both their sequence similarity yet divergence for a species belonging to the *Ilex* versus *Quercus* taxonomic group (Lepoittevin *et al*. 2015; see Table S1 column Q, and see Appendix S5, Supporting information, for *Q. ilex* genomic sequences).

Annotations and GO term distributions

BlastX matches with *E*-values below 10^{-30} were found for ~97% (738/764) of the contig consensus, only 11 sequences (1.4%) having hits with *E*-values above 10^{-10} that were all among the reference random sample (see BlastX criteria in Table S2, Supporting information). The most represented species among the best hits with informative annotations were *Prunus persica (111)*, *Theobroma cacao (91)*, *Morus notabilis (57)* and *Populus trichocarpa (45)* (Appendix S6-A, Supporting information), which probably illustrates both the close phylogenetic relationships among *Quercus* and *Prunus* genera, consistently with results obtained on the larger *ocv4* assembly (Lesur *et al.* 2015), and the quality and availability of *P. persica* genome annotation (Verde *et al*. 2013, 2017).

Between 1 to 30 GO terms could be assigned to 761 sequences, with EC codes and InterProScan identifiers for 343 and 733 of them respectively (Fig. 1, and Table S2, Supporting information). The most relevant GO terms were then retained using the Blast2GO "annotation rule" (Conesa *et al*. 2005) that applies filters from the Direct Acyclic Graph (DAG) at different levels (Fig. 2, Fig. S4-A- to-F, Supporting information).

Figure 2 Distributions of GO terms across different gene lists (*bud*, *abiotic* and *biotic*) at biological process level 3, and Fisher exact tests across pairs of sequence clusters with the same GO terms between the random list and other lists. Significance levels *: P<0.05, **: P<0.01.



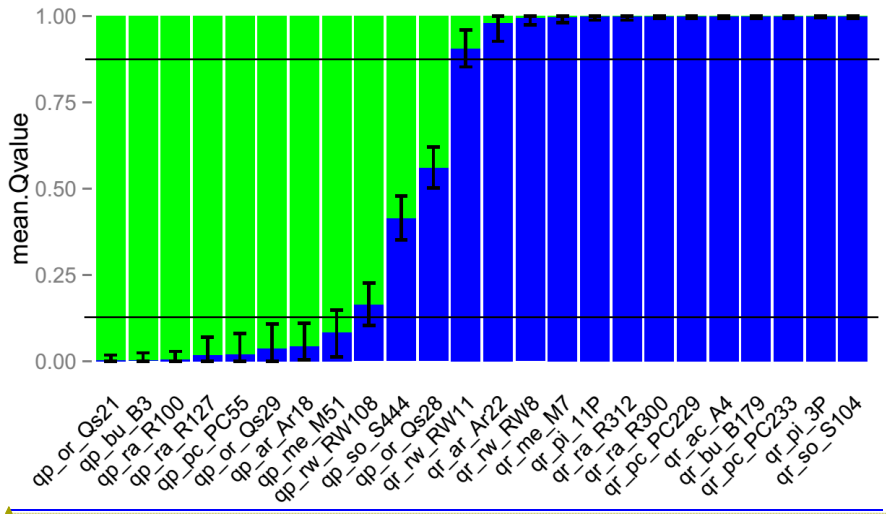
At biological process (BP) level ~~(BP)~~ 3, apart from general terms involving "metabolic processes", a large number of sequences (between ~100 and ~150) were mapped to "response to..." either "...stress", "...abiotic stimulus" or "...chemical", and also to categories linked to developmental processes (Fig. S4-D, Supporting information).

Enrichment tests also revealed a significant increase at both BP levels 2 and 3 for the following GO categories: "response to stress" or "external stimulus" for *bud* and *biotic* gene lists, "response to abiotic stimulus" for the *bud* list, and "immune" and "biotic stimulus" responses for the *biotic* list (see Fig. 2-B to 2-D compared to Fig. 2-A, and Fig. S5, Supporting information). Most of these exact tests (>80%) were still significant when selecting genes attributed exclusively to one particular list (in Table S1, Supporting information), which adds to the relevance of our original gene lists in targeting particular functional categories.

Species assignment and introgressed individuals

In both species, the proportion of significant association tests among the loci used for clustering (> two million within each species) was generally one order of magnitude below the type-I error rates at 5% or 1%. This indicates a very low background LD within species at their range levels, consistently with the underlying model assumptions used in STRUCTURE. Based on both ln Pr(X/K) and ΔK statistics and as expected, the optimal number of genetic clusters inferred was 2, whatever the number of polymorphisms and type of sampling (Fig. 3, Fig. S3 and S6, Supporting information).

Figure 3 Posterior assignment probabilities of individuals into two optimal clusters from STRUCTURE analyses, sorted in increasing order of belonging to cluster 2 (here *Q. robur* (Qr, in blue/dark grey), the alternative cluster 1 matching *Q. petraea* (Qp, in green/light grey), apart from individuals with higher introgression levels. Each bar represents one individual and includes mean upper and lower bounds of 90% Bayesian confidence intervals around mean *Q*-values across 10 replicates. Each replicate is a different random sample of 1785 polymorphisms. Horizontal black lines represent the 0.125 and 0.875 ~~se~~ values, which can be considered as typical thresholds for back-crosses and later-generation hybrids (Guichoux *et al.* 2013), values within those thresholds suggesting a mixed ancestry with the other species for a small number of generations in the past.



Most individuals (20) clearly belonged to either cluster with a mean probability of cluster assignment above 0.9 , which was not significantly different from 1, based on mean values of 90% Bayesian credible intervals (BCI) bounds across replicates, and for different types of sampling or SNP numbers (Fig. 3 and Fig. S6, Supporting information). Two individuals from Roudsea Wood in UK, the most northerly forest stand of this study, were considered to be significantly introgressed, each from a different cluster, since both showed a BCI that did not

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include the value "1" across other replicated runs and SNP sampling (Fig. S6, Supporting information), RW108 also having ~~with~~ a mean probability above~~ies within~~ 0.125 (Fig. 3) ~~and 0.875~~. Although M51 has a mean assignment value close to that of RW11 in the particular run shown in Fig.3, its BCI was larger and often included the zero value in other runs (Fig. S6, Supporting information), so it was assigned to the *Q. petraea* cluster. ~~These values can be considered as typical for back-crosses and later-generation hybrids (Guichoux *et al.* 2013), suggesting a mixed ancestry with the other species for a small number of generations in the past.~~ In the initial morphological *Q. petraea* group, two individuals were also clearly of recent mixed ancestry: one from the easternmost forest stand of Sopron (S444), and another one (Qs28) from central France, considered previously to be a *Q. petraea* parental genotype in two oak mapping pedigrees (Bodénès *et al.* 2012, 2016; Lepoittevin *et al.* 2015). However, Qs28 shows here a clear F1 hybrid pattern, given its probability values close to 0.5 and its BCI maximum upper and minimum lower bound values of 0.30 and 0.61 respectively across runs (Fig. 3 and Fig. S6-A to S6-J, Supporting information). Testing 3 or 4 possible clusters showed the same ancestry patterns for the introgressed individuals with 2 main clusters and similar *Q*-values (data not shown), which does not support alternative hypotheses of introgression from different species in those individuals.

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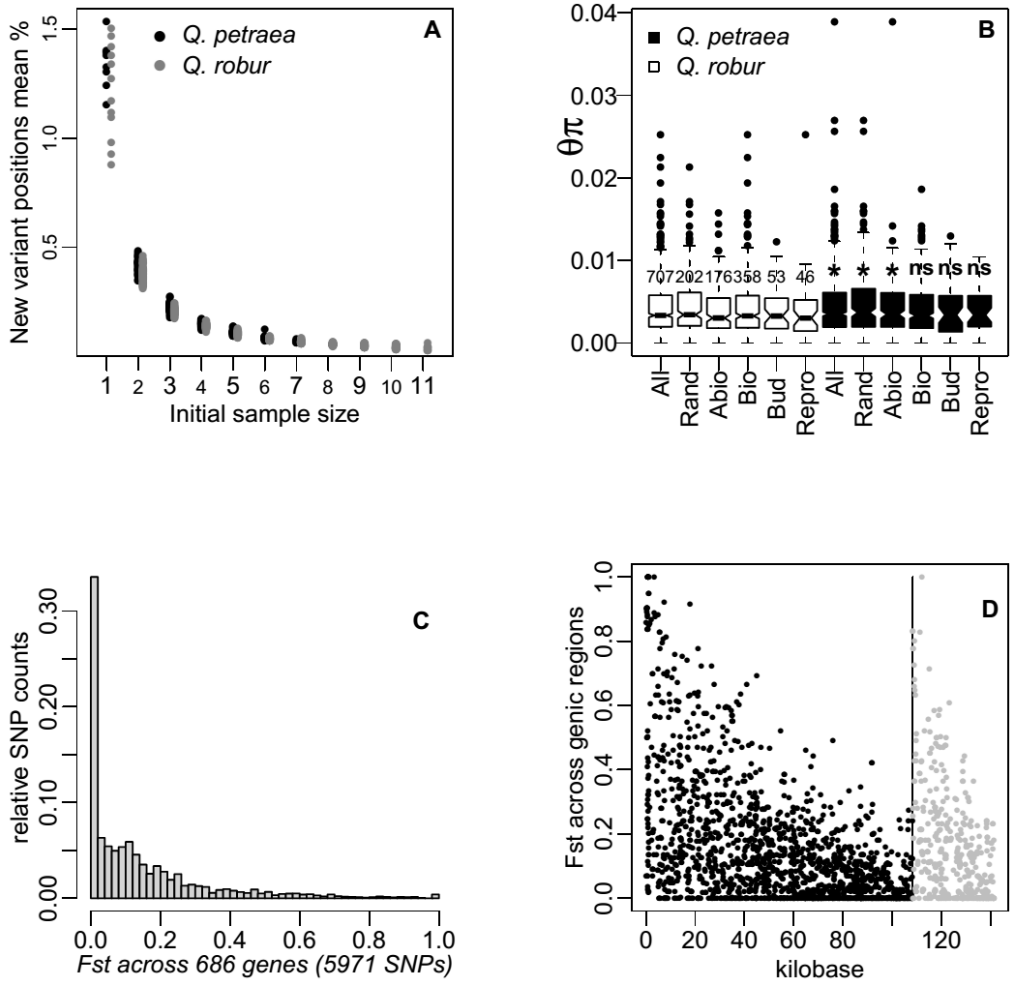
Large heterogeneity of diversity and differentiation across genes

Nucleotide diversity was thus estimated in each parental species after excluding Qs28, RW108, S444 and RW11, which were considered to be the 4 most introgressed individuals ~~from each the initial morphological groups~~ (see Fig. 3 above). We then checked how the remaining samples represented species' diversity. Starting with one individual, we observe a dramatic drop in the mean proportion of new variant positions brought by each new individual in any species (*Mpn*) as a function of the initial sample size, followed by a subsequent stabilization (Fig. 4-A, and see Fig. S7-A, Supporting information). Indeed, *Mpn* was only around 11% when going from 4 to 5 individuals in both species, and stabilized below 5% after 8 individuals in *Q. robur* **(**Fig. 4-A). We thus decided to retain 726 gene regions with at least 8 gametes per species (listed in column L in Table S1, Supporting information). The larger *Q. robur* sample after excluding the most introgressed individuals (24 versus 16 gametes in *Q. petraea*) only exhibited slightly higher polymorphism counts than in *Q. petraea* overall (Table 3).

Also, 48% and 52% of the polymorphisms observed were exclusive to *Q. petraea* and *Q. robur* respectively in our panel, the rest being shared among species (Table 3). Among

exclusive polymorphisms, 46% and 44% were singletons in *Q. petraea* and *Q. robur* respectively, suggesting that they might be either rare in both species, or more polymorphic in local populations from which few individuals were sampled across the species wider ranges. Overall and within both species, we observed a large variation in the numbers of segregating sites per gene size (Fig. S7-B, Supporting information).

Figure 4 Mean proportion of new variant sites brought by each new distinct individual added to all possible initial sample size combinations (A); Mean nucleotide diversity (considering all polymorphisms) in both species across genic regions, and different functional categories (B) compared between species with Wilcoxon signed-rank tests: significant at Pr<5% (*), non-significant (ns); Histogram of *Fst* estimates across polymorphic gene regions with a minimum of 8 gametes per species, after excluding singletons and grouping negative with null values (C); Manhattan plot of *Fst* estimates sorted by mean *Fst* values across randomly chosen (black dots) and Bud phenology (grey dots) genic regions (D).



The mean nucleotide diversity estimates ($\theta\pi$) across genic regions when considering all polymorphisms were 0.00447 and 0.00425 in *Q. petraea* and *Q. robur* respectively, with up to a 10-fold variation among polymorphic genes overall and in different functional categories (Fig. 4-B and Table 3).

Table 3 Polymorphism counts and nucleotide diversity in parental species across genic regions with larger sample sizes.

Polymorphism in 726 gene fragments	both species	*Q. petraea*	*Q. robur*
Number of individuals considered	20	8	12
Monomorphic gene fragments	17 (2.34%)	19 (2.63%)	20 (2.87%)
Total number of polymorphisms	11089	7061	7721
SNPs only	9867	6226	6830
All Indels and SSRs	1222	835	891
Exclusive polymorphisms	-	3359	4024
Singletons among them (%)	-	0.456	0.437
Shared polymorphisms	3696	-	-
Mean nucleotide diversity estimates*			
SNPs only	3.849E-03	**3.957E-03****	**3.740E-03**
" " diversity range		0-0.03823	0-0.02525
Tajima's evolutionary standard deviation	2.549E-03	2.632E-03	2.465E-03
SNPs only (509 chosen genes)	3.752E-03	3.821E-03	3.682E-03
SNPs only (202 random genes)	4.103E-03	**4.306E-03**	**3.900E-03**
All polymorphisms	4.359E-03	**4.471E-03**	**4.247E-03**
" " diversity range		0-0.03893	0-0.02525
Tajima's evolutionary standard deviation	2.816E-03	2.903E-03	2.729E-03
All polymorphisms (509 chosen genes)	4.214E-03	4.278E-03	4.150E-03
All polymorphisms (202 random genes)	4.716E-03	**4.944E-03**	**4.488E-03**

The 4 most introgressed individuals from Fig. 3 (Qs28, S444, RW108, RW11) are excluded for computations. Monomorphic regions are defined as in Table 2. *: Diversity is~~are~~ computed for regions with a minimum of 200 bp overall and at least 8 gametes per species at variant positions. The 509 chosen genes belong to the different functional categories listed in Table S1. Values in the "both species" column for diversity estimates are means across all genes, of both ~~each~~ species' values. **: Values in bold indicate significant Wilcoxon paired ranked tests for a higher *Q. petraea* nucleotide diversity compared to *Q. robur* across genes.

When including SNPs only, mean $\theta\pi$ decreased overall by more than 10% (Table 3, and see column D in Table S4, Supporting information). The large variation among genes is also illustrated by the absence of significant differences between mean diversity among functional categories *within species*, in most comparisons using non-parametric Wilcoxon rank sum tests (*Wrs*) with similar number of genes. Two notable exceptions were observed when considering all polymorphisms: the *biotic stress* category (358 genes) had on average a lower $\theta\pi$ in *Q. petraea* than in the random gene list (211 genes, *Wrs* Pr<0.042), and the mean $\theta\pi$ of the

reproductive phenology category was significantly lower in both species than that of the *Bud phenology* category (*Wrs* Pr<0.040 and Pr<0.013 in *Q. petraea* and *Q. robur* respectively, considering exclusive categories from Table S2, Supporting information). Genes with $\theta\pi$ estimates above 0.02 were found across most categories, whether considering all polymorphisms (Fig. 4-B) or SNPs only. The 8 genic regions showing the highest $\theta\pi$ values in both species were annotated for example as disease resistance, transcription factor or membrane transport proteins, half of them being from the original random list.

Comparing nucleotide diversity between individuals according to their main cpDNA lineages B *versus* A or C (Table 1), no significant differences were found between lineages within both species, using *Wpr* tests across all genes (see also the lineage-associated distributions of genes' diversity in Fig. S8, Supporting information). This was also true for all functional categories. In both species, the mean differentiation across genes among lineages was very low (<0.015, each gene estimate being the mean F_{ST} across all polymorphisms at this gene), with very few genes (~1%) having much higher mean F_{ST} (ranging from 0.21 to 0.41 or 0.56 within *Q. petraea* and *Q. robur* respectively).

Mean $\theta\pi$ comparison tests *between species* across all gene regions were not significant (Table 3, *Wrs* Pr>0.15 for all polymorphisms or SNPs only), nor were they across different categories and between gene pairs, using a 95% confidence interval based on Tajima's evolutionary variance for $\theta\pi$ (Tajima 1983) while assuming underlying Gaussian distributions. Indeed for the same genic regions, many examples can be found of higher $\theta\pi$ estimates in one species or the other. However, comparing diversity estimates across the exact same positions and performing Wilcoxon paired ranked tests (*Wpr*) across all genes, there was a significant pattern of a slightly higher diversity in *Q. petraea* (see Table 3 and Fig. 4-B), whether considering all polymorphisms (*Wpr* Pr<0.028) or SNPs only (*Wpr* Pr<0.036). This pattern remained significant across the 202 polymorphic genes chosen randomly (*Wpr* Pr<0.037, all polymorphisms, Table 3), even when excluding the 5% or 10% of genes having the highest $\theta\pi$ values. This pattern of a significantly higher $\theta\pi$ in *Q. petraea* ~~, but it~~ was not observed when~~robust to~~ considering the ~~other~~ 509 polymorphic gene~~ic~~ regions chosen in functional categories, either together or separately in the different categories (Fig. 4-B), except for the *Abiotic stress* category.

We also observed a very large variation for F_{ST} estimates across gene regions and functional categories, which covered the full range of possible values [0,1], with mean values of ~0.13

whether considering all polymorphisms or SNPs only (Fig. 4-C, and Fig. 4-D for the random genic regions and a representative example in one category). The very few segregating sites with F_{ST} values equal to one had either missing individuals' or strands, possibly caused by polymorphisms within primer regions. Among the sites sequenced for the full sample of gametes, the 20 highest F_{ST} values ranged from 0.6 to 0.9 and belonged to 10 genic regions, many of which also showed null or very low F_{ST} values within 100 bp. This large variation in differentiation was observed between very close variant sites in many genes, suggesting very high recombination rates at genome-wide and range-wide scales, and consistently with the very low expected background LD (see above). Additionally, a large variance is expected around F_{ST} estimates due to the relatively low sample size in both species, in particular for bi-allelic loci (Weir and Hill 2002; Buerkle *et al*. 2011; e.g. Eveno *et al.* 2008).

Discussion

In the NGS era, non-model tree species such as many *Fagaceae* still lag behind model species for easy access to sequence polymorphism and SNP data (but see Gugger *et al*. 2016 for *Quercus lobata*). These data are needed for larger scale studies addressing the many diversity issues raised by their combined economic, ecological and conservation interests (Cavender-Bares 2016; Fetter *et al*. 2017; Holliday *et al*. 2017). Recent achievements and data availability from the *Q. robur* genome sequence project (Plomion *et al*. 2018) opens a large range of applications in many related temperate and tropical *Fagaceae* species due to their conserved synteny (Cannon *et al.* 2018). In this context, we discuss below the representativity of our data in terms of species genomic diversity as well as the robust patterns observed across genes, and further illustrate their past and future usefulness for *Quercus* species.

Genic resources content, quality, and representativity

We provide a high-quality polymorphism catalog based on Sanger resequencing data for more than 850 gene regions covering ~530 kb, using a discovery panel (*DiP*) from mixed *Q. robur* and *Q. petraea* populations located in the western and central European ~~across a large~~ part of their geographic range. This catalog details functional annotations, previous published information, allele types, frequencies and various summary statistics within and across species, which can assist in choosing novel polymorphic sites (SNPs, SSRs, indels…) for genotyping studies. Among genomic SSRs, more than 90% (~200) are new (17 already detected in Durand *et al*. 2010; 3 in Guichoux *et al.* 2011), so they constitute an easy source of potentially polymorphic markers in these oak species. Standard formats for high-density genotyping arrays and primer information are also provided, making these resources readily

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operational for medium scale molecular ecology studies while avoiding the burden of bioinformatics work needed for SNP development (Tables S1 to S5, Supporting information, and see also https://github.com/garniergere/Reference.Db.SNPs.Quercus for additional information). This catalog corrects and largely extends the SNP database for Q. petraea/robur at https://arachne.pierroton.inra.fr/QuercusPortal/ which was previously used to document a SNP diversity surrogate for both *Quercus* species in the oak genome first public release (Plomion *et al*. 2016).

Thanks to a high quality dedicated pipeline, we could perform a quasi-exhaustive characterization of polymorphism types in our *DiP* and across part of the genic partition of these *Quercus* species (see Fig. 1). Although base call error rates below 1/1000 were used (as originally developed for Sanger sequencing), most variant sites were located in regions with lower error rates (below 1/10000) so that true singletons could be identified. At the genotypic level, a Sanger genotyping error rate below 1% was previously estimated using a preliminary subset of around 1200 SNPs from this catalog (corresponding to around 5800 data points in Lepoittevin *et al.* 2015). This rate can be considered as an upper bound for the present study, given all additional validation and error correction steps performed. Although little produced now with the advent of NGS methods, Sanger data have served for genome sequencing projects in tree species before 2010 (Neale *et al*. 2017), and have been instrumental, in combination to NGS for BAC clones sequencing, in ensuring assembly long-distance contiguity in large genomes such as oaks (Faivre-Rampant *et al*. 2011, Plomion *et al*. 2016). Sanger sequencing has also provided reference high-quality data to estimate false discovery or error rates, and validate putative SNPs in larger scale projects (e.g. Geraldes *et al*. 2011 in *Populus trichocarpa*; Sonah *et al*. 2013 in Soybean; Cao *et al*. 2014 in *Prunus persica*).

Finding an optimal balance between the number of samples and that of loci is critical when aiming to provide accurate estimates of diversity or differentiation in population genetics studies. Given the increasing availability of markers in non-model species (usually SNPs), it has been shown by simulation (Willing *et al*. 2012, Hivert *et al*. 2018) and empirical data (Nazareno *et al*. 2017) that sample sizes as small as 4 to 6 individuals can be sufficient to infer differentiation when a large number of bi-allelic loci (> 1000) are being used. A broad-scale geographic sampling is however required if the aim is to better infer genetic structure and complex demographic scenarios involving recolonization and range shifts due to past glacial cycles, such as those assumed for many European species (Lascoux and Petit 2010, Keller *et al*. 2010, Jeffries *et al*. 2016, Sousa *et al*. 2014). Our sampling design is likely to

have targeted a large part of both species overall diversity and differentiation across the resequenced genic regions. This is first suggested by the small proportion of additional polymorphisms once an initial sample of 8 gametes was included for each species (i.e. ~10% and decreasing as sample size increases, Fig. 4-A and Fig. S7-A, Supporting information). Considering the *DiP* within each species, each individual brings on average ~166 new variants (~1% of the total). Second, the large variance observed across gene nucleotide diversity estimates (see Table 3) is mostly due to stochastic evolutionary factors rather than to sampling effects so unlikely to be impacted by sample sizes over 10 gametes (Tajima 1983). Third, sampling sites are located in regions which include 4 out of the 5 main cpDNA lineages reflecting~~along~~ white oaks recolonization routes (lineages A to C and E in Petit *et al.* 2002a), the likely haplotypes carried by the *DiP* individuals being A to C (Table 1).~~, so only the less frequent *D* lineage from South-western Spain might not be represented in our *DiP*.~~

Therefore, if new populations were being sampled within the geographical range considered, they would likely include many of the alleles observed here within species and at other genes across their genomes. For differentiation patterns, older and more recent reports showed a low genetic structure among distant populations within each species, and a relatively stable overall differentiation among species compared to possible variation across geographical regions (Bodénès *et al.* 1997; Mariette *et al.* 2002; Petit *et al.* 2003; Muir and Schlötterer 2005; Derory *et al.* 2010; Guichoux *et al.* 2013; Gerber *et al.* 2014). For new populations sampled outside the *DiP* geographic range, a recent application to *Q. robur* provenances located in the low-latitude range margins of the distribution (where 3 main cpDNA lineages occur) showed a high rate of genotyping success, a high SNP diversity, and outliers potentially involved in abiotic stress response (Temunovic *et al.* 2020). ~~However, more exhaustive sampling would be required to explore whether data from one particular region could be extrapolated to overall genomic patterns across a larger geographical range.~~

We further tested the frequency spectrum representativity of our range-wide *DiP* by comparing genotypic data for a set of 530 independent SNPs (called *sanSNP* for Sanger data) with data for the same set of SNPs obtained in Lepoittevin *et al.* (2015, called the *illuSNP* set since it used the Illumina infinium array technology) for larger numbers of ~70 individuals per species from Southern France natural stands. The SNPs were chosen so that the *illuSNP* set excluded SNPs showing compressed clusters (*i.e.* potential paralogs) and those showing a high number of inconsistencies with control genotypes, as recommended by the authors. Comparing between datasets, for SNPs exclusive to one species in the *sanSNP* set, more than

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68% either show the same pattern in the *illuSNP* set, or one where the alternative allele was at a frequency below 5% in the other species. Less than 8% of those SNPs are common in both species in the *illuSNP* set. Similarly, for singletons in the *sanSNP* set, more than two-third of the corresponding SNPs in the *illuSNP* set showed very low to low frequency (<10%), while only 11% in *Q. petraea* and 9% in *Q. robur* showed a *maf* above 0.25. This further confirms the reality of singletons in our *DiP*, and also that some may represent more frequent polymorphisms in larger samples of local populations. The correlations among *maf* in both datasets were high and significant (0.66 and 0.68 respectively for *Q. petraea* and *Q. robur*, both Pr< 0.0001).

Finally, various methodological steps and obtained results tend to demonstrate that we avoided a bias towards low-diversity genic regions: (i) an initial verification that very low BlastX *E*-values (< 10^{-80}) did not target more conserved regions, (ii) a primer design optimizing the amplification of polymorphic fragments, both (i) and (ii) using potential variants in ESTs data assembled across both species (Fig. S2-B steps 1 and 3; Appendix S1, Supporting information), (iii) a high nucleotide diversity across genes and ~50% of shared variants (Table 3 and Fig. 4), (iv) a very low proportion of fragments with no detected variants, and a substantial part (~30%) of variant positions due to Indels and SSRs (Table 2), (v) additional results showing that, across ~100 kb of more than 150 independent fragments amplifying in one species only and thus with possible more divergent primer pairs, the number of detected heterozygotes was twice smaller compared to fragments amplifying in both species (more details in Appendix S1, Supporting information).

These results altogether suggest a small risk of SNP ascertainment bias if these new resources were to be used in populations both within and/or outside the geographic distribution surveyed, in contrast to panels with much less individuals than here (see respectively Lepoittevin *et al*. 2015 for a discussion on the consequences of such bias in *Quercus* species, and Temunovic *et al*. 2020 cited above).

Overall, we obtained sequence data for 0.072% (~530 kb) of the haploid genome of *Q. robur* (size of ~740 Mb in Kremer *et al*. 2007). We also targeted ~3% of the 25808 gene models described in the oak genome sequencing project (www.oakgenome.fr), and around 1% of the gene space in length. Interestingly, both randomly chosen genic regions and those covering different functional categories have been mapped across all linkage groups (columns F and X in Table S1, Supporting information). Due to the absence of observed background LD, their diversity patterns can be considered independent. The genes studied represent a large number

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of categories, as illustrated by very similar distributions for level 2 GO terms to those obtained with the larger *ocv4* assembly (Lesur *et al.* 2015, comparing their Figure 2 to Fig. S4-A to S4-C, Supporting information).

Diversity magnitude and heterogeneity highlight species integrity and introgression patterns

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Using a detailed polymorphism typology, we characterized for the first time in two oak species a high proportion of variant positions (30%) that included 1 bp to medium-sized indels and sequence repeats, compared to the more common and commonly reported SNP loci (Table 2). The proportions of indels observed (11.5% of all polymorphisms) is in the range of results available in model tree species (e.g. 13.8% across the genome in *Prunus avium*, Shirasawa *et al.* 2017; 19% in *Prunus persica,* Cao *et al.* 2014; a lower estimate of 1.4% in *Populus trichocarpa*, Evans *et al.* 2014). Although less abundant than SNPs, they represent an important component of nucleotide variation, often having high functional impacts when located within coding sequences, and they have been proposed as an easy source of markers for natural populations studies (Väli *et al.* 2008). Larger-sized indels are also likely to be relatively frequent in intergenic regions of the *Quercus* genome and have been linked to transposable elements (TE, see the BAC clones overlapping regions analyses in Plomion *et al.* 2016). Similarly, large indels and copy number variation linked to TE activity were identified as an important component of variation among hybridizing *Populus* species (Pinosio *et al.* 2017). Here when considering variant positions involved in complex polymorphisms, we observed one variant position per 48 bp on average within species (resp. one per 30 bp in both), compared to the one SNP per 68 bp statistic (resp. one SNP per 42 bp across both species). Also, some of the SNPs observed were located within complex polymorphic regions that would have been classically filtered out, and nucleotide diversity (π) estimates were higher by 12% when including all polymorphisms (from 0.0038 to 0.0044 if averaging across both species and all genes, Table 3). These nucleotide diversity estimates are provided for the first time in *Q. petraea* and *Q. robur* across a large number genic regions (> 850), compared to previous candidate genes studies across much smaller numbers (< 10) of gene fragments (Kremer *et al.* 2012 in *Q. petraea*; e.g. Homolka *et al.* 2013).

Based on these data, there is an interest in attempting to estimate SNP numbers across the full genome of the studied species for range-wide samples, as it may impact filtering strategies in pipelines for future NGS haplotype-based data production, or decisions to develop or not SNP arrays in these species. In order to do that, a few realistic assumptions can be made from both the exhaustive description of variants provided, and the mean proportions of SNP numbers in

new individuals that we computed for increasing across sample sizes. First ~10% additional rare SNPs per sample could be observed for a *DiP* twice as large as ours (based on Fig. S7-A data, Supporting information). Thus given the representativity of our data compared to the *ocv4* unigene (Lesur *et al.* 2015), we would expect around 1.36 million SNPs on average within species by applying our statistics to the full genic partition of *Q. robur* or *Q. petraea* (~80 Mb, www.oakgenome.fr, Plomion *et al.* 2018). Another reasonable assumption is that shared and exclusive polymorphisms proportions across genic regions would be around 30% and 70% respectively, for these closely related oak species (based on both our *DiP* and Lepoittevin *et al*. 2015 results), which translates into the presence of ~2.32 million SNPs for the genic partition in a sample including both *Q. petraea* and *Q. robur* (resp. ~4.22 if including also *Q. pubescens* and *Q. pyrenaica*). Finally, if we apply to the *Quercus* genome a range of ratios for SNPs counts in intergenic over genic regions estimated from several tree species natural population samples (2.03 in *Populus trichocarpa*, Zhou and Holliday 2012; 2.25 in the "3P" *Q. robur* reference genotype, Plomion *et al.* 2016; 2.57 in *Prunus persica* wild accessions, Cao *et al.* 2014), we obtain an estimate of between 34 to 42 million SNPs within species across a large spatial range (resp. 41 to 51 million SNPs in both Q. petraea and robur species, and 75 to 94 million SNPs considering the 4 species previously cited). All these figures could be at least 30% higher if one considers all possible variants involved in indels, SSRs and complex polymorphisms, as shown in our results. Although of the same order of magnitude, the contrast with the twice smaller number of SNPs identified in Leroy *et al.* 2019 (~32 millions) across the same ~~4~~ four species with similar sample sizes than ours, could be explained by different factors. First their filtering strategy applied on Pool-seq data in order to minimize errors basically excludes all singletons. However, we have seen that verified singletons which could represent rare or local variants amounted to more than 20% of all polymorphisms (see Results). Indeed, very stringent filters are often applied in practice to limit error rates and avoid false-positives, hence limiting the impact of variable read depth and possible ascertainment bias risks, which altogether significantly decrease the number of informative loci compared to either initial fixed amounts (in genotyping arrays, e.g. Lepoittevin *et al.* 2015) or potential amounts (in reference genomes, e.g. Pina-Martins *et al.* 2019 in *Quercus* species; see also Van Dijk *et al*. 2014). Second, no cross-validation step is available in Leroy *et al.* (2019) for data quality, that would have permitted to have a better grasp of possible bias and error rate expected in such a dataset, and its consequences on allele frequency estimates and inference methods (see Hivert *et al*. 2018 and discussion below). Also, we can't exclude that a regional sampling strategy such as the one used in Leroy *et al.*

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(2019) might miss allelic variants with a higher *maf* in other regions for the two~~2~~ species having the wider geographical range.

Our nucleotide diversity estimates are consistent with those obtained from genome-wide data and range-wide panels in angiosperm tree species, available mostly from the model genus *Populus* (e.g. *P. trichocarpa*: 1 SNP per 52 bp and π~0.003 across genic regions, Zhou and Holliday 2012, Zhou *et al.* 2014, Evans *et al*. 2014, Wang *et al*. 2016; *P. tremula*: π~0.008, P. tremuloides: π~0.009 across genic regions, Wang *et al*. 2016; π ~0.0026 to 0.0045 in a panel including wild *Prunus persica* accessions, Cao *et al.* 2014). These diversity levels are also within the range estimated for the long-term perennial outcrosser category in Chen *et al*. (2017, see Fig. 1-D with a mean value of silent π close to ~0.005) and can be considered relatively high in the plant kingdom if excluding annual outcrosser estimates or intermediate otherwise. In oaks as in many other tree species with similar life history traits, these high levels would be consistent with their longevity, large variance in reproductive success and recolonization or introgression histories, which could have maintained deleterious loads of various origins (Zhang *et al*. 2016, Chen *et al*. 2017, Christe *et al.* 2016b).

Comparing the nucleotide diversity distributions and examining the range of differentiation across genic regions in our *Dip* reveal several robust patterns that altogether illustrate historical introgression among both *Quercus* species. These two species have long been considered as iconic examples of species exhibiting high levels of gene flow (e.g: Petit *et al*. 2003; Arnold 2006), despite more recent evidence of strong reproductive barriers (Abadie *et al.* 2012). What has been referred to as "strong species integration" seems nevertheless clearer in our *Dip* for *Q. robur* than for *Q. petraea*, according to genetic clustering inference without any *a priori*. Three individuals (27%) considered as typical morphological *Q. petraea* adults (Kremer *et al* 2002a) showed significant levels of introgression (Fig. 3). In contrast, only one *Q. robur* based on morphology was introgressed to a level matching the least introgressed *Q. petraea* individual. Discussing species delimitation, Guichoux *et al*. (2013) also showed more robustness in assigning morphological *Q. robur* individuals to their genetic cluster, illustrating an asymmetry in their introgression levels. We note that among our *Dip* individuals, Qs28, one parent from two mapping pedigrees (Bodénès *et al*. 2016) is a clear F1 hybrid among both species (Fig. 3), making those pedigrees two back-crosses instead of one cross within species and one between species.

Moreover, after excluding the four most introgressed individuals, nucleotide diversity in *Q. petraea* was significantly higher (by ~5% on average) than in *Q. robur*. This effect is small,

detectable only with Wilcoxon paired ranked tests, mostly across the same ~200 regions sampled randomly and in the *Abiotic stress* category, despite the very large diversity variance across regions, and robust to excluding the highest diversity values. We also sequentially removed the three individuals with the highest *Q*-values from the *Q. petraea* cluster (Fig. 3), since they could still harbor residual heterozygosity due to recent back-crossing events and generate the pattern observed. Remarkably, the same significant patterns of higher diversity in *Q. petraea* were observed. Therefore, with 8 to 10 gametes in *Q. petraea* instead of 8 to 24 gametes in *Q. robur*, and with twice less natural stands sampled, the nucleotide diversity in *Q. petraea* was still slightly and significantly higher than in *Q. robur* (Pr<0.011 and Pr<0.026, using all polymorphisms or SNPs only respectively). Although the magnitudes of range-wide population structure within both species could differentially affect both species global diversity across our *Dip*, published results show that these are very small with similar values (~1% across SNPs, Guichoux *et al.* 2013).

The main hypotheses proposed so far to explain this difference in extent of diversity between species relate to their disparities in life-history strategies for colonizing new stands and associated predictions (Petit *et al*. 2003, Guichoux *et al*. 2013). The colonization dynamics model and patterns observed also assumes very similar effective population sizes in both species, which is a reasonable assumption due to their shared past history and the strong introgression impact at the genomic level. However, given increasing and recent evidence of pervasive effects of different types of selection across genic regions with high-throughput data (e.g. Zhang *et al.* 2016; Christe *et al.* 2016b in *Populus*; Chen *et al* 2017 for long-term perennials), alternative (and non-exclusive) hypotheses worth considering are ones of a higher genome-wide impact of selective constraints in *Q. robur (*Gillespie 2000; Hahn 2008; Cutter and Payseur 2013; Kern and Hahn 2018; e.g. Grivet *et al.* 2017). Since *Q. robur* is the most pioneering species, it has likely been submitted to very strong environmental pressures at the time of stand establishment. Selection might be efficient, given oak tree reproductive capacities, and affect variation across a large number of genes involved in abiotic and biotic responses. This would be consistent with significantly lower levels of diversity (*He*) in *Q. robur* at SNPs located in genes that were specifically enriched for abiotic stress GO terms (Guichoux *et al*. 2013, see their Table S5). Redoing here the same tests across a larger number of independent SNPs (> 1000), *Q. petraea* systematically showed the same trend of a slightly higher diversity overall, and significantly so only for the *Abiotic stress* category (*Pr*<0.01) and for a similar outlier SNP category (F_{ST} >0.4, mean He>0.15, *Pr*<0.001) than in Guichoux

766 *et al*. (2013). In summary, the absence of the same pattern in any other functional categories
767 might suggest that these are too broad in terms of corresponding biological pathways, hence
768 mixing possible selection signals of opposite effects among species, while we still detect an
769 overall effect due to linked selection on a random set of genes, and on genes involved in
770 abiotic stress.

771 Within both species, no differences in nucleotide diversity, and a very small differentiation
772 (below 1.5%) were found on average across genes among the main cpDNA lineages (B *versus*
773 A or C) that indicate past refugial areas and migration routes. These patterns were expected,
774 given oaks' life history traits (e.g. high fecundity and dispersal rates), large population sizes,
775 and plausible recolonization scenarios throughout Europe leading to current adaptive
776 differentiation among populations at both nuclear genes and traits (Kremer *et al*. 2010). Only
777 cpDNA ancient differentiation signals among isolated historical refugia were retained, while
778 other putative adaptive divergence effects due to different environments were erased, as
779 illustrated by an absence of correlations between cpDNA and nuclear or phenotypic traits
780 divergence across populations (Kremer *et al*. 2002b). This is consistent with many events of
781 population admixture during the last ~6000 thousands years after European regions were
782 recolonized, as well as a very low genetic differentiation among distant populations (e.g.
783 Guichoux *et al*. 2013), which contrasts with a much higher differentiation often observed for
784 adaptive traits (e.g. Kremer *et al*. 2014; Sáenz-Romero *et al*. 2017). Interestingly, the very
785 few genes with mean F_{ST} between 0.21 and 0.56 among lineages are not the same in *Q.*
786 *petraea* and *Q. robur* (five and seven genes respectively). Seven of them have GO terms
787 indicating their likely expression in chloroplasts, or their interaction with chloroplastic
788 functions. They are either housekeeping genes for basic cellular functions, or belong to biotic
789 or abiotic stress functions (seven of them), and could be involved in local adaptation between
790 ecologically distant populations, calling for further research in larger samples.

791 More generally, analyses comparing the nucleotide diversity patterns at genes involved in
792 both species relevant biosynthesis pathways for ecological preferences (e.g. Porth *et al*. 2005;
793 Le Provost *et al*. 2012, 2016) are clearly needed in replicated populations, for example to
794 estimate the distribution and direction of selection effects and putative fitness impact across
795 polymorphic sites (Stoletzki and EyreWalker 2011), or to study the interplay between
796 different types of selection and variation in local recombination rates on both diversity and
797 differentiation patterns (Payseur and Rieseberg 2016).

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A large proportion of shared polymorphic sites (~50% in any species) highlights the close proximity of species at the genomic level, consistently with a low mean differentiation across polymorphic sites (F_{ST}~0.13, Fig. 4-C), and despite the very large heterogeneity observed across differentiation estimates. This has now been classically interpreted (and modeled) as reflecting a strong variance in migration and introgression rates, in oaks in particular (Leroy *et al*. 2017), with islands of differentiation assumed to represent regions resistant to introgression. However, interpretations of such patterns remain controversial and multiple processes might be involved and worth exploring further in oaks, such as the effects of heterogeneous selection (both positive and background) at linked loci (Cruickshank and Hahn 2014; Wolf and Ellegren 2017). These effects could be particularly visible in low-recombination regions (Ortiz-Barrientos *et al*. 2016), and would further interact with the mutational and recombination landscapes during the course of speciation (Ortiz-Barrientos and James 2017) and during their complex demographic history.

Applications and usefulness as reference data

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During this project, several studies valued part of these resources, hence illustrating their usefulness. For example, good quality homologous sequences were also obtained for ~50 % of the gene fragments in one individual of *Quercus ilex*. This species is relatively distant genetically to both *Q. petraea* and *Q. robur*, belonging to a different section, so these data guided the choice of nuclear genes for better inferring phylogenetic relationships across 108 oak species (Hubert *et al.* 2014). Bioinformatics tools and candidate genes annotated during the project were also useful to similar genes and SNP discovery approach in *Quercus* or more distant *Fagaceae* species (Rellstab *et al*. 2016, Lalagüe *et al.* 2014 in *Fagus sylvatica*, El Mujtar *et al*. 2014 in *Nothofagus* species). Given the low ascertainment bias and good conversion rate expected within the range surveyed, those genomic resources would be directly applicable to landscape genomics studies at various spatial scales (reviewed in Fetter *et al.* 2017) in both *Quercus* species. Indeed, easy filtering on provided SNP statistics in the catalog would allow distinguishing among different classes of SNPs (e.g. exclusive to each species, common and shared by both, linked to particular GO functional categories), delimiting and tracing species in parentage analyses and conservation studies (e.g. Guichoux *et al*. 2013; Blanc-Jolivet *et al.* 2015), or improving estimates of lifetime reproductive success and aiming to understand how demographic history and ecological drivers of selection affect spatial patterns of diversity or isolating barriers (Andrew *et al*. 2013; e.g. Geraldes *et al.* 2014). This type of spatial studies are surprisingly rare in these oak species, they usually

include a small number of SSR markers, and all suggest complexity in geographical patterns of genetic variation and importance of the ecological context (e.g Neophytou *et al*. 2010; Lagache *et al*. 2014; Klein *et al*. 2017, Beatty *et al.* 2016 for local or regional studies; Muir and Schlötterer 2005; Gerber *et al*. 2014, Porth *et al.* 2016 for range-wide studies). Their power and scope would likely be greatly improved by using medium-scale genotyping dataset including a few thousands SNPs such as those described in our study.

The robust patterns described above of differentiation heterogeneity and consistent differences in diversity magnitude among species call for more studies at both spatial and genomic scales for unraveling these species evolutionary history, in particular regarding the timing, tempo, dynamics and genetic basis of divergence and introgression. Practically, in order to address those questions in oaks, genomic data on larger samples of individuals could be obtained from either genome complexity reduction methods such as RAD-seq and similar approaches (e.g. ~~Elshire *et al.* 2011~~Andrews *et al.* 2016) or previously developed SNP arrays (e.g. Silva-Junior-*et al.* 2015). ~~might be fairly limiting for the research questions mentioned above (Arnold *et al.* 2013; Henning *et al.* 2014; Zhou and Holliday 2012), especially given the large variance in nucleotide diversity and low overall differentiation characterized here.~~ We ~~therefore~~ do not recommend the development of a very large SNP array in oaks since it is likely to be very costly for the actual~~a minimal~~ return, especially given the very large and range-wide panel that would be needed to significantly limit ascertainment bias (see Lepoittevin *et al*. 2015). The very low overall levels of LD observed here indicate also potentially high recombination rates, and thus that a very high SNP density would be required for targeting functional variants, which would not be compatible with technical constraints for controlling for genotyping error rates (previously shown to be high in SNP array). Indeed, these rates would probably be stronger for high diversity, complex, duplicate or multiple copy genic regions (as those observed in this study in Tables S1 and S4, Supporting information, and shown recently to have an evolutionary impact on the *Q. robur* genome structure, Plomion *et al*. 2018), preventing these regions to be included in SNP arrays. The very short LD blocks observed in this study might also limit the utility of RADseq data alone to uncover many loci potentially under selection in genome scans for local adaptation studies (Lowry *et al.* 2016; McKinney *et al.* 2017). In contrast, targeted sequence capture (TSC) strategies for resequencing (Jones and Good 2016), and the more recent advances in RADseq approaches that deal with previous limitations (Arnold *et al*. 2013; Henning *et al*. 2014; and see Rochette *et al.* 2019), although still uncommon in forest tree species evolutionary studies, might be

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more useful and efficient since they can be oriented towards recovering long genomic fragments. ~~, and t~~They would thus allow more powerful site frequency spectrum and haplotype-based inferences to be pursued, therefore avoiding most of the SNP arrays technical issues (e.g. Zhou *et al.* 2014; Wang *et al*. 2016), ~~while at the same time avoiding most of the above technical issues.~~ especially given the large variance in nucleotide diversity and low overall differentiation characterized here. TSC approaches will surely be encouraged and tailored to specific evolutionary research questions in oaks in the next decade, given the new *Q. robur* genome sequence availability (Plomion *et al*. 2018; Lesur *et al*. 2018 for the first TSC in oaks). However, the bioinformatics pipelines needed for validating haplotype-based or quality data for population genetics inferences also need constant reassessment according to research questions and chosen technology.

We thus propose, in addition to direct applications to landscape genetics (detailed above) and transferability to other *Quercus* species (for example using primer information in Table S1, Supporting information, and see Chen *et al*. 2016), that the high-quality data characterized in this study serve as a reference for such validation purposes. They could not only help for adjusting parameters in pipelines for data outputs, but also allow estimating genotyping error rates for SNP and more complex classes of variants, either by comparing general patterns (e.g *maf* distribution from Tables S3, S4 Supporting information) or using the same control individuals maintained in common garden that could be included in larger-scale studies. Such a reference catalog of SNPs and other types of polymorphisms within gene fragments could also be very useful for solid cross-validation of variants identification, allele frequency and other derived summary statistics in alternative strategies such as *Pool-Seq*, which allow increasing genomic coverage while sampling cost-effectively by pooling individuals (Schlötterer *et al.* 2014). Indeed, the drawback of *Pool-Seq* approaches, despite dedicated software (PoPoolation2, Kofler *et al*. 2011) is that they can give strongly biaised estimates, or ones that do not consider evolutionary sampling (Hivert *et al*. 2018). Therefore, they require further validation methods which usually value previously developed high-quality and lower-scale data (e.g. Pool-Seq *versus* Sanger and Rad-Seq in Christe *et al*. 2016b; Illumina GA2 *versus* Sanger in Cao *et al*. 2014; EUChip60K *versus* deep-whole genome resequencing in Silva-Junior *et al.* 2015). Finally such a reference dataset would help optimizing the amount of data recovery from either TSC or whole-genome resequencing experiments in future research challenges by fine-tuning dedicated data processing bioinformatics pipelines.

Data Accessibility

The original assembly used for selecting contigs is in Appendix S2 (Supporting information). For Sanger trace files (with data on at least 2 individuals), see the Dryad repository (at the https://doi.org/10.5061/dryad.4mw6m906j link ~~will be available once data have been curated, and the reviewer URL is https://datadryad.org/stash/share/klvEAfXP-GQytODunTk1m1g1BHc7HtTdETj7SlN-OfY~~). Consensus sequences are respectively in appendices S3 (used to design primers and for functional annotation, see also Table S2), S4 (genomic sequences obtained), and S5 (genomic sequences obtained for *Q. ilex*). Tables S1 and S2 correct and extend the oak Candidate Genes Database of the Quercus Portal (www.evoltree.eu/index.php/e-recources/databases/candidate-genes). SNP, indel and SSR catalogs and positions within genomic consensus sequences, and ready-to-use format for genotyping essays are provided in Tables S3 to S5 (Supporting information), and at https://github.com/garniergere/Reference.Db.SNPs.Quercus with additional information.

Bioperl scripts from the SeqQual pipeline are given at https://github.com/garniergere/SeqQual, example of parameter files and scripts for STRUCTURE analyses and parsing MREPS software are given at https://github.com/garniergere/Reference.Db.SNPs.Quercus

Acknowledgments

The authors thank Alexis Ducousso, Jean-Marc Louvet, Guy Roussel, Pablo Goicoecha, Hervé le Bouler, Félix Gugerli, Csaba Matyas, Sandor Bordacs, Hans P. Koelewijn, Joukje Buiteveld, Stephen Cavers, Bernd Degen and Jutta Buschbom for choosing trees and providing dried leaves of individuals from various Intensive Study Populations of previous European projects populations. We are grateful to H. Lalagüe, G. Vendramin, I. Scotti, and L. Brousseau for testing earlier scripts of SeqQual and to I. Lesur for help in using the *ocv4* oak resources. The sequencing work was funded by the EVOLTREE network of Excellence (EU contract n°016322). TL post-doc fellowship was funded by the ANR TRANSBIODIV (06-BDIV-003-04) and LINKTREE (contract n°2008-966). TD salary was funded by the ANR REALTIME (N°59000256). Computing facilities of the Mésocentre de calcul Intensif Aquitain des Universités de Bordeaux, de Pau et des Pays de l'Adour are thanked for providing computer time for this study. We also thank Rémy Petit for funding part of TL fellowship and support in developing SeqQual tools. PA received a Ph.D. grant (2009-2011) from the « Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche » of France, and additional funding from EVOLTREE. We thank Oliver Brendel,

Ricardo Alia, Komlan Avia and Hilke Schröder for reviewing the manuscript and for their constructive comments.

Conflict of interest disclosure

The authors of this article declare that they have no financial conflict of interest with the content of this article.

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Author contributions

Funding acquisition: AK, PGG, CP, and MLDL; Initial conception and individuals sampling: PGG, AK, CP, MPR, VL; Bioinformatics strategy and experimental design: PGG, TL; DNA extraction and quality check: VL; Sequence Data acquisition: PGG, CP, TL, VL; Individuals' identification checks for quality control VL, CL, PL; Pilot study: VL, PGG; Working assembly: JMF, PGG: Primer design and amplicon choice: PGG, VL, TD; Original candidate gene lists choice: PGG, TL, JMF, CP, AK, TD, CR, MLDL, GLP, ChB, EG, CaB, NT, PA; Bioinformatics tools: TL and PGG (SeqQual pipeline and R scripts), JMF and AF (Bioperl and R scripts), PA, CL, VelM**,** JT**,** FH, TD (SeqQual tests), FR (website); Visual Chromatogram checks, SNP/assembly validations: PGG, VL, TD, PA, TL, MLDL, CaB, ChB, CL, CR and EG; Bioinformatic and population genetic analyses: PGG, TL, SM, ChB: Functional annotation: TL, PGG, VelM, PA; Manuscript draft: PGG; Manuscript review and edition: PGG, SM, CL, ChB, TL; all authors agreed on the manuscript.

Supporting Information

Fig. S1 Sampling site locations within the natural geographic distribution of *Q. petraea* and *Q. robur*. Vector map is from *http://www.naturalearthdata.com* and distribution areas from Euforgen (*http://www.euforgen.org/distribution-maps/)*

Fig. S2 Working assembly steps and softwares (A), and bioinformatic strategy for search of candidate genes and amplicon choice (B).

Fig. S3 Plots of the ΔK values from the Evanno *et al*. (2005) method (S3-A, -B, -C, -D, -E), and of the mean values of the estimated probability ln (of the data given K) with standard deviations for K ranging from 1 to 5 (S3-F to S3-J), which show support for K=2. Plots are from the STRUCTURE HARVESTER program.

Fig. S4 Distributions of Gene Ontology (GO) terms for the consensus sequences in Appendix S3, at level 2 (-A, -B, -C) and level 3 (-D, -E, -F): A- and D- for Biological Process, B- and E- for Molecular Function, C- and F- for Cellular Component. Annotation rules: E-value<10^{-30}, annotation cut-off 70, GO weight 5, HSP coverage cutoff 33%. Filtering applies for at least 5 sequences and a node score of 5 per GO term (but see rare exceptions in Table S2).

Fig. S5 Distributions of GO terms across different gene lists (*bud*, *abiotic* and *biotic*) at Biological Level 2, and Fisher exact tests across pairs of sequence clusters with the same GO terms between the random list and other lists. Significance levels *: P<0.05.

Fig. S6-A to S6-J Posterior assignment probabilities (*Q*-values) of 24 individuals attributed to 2 clusters (STRUCTURE analysis) for different numbers of polymorphisms, different sampling of SNP data, and different plots of credible intervals.

Fig. S7 Mean number of new variants brought by each new distinct individual added to all possible initial sample size combinations (**-A**); Number of high-quality variant positions per 100 base pair (bp) across 852 gene fragments ranked by their length (bp), overall and for each species (**-B**).

Fig. S8 Comparison of nucleotide diversity (*theta.pi*) distributions between main cpDNA lineages (B and A or C) for *Q. robur* (586 genes) and *Q. petraea* (449 genes). The histogram represents lineage B for *Q. robur*. Data are available in both lineages within each species for at least 8 gametes per lineage, and a minimum of 200 bp per gene fragment.

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Table S1 Description of amplicons: primer sequences, original candidate gene list, targeted biological functions (see references), candidate gene type, fragment expected size and position in the *orict* original working assembly, preliminary results based nucleotide quality for obtained sequences, and validation decision after excluding paralog amplification.

Table S2 Functional annotation results from Blast2GO (-A), comparison of BlastX best hits results (according to *E-values*) between consensus sequences of the *orict* working assembly and the *ocv4* assembly (-B), and comparisons of BlastN results of consensus sequence for both *orict* and corresponding expected amplicon (*orict-cut)* onto *ocv4* (-C).

Table S3 Description of all variants single base positions, with sample sizes, alleles, genotypes counts, various statistics, and generic format for genotyping essays input data. Species samples exclude the 2 most introgressed individuals.

Table S4 Description of all polymorphisms as in Table S3, but with a characterization of the length, sequence motifs, contiguous base positions for complex polymorphic regions including indels, SNPs and SSRs (see also Table S5 for SSR positions).

Table S5 SSR patterns as detected from the *mreps* software.

Appendix S1 Additional method details.

Appendix S2 Contigs of the original working assembly used for selecting candidate gene regions and design amplicon primers, including consensus sequences and reads where nucleotides with Phred score below 20 have been masked.

Appendix S3 Sequences of chosen contig consensus and singletons sequences for functional annotation analyses.

Appendix S4 Consensus sequences of 852 genomic regions obtained in this study for *Quercus petraea* and *Q. Robur* individuals. "$(N)^{9}$" : represents a low-quality fragment of a length below ~1 kb separating Forward and Reverse amplicons; "n" represents positions with a majority of nucleotides with phd score below 30. "$(-)^{x}$": means that the insertion is a minor allele at that position, x being the size of the indel.

Appendix S5 Nucleotide sequence data of 394 gene regions for one *Quercus ilex* individual, heterozygote sites being indicated by IUPAC codes.

Appendix S6 Outputs from Blast2GO analyses**.**

High-quality SNPs from genic regions highlight introgression patterns among European white oaks (*Quercus petraea* and *Q. robur*).

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Appendix S1 - Supporting Methods

Original assembly description (see also Figure S2, supporting information)

Sequences from the 14 cDNA libraries were obtained from various tissues and developmental stages (bud, leaf, root and wood-forming tissues) from a total of 146 individuals identified as belonging to both species, and that were sampled in 3 different French regions (South-West, North-East and North-West). These sequences were thus likely to target a large range of expressed genes. We performed the first working assembly for those sequences, with the main aim of avoiding paralog assembly while limiting split contigs with overlapping homolog sequences (Figure S2, supporting information)~~Briefly~~, we initially pre-processed all sequences by removing low-quality EST, keeping those with a PHRED score above 20 (PHRED software, Ewing *et al.* 1998) for at least 90% of base pairs (bp) within a minimum of 100 bp. Vector-related sequences were trimmed or masked using Cross_match (www.phrap.org/phredphrapconsed.html) and BLAST analyses (Altschul *et al.* 1990, 1997) against the UniVec database (https://www/ncbi.nlm.nih.gov/tools/vecscreen/univec/). The ~90 000 sequences so obtained were assembled with the STACK_PACK pipeline (Miller *et al.* 1999) with the aim of avoiding the assembly of paralogs while at the same time limiting split contigs belonging to homolog sequences. The 3 main steps followed were 1) the "loose" clustering with the d2_cluster program (Burke *et al.* 1999), 2) the contig assembly within clusters with Phrap (www.phrap.org/phredphrapconsed.html) and 3) the final alignment and consensus sequence generation using STACK_Analysis and CRAW that accounts for alternative splicing variation (Burke *et al.* 1998). An iterative PHRAP step was also used for the largest contigs (including one or two orders of magnitude more reads than the average

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contig size), splitting contigs when quality of alignments was poor in low-depth regions to avoid possible paralog assembly. The final assembly includes 13477 contigs and 74 singletons is given in Appendix S2 with nucleotides having a Phred scored below 20 being masked with "?" using the SeqQual pipeline (https://github.com/derfR/SeqQual/). The libraries used in this assembly have since been named A, B, F to O, and S, and were included in larger transcriptome resources for *Quercus* species (Ueno *et al*. 2010).

Choice of fragments for re-sequencing ~~BlastX and BLAST2GO analyses~~

Expressional and functional candidate genes information was compiled for targeting those potentially involved in white oaks' divergence and/or local adaptation (Fig. S2-B and Table S1, Supporting information). Briefly, model species databases were searched for gene accessions by gene ontology (GO) and metabolic pathways keywords. Those sequences were first Blasted (Altschul et al. 1990, 1997) against our working oak assembly. Second, the sequences from their best hits were extracted (see filtering criteria in Fig. S2-B, Supporting information) and re-Blasted against the non-redundant protein (NR) database at NCBI. Third, their annotation was compared to those of the initial gene accessions, allowing 95% of hits from the oak assembly to be validated (step 2 in Fig. S2-B, Supporting information). Expressional candidate genes sequences from bud tissues or stress treatment libraries and a random set of ESTs were also directly sampled across the oak assembly generated above (see Table S1, column F, Supporting information). Primers were designed with the OSP software (Hillier and Green 1991) by setting up homogenous melting temperatures constraints and excluding low-complexity propositions. We also checked that they were located preferentially in the 3' ends of large contigs, but in regions where putative variants were absent compared to contiguous regions that could include variants (see Step 3 in Fig. S2-B, Supporting information, and primers provided in columns V and W of Table S1, Supporting information). At this stage, we also wanted to avoid targeting more conserved genes *a priori*. Thus we visually examined, among pre-selected contigs from the oak assembly, the ~100 providing BlastX results with lowest E-values (below $<10^{-80}$), in order to compare their putative polymorphisms patterns with another set of contigs with higher E-values (~ 10^{-30}, see Fig. S2-B, supporting information)~~-~~. We verified that the lowest E-values contigs did not correspond to those with the lowest numbers of polymorphisms, or with an absence of putative polymorphisms. Predicted amplicons were Blasted against each other and onto our assembly to exclude those with potential amplification problems and multiband patterns. They were

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also checked for their depth and presence of putative polymorphisms in contigs alignment, yielding finally 2000 amplicons for resequencing (Fig. S2-B, Supporting information).

Preliminary analyses of the 1968 amplicons (Fig. 1-A)

Overall, more than 85% of the designed amplicons were successful in both individuals, one from each species. We tested whether fragments amplified only in one individual or the other (called fragments A) were more polymorphic overall than those amplifying in both individuals (called fragments B): using a minimum Phred score of 30 (i.e. error rate below 0.001), we extracted fragments with a minimum length of 200 bp and maximum mean proportion of missing data of 50%, as computed across overlapping windows by 50% across fragments. Among these, a similar amount of fragments A were found in both individuals (158 in *Q. robur* 11P individual versus 155 in *Q. petraea* Qs21 individual). One SNP (or heterozygote here) per 654 bp was observed on average across the ~99 kb amplified in Q. robur/11P only, compared to one SNP per 340 bp (across ~ 500 kb) using data on the same individual for fragments B. For the Q. petraea/Qs21 individual, the same statistics are respectively: one SNP per 926 bp across ~98 kb, and one per 342 bp across ~500 kb). Filtering more strongly on quality with a maximum proportion of missing data of 25% slightly increased the number of fragments which can be considered as being amplified only for one individual or the other, but the same trend remains (although with more similar values in Q. robur/11P and Q. petraea/Qs21) of around twice less heterozygote at SNPs compared to fragments B with similar quality.

Although it is difficult to conclude on the basis of one individual per species, there is no evidence that fragments A are more polymorphic than fragments B. We also need to be prudent since with the Sanger technique used here, the quality filtering may also have masked some heterozygote indels in diploid sequences (see the part *Treatment of diploid sequences…* below for the full data obtained), and thus also subsequent parts in the fragments, yielding stretches of low-quality positions due to the frame shift of the second strand, which might also harbor polymorphic sites. Indeed, 24% of fragments A had proportion of missing data above 25%, compared to 9% for fragments B, indicating an overall lesser apparent quality and thus the possibility that some polymorphisms may have been missed. However, the same treatment was used for Fragments A and B here, and thus polymorphic sites may have been missed also in fragments B. Overall, we can consider that given the strategy followed for choosing the fragments and designing the primers, given the preliminary results above on the 1968 amplicons, and given the results showing a large nucleotide diversity overall in these species

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(see Results of the main text), there is no strong evidence that we targeted genic regions that were particularly conserved.

Functional annotation of re-sequenced genic regions using BlastX and BLAST2GO analyses

BlastX search (using BlastX 2.6.0+ program at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was finally performed on 396 sequences from *orict* (our working assembly original contigs) and 368 sequences from the most recent oak assembly *ocv4* (Lesur *et al*. 2015, and see Table S2-A,-B,-C and Appendix S3, Supporting information, for contig consensus sequences), based on their annotation consistency, BlastN and BlastX lowest *E-values* and highest % of identity, consensus length (below 6 kb) and a minimum number of IUPAC ambiguity codes (below 50) scattered across sequences. We excluded ~60 *ocv4* consensus sequences (~8%) with stretches of such codes (from around 50 to 430 bp) that indicate possible paralogs or alternative spliced exons in the *ocv4* assembly (Lesur *et al*. 2015, and see column P "*nb.pol.2c"* in Table S2-B, Supporting information).

For Blast2GO analyses, based on the studied genes hits similarity distribution (Appendix S6-B, Supporting information), we used 2 cut-off values: 55% for similarity and 33 (~100 bp) for high scoring segment pair (HSP). This allowed retrieving Genbank identifiers and corresponding Gene Ontology (GO) terms, which were mainly from the UniProtKB and TAIR databases. In order to examine the relevance of the original gene lists in targeting broad functional traits (column F in Table S1, Supporting information), we tested whether they contained an enrichment of particular GO terms in comparison to the randomly chosen contigs, using GO data across all sequences in each gene list.

Treatment of diploid sequences obtained in the discovery panel with SeqQual for polymorphism discovery

Sequence data of amplicons from the same original contig were assembled together and consensus sequences were obtained with the Phred/Phrap (www.phrap.org) suite of programs called by SeqQual. Ambiguous codes not detected as valid polymorphisms by Polyphred (https://droogs.gs.washington.edu/polyphred/) were considered as missing data and masked. Overall, the time needed to call polymorphisms with SeqQual scripts and to validate by visual examination the traces or alignments in amplicons identified with possible problems was much smaller (by a factor of at least 50) than the time needed to correct data in BIOEDIT (Hall 1999) or CodonCode Aligner (CodonCode Corporation, www.codoncode.com/aligner/ and see parameter examples at

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https://github.com/garniergere/SeqQual/tree/master/SeqQual_shell_ex). Using print_source-SNP-statistic.pl script output for diploid sequences (https://github.com/garniergere/SeqQual/tree/master/SeqQual_pdf/SeqQual-part3-fastools-usage.pdf), we could easily point to amplicons with simple insertion-deletion polymorphisms (*inde*ls), more complex *indel* patterns that included simple sequence repeats (*SSRs*), and rare or outlying patterns such as heterozygote excess or deficit (see Results). Visual examination then allowed alignments in those complex regions to be corrected if needed. From mismatch cases that were revealed automatically when merging forward and reverse amplicons, we confirmed that more than 99% of identified heterozygotes were correct with chosen Polyphred parameter values (60 and 90 for threshold overall and genotype scores respectively), and so their absence in one of the alternative strand was due to a clear but too weak second peak for a positive heterozygote call. Also, cases of heterozygote excess that mostly showed double peaks (*DoP*) were considered as paralog amplifications and excluded (column L in Table S1, Supporting information and Fig. 1-D). Additionally for each diploid sequence with a clear automatic heterozygote indel (*HI*) pattern (i.e. a trace with mostly single peaks becoming clear DoP after a particular position and the presence of at least one homozygote individual for the deletion at that position, or with *DoP* patterns that were consistent with a particular deletion), we coded the heterozygotes at the first position with corresponding IUPAC codes (http://www.chem.qmul.ac.uk/iubmb/misc/naseq.html), followed by missing data. This allowed minimizing the amount of missing data produced by superimposed allele traces. In several cases, a second *HI* further along the sequence allowed getting back a clear open reading frame and diploid sequence information. The putative rare variants in some individuals that we missed would be located in those DNA stretches assigned to missing data due to overlapping traces after such *HI* recoded positions. These *HI* positions, because there were coded and characterized in the lists provided, homopolymers excepted for thus allow more accurate diversity estimates (Tables S3 and S4, supporting information).

Treatment of homopolymers in diploid sequences ~~amplicons~~

Homopolymers, mostly from 8 to 10 A or T repeats depending on the amplicons, often stopped the correct functioning of the polymerase during sequencing after their positions and were observed in 104 amplicons (Tables S1 and S5, supporting information, for their description, position and presence across genes), so heterozygote indels in those regions were generally masked before polymorphism counts.

STRUCTURE analyses and runs

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We used STRUCTURE v2.3.3 (Pritchard *et al*. 2000, Falush *et al.* 2003) to infer genetic clusters and test for possible levels of introgression across individuals. Following recommended defaults, we used the admixture model allowing for mixed ancestry and the correlated allele frequencies assumption for closely related populations. We first drew one polymorphic locus at random per genic region and simulated 10 replicates for each value of K clusters (1 to 5) with burn-in and post- burn-in periods of 100,000 and 1,000,000 iterations respectively. Due to very low standard deviation across replicates of the data log likelihood given K (ln Pr(X/K)), we further tested the robustness of the results to genetic stochasticity by resampling loci at random for each of 10 replicate datasets in 3 different manners: 1) one per region, 2) one per 100 bp block, and 3) one per 200 bp block along genes. The block sizes were chosen to sample more loci while keeping low levels of background linkage disequilibrium (LD) *a priori*, given the already high number of independent gene regions (>800), and given that STRUCTURE permits the inclusion of weakly linked markers (Falush *et al.* 2003). Examples of STRUCTURE data and parameter files are archived as recommended by Gilbert *et al.* (2012) along with R scripts for plots including Bayesian confidence intervals in the K=2 case (https://github.com/garniergere/Reference.Db.SNPs.Quercus/tree/master/STRUCTURE.files). Missing data were below 20% across loci, at least 12 gametes were present in the original morphological species, and an arbitrary *maf* of at least 9% across polymorphisms allowed singletons to be excluded. We examined both ln(Pr(X/K)) and ΔK (Evanno *et al*. 2005) statistics using STRUCTURE HARVESTER (Earl and von Holdt 2012).

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