

Massive postglacial gene flow between European white oaks uncovered genes underlying species barriers

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Summary

- Oaks are dominant forest tree species widely distributed across the Northern Hemisphere, where they constitute natural resources of economic, ecological, social and historical value. Hybridisation and adaptive introgression have long been thought to be major drivers of their ecological success. Therefore, the maintenance of species barriers remains a key question, given the extent of interspecific gene flow.
- In this study, we made use of the tremendous genetic variation among four European white oak species (31 million single nucleotide polymorphisms (SNPs)) to infer the evolutionary history of these species, study patterns of genetic differentiation and identify reproductive barriers.
- We first analysed the ecological and historical relationships among these species and inferred a long-term strict isolation followed by a recent and extensive postglacial contact using approximate Bayesian computation. Assuming this demographic scenario, we then performed backward simulations to generate the expected distributions of differentiation under neutrality to scan their genomes for reproductive barriers. We finally identified important intrinsic and ecological functions driving the reproductive isolation.
- We discussed the importance of identifying the genetic basis for the ecological preferences between these oak species and its implications for the renewal of European forests under global warming.

Introduction

Oaks are a diverse group of *c.* 350–500 species widely distributed throughout the Northern Hemisphere (Hubert *et al.*, 2014; Denk *et al.*, 2017). The variability in the number of recorded oak species highlights the challenge of delineating species limits within a genus displaying a high degree of morphological diversity, sometimes described as a ‘botanic horror’ by taxonomists (Darwin, 1859; Palmer, 1948; Rieseberg *et al.*, 2006; Leroy *et al.*, 2020). Genetic markers have corroborated these taxonomic concerns, particularly in European white oaks, which have been the subject of a large number of genetic surveys. Studies based on nuclear DNA markers have reported unambiguously high levels of admixture between European white oak species, confirming the reported taxonomic issues for oaks (Lepais *et al.*, 2009). Several detailed empirical studies based on chloroplast DNA markers have revealed an absence of private chlorotypes between European white oak species, but congruent associations between chlorotypes and expansion routes during the last postglacial

recolonisation, suggesting cytoplasmic capture via recurrent hybridisation and backcrossing (Petit *et al.*, 1997, 2002). Recent advances in oak genomics (Plomion *et al.*, 2016, 2018) have made it possible to investigate interspecific gene flow at the whole-genome scale. Therefore, Leroy *et al.* (2017) have provided evidence suggesting that extensive secondary contacts have occurred between four European white oak species, probably at the start of the current interglacial period. These results reconcile earlier findings of contrasting species differentiation at the nuclear and organelle levels. Indeed, secondary contacts explain present-day patterns of species differentiation, including complete sharing of haplotypes in mixed oak stands (Petit *et al.*, 2002) and the partial maintenance of nuclear genetic divergence at some loci (e.g. Scotti-Saintagne *et al.*, 2004). The inferences drawn are also consistent with the persistence of genomic regions impermeable to gene flow due to reproductive barriers, corresponding to a typical case of semi-isolated species (Leroy *et al.*, 2017). However, the genetic basis of these barriers remains unknown.

Controlled pollination trials have provided empirical evidence for the existence of strong reproductive barriers in these four European white oak species (Abadie *et al.*, 2012; Lepais *et al.*, 2013). Ecological preferences *in situ* have also been previously reported, with tolerance to dry (*Q. petraea*) or wet (*Q. robur*) sites (Eaton *et al.*, 2016), or acidic (*Q. pyrenaica*) or limy (*Q. pubescens*) soils (Timbal & Aussenac, 1996) but fine-grained ecological surveys do not yet exist for all these four species. The four species occupy different geographic ranges (Fig. 1a): extending up to Scandinavia for *Q. petraea* and *Q. robur*, whereas the other two species are present mostly in Mediterranean and sub-Mediterranean regions. However, the distribution ranges of these species overlap in some areas, mostly in south-west France, but the four species are rarely found together in the same stand (but see Lepais *et al.*, 2009). The overlapping species ranges in south-west France therefore provide an ideal 'natural laboratory' (Hewitt, 1988) for investigating reproductive barriers between these European white oak species.

Here we combined state-of-the-art methods in population genomics to explore the genomic patterns of species differentiation (Fig. 2): (1) we used approximate Bayesian computation (ABC) to perform ascertainment bias-free demographic inferences in order to refine estimates of the timing of secondary contacts; and (2) scanned genomes for reproductive barriers. Our findings identified important intrinsic and ecological functions driving the reproductive isolation of these four oak species including tolerance to biotic and abiotic constraints, and intrinsic mating barriers.

Materials and Methods

Ecological niche of the four species

French data We delineated the extant ecological niche of the four oak species in France (Fig. 1b, Supporting Information Figs S1, S2) by using their distribution maps based on the

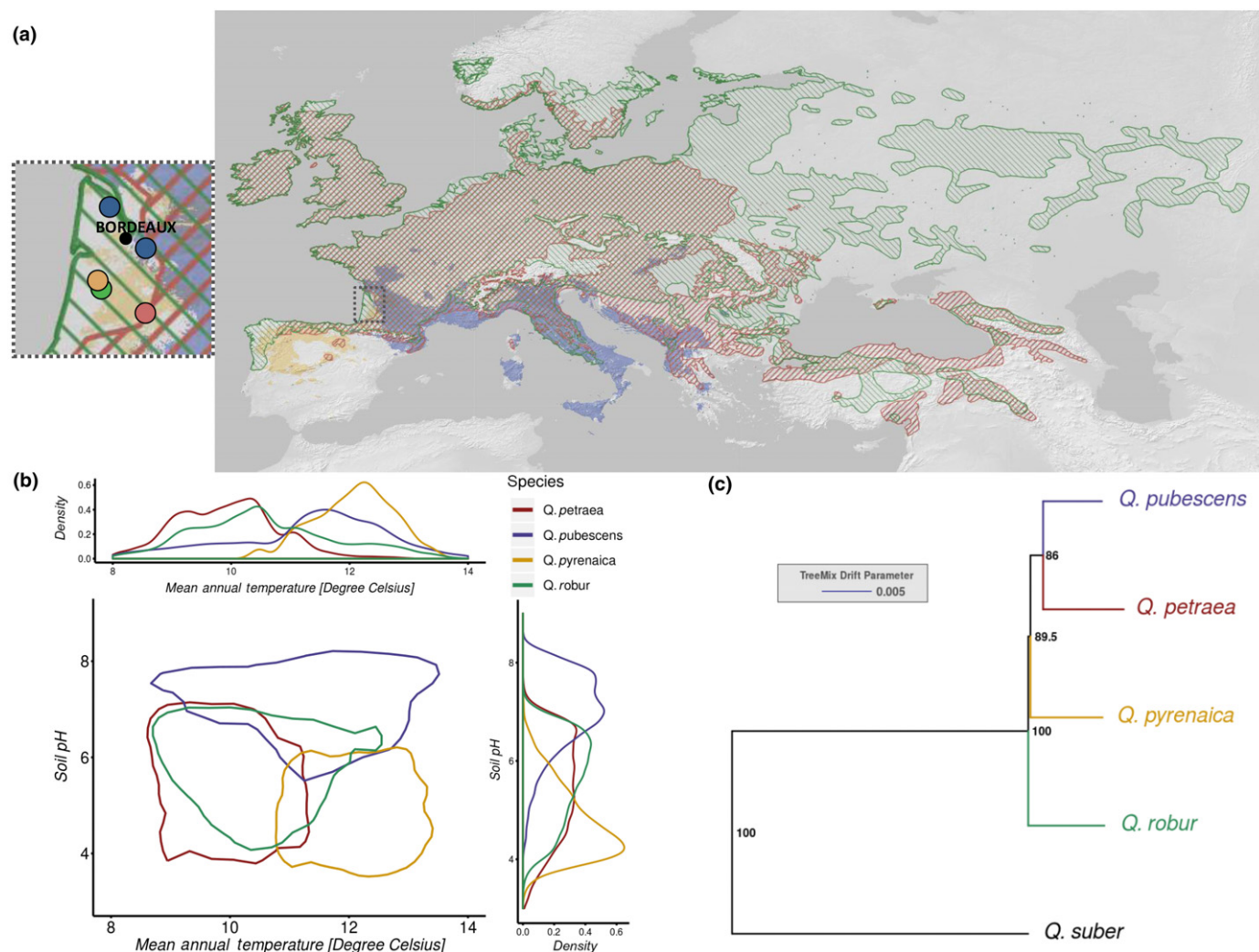


Fig. 1 Continental-scale species distributions and origin of the study material (a) and, ecological (b) and phylogenetic relationships of the four European white oak species under investigation using TreeMix (c). (b) The central plot shows the contour of the two-dimensional density between the soil pH and the mean annual temperature enclosing 95% of the ecological data values, based on the French data only. The above and right charts show the one-dimensional density curves for mean annual temperature and soil pH, respectively.

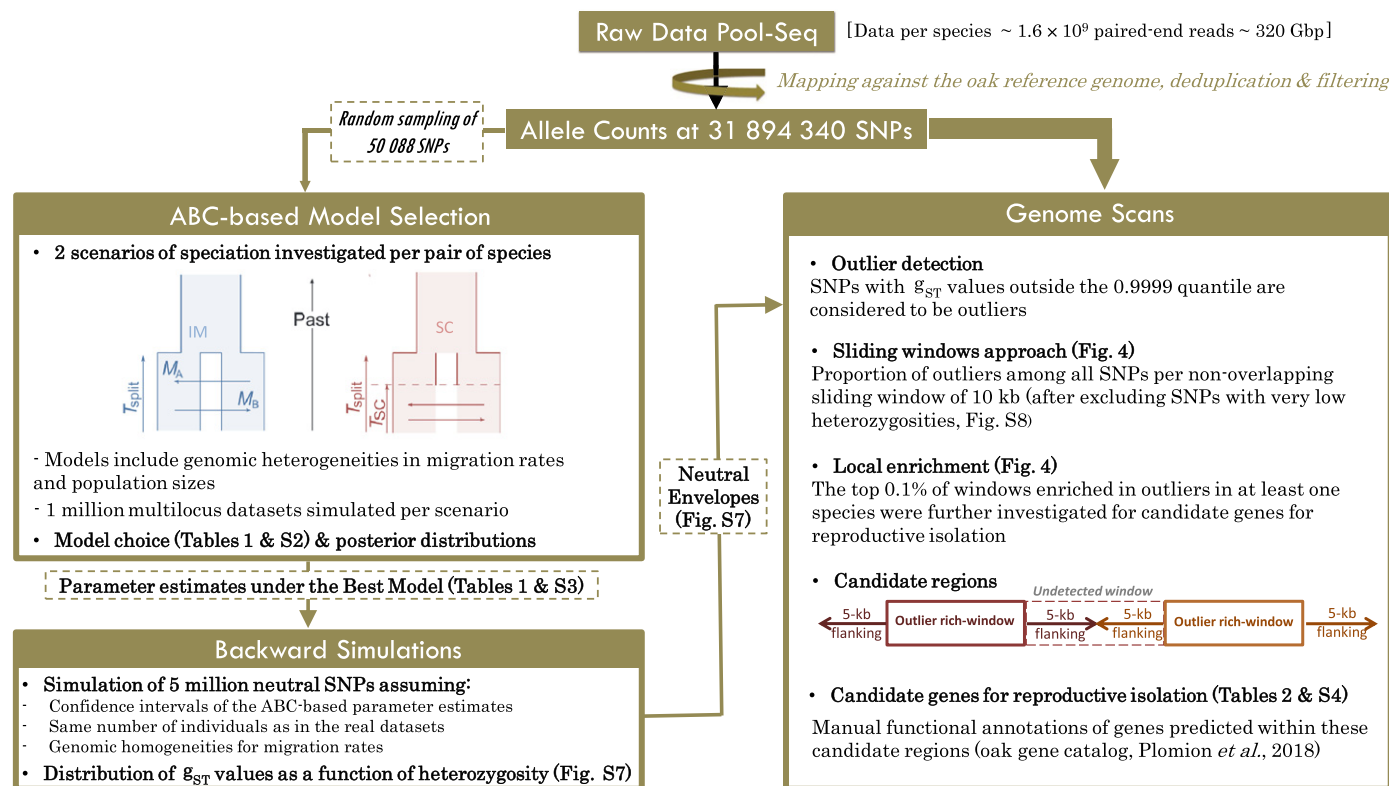


Fig. 2 Workflow used to identify genes contributing to reproductive isolation between four European white oak species. A subset of 50 000 of the called single nucleotide polymorphisms (SNPs) was selected at random and used for model selection under an ABC framework and the generation of parameter estimates under the best model. Large neutral datasets were then simulated to create null envelopes for the identification of SNPs displaying significant departure from expectations under neutrality. We searched for candidate genes in regions enriched in outliers.

National Forest Inventory (Fig. S3) and climatic data extracted from the Chelsa database (Karger *et al.*, 2017). In addition to the climatic data we added pH values of the soil. Proxies of pH values were derived from National Forest Inventory floristic plots installed since 2005. Floristic composition of these inventory plots was compared with existing database (comprising floristic and physical data) to calculate proxies of pH values (Gégout *et al.*, 2005). We intersected the distribution maps with the climatic rasters (30-inch resolution) and using the R package 'GGPLOT2' v.2.2.1 (Wickham, 2009) calculated a 2D density plot of species presence in the ecological space as defined by climate (mean temperature) and soil pH.

European data European distributions maps were constructed based on the presence data of species made available by the European Forest Genetic Resources Programme (EUFORGEN, for *Q. robur* and *Q. petraea*, de Vries *et al.*, 2015) and the European atlas of forest tree species (JCR, San-Miguel-Ayán *et al.*, 2016, for *Q. pyrenaica* and *Q. pubescens*). Climatic data are based on the Chelsa database (Karger *et al.*, 2017) and soil pH were derived from the European atlas of forest tree species data (JCR, San-Miguel-Ayán *et al.*, 2016). As these data were collected from different sites, we computed univariate (rather than bivariate) density distributions using GGPLOT2, using a procedure similar to that used for the French data.

Sampling and sequencing

We sampled populations of the four *Quercus* species in stands of natural origin located in south-west France. We sampled 13 *Q. petraea* trees from Laveyron (Landes, France), and 20 *Q. robur* and 20 *Q. pyrenaica* trees from the Landes EVOLTREE 'Intensive Study Site' (ISS), 18 *Q. pubescens* trees from two sites in Gironde: 12 in Branne and six in Blagnan (Gironde, France) (see Table S1 for details). Samples of reference species populations came from the same geographic region. Such sampling strategy does not bias species comparison, as shown by an earlier methodological study showing that species differentiation is only moderately impacted by the geographical origin of populations (Bodénès *et al.*, 1997).

DNA was extracted from individual trees using the Invisorb Spin Plant Mini kit, according to the manufacturer's specifications (Startec Molecular, GmbH, Berlin, Germany). DNA yields were evaluated using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and DNA samples were mixed in equimolar amounts to obtain a single pool for each species.

In this study, we sequenced libraries of pooled DNA samples. Such a pool-seq approach is indeed a cost-effective and time-effective alternative to individual sequencing. The main advantages include the reduction in the total sequencing effort and the costs

for the preparation of the genomic libraries (e.g. Gautier *et al.*, 2013; Schlötterer *et al.*, 2014). If pool-seq data are expected to generate efficient allele frequency estimates under various experimental designs (Gautier *et al.*, 2013), a pool-seq strategy has also some limitations, especially the difficulty to reconstruct haplotype blocks and estimate linkage disequilibrium, at least for short reads, as used in this study. To meet the requirement of independence in some analyses (see the ABC section below), we randomly selected a low proportion of single nucleotide polymorphisms (SNPs) (one SNP every 15 kb, on average), a far larger physical distance than the level of linkage disequilibrium generally assumed for oaks (e.g. < 500 bp for the *Q. mongolica* var. *crispula* oak, Kremer *et al.*, 2012). For each pool, a paired-end DNA genomic library was generated with a Paired-End DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). The library was then sequenced on a HiSeq 2000 sequencer (Illumina) with 2×100 paired-end reads. For each pool, we used nine or 10 sequencing lanes.

Raw reads were trimmed to remove low-quality bases (< 20) from the ends, and sequences between the second unknown nucleotide and the end of the reads were removed. Reads < 30 nucleotides long after trimming were discarded.

Overall, between 1617 465 418 and 1813 403 677 reads per pool were retained for analysis, corresponding to 313 Gb ($425 \times$) to 356 Gb ($483 \times$) of raw data. Raw data were deposited in the Sequence Read Archive (SRA): PRJEB23847.

Mapping and calling

All reads were then mapped against the v.2.3 oak haplome assembly (Plomion *et al.*, 2018), using BOWTIE2 v.2.1.0 (Langmead & Salzberg, 2012), using standard parameters for the 'sensitive end-to-end' mode. PCR duplicates were removed using PICARD v.1.106 (<http://broadinstitute.github.io/picard/>). SAMTOOLS v.1.1 (Li *et al.*, 2009) and POPOOLATION2 v. 1.201 (Kofler *et al.*, 2011) were then used to call biallelic SNPs with at least 10 copies of the alternate allele and a depth between $50 \times$ and $2000 \times$ at each position. To ensure a reasonably low rate of false positives due to Illumina sequencing errors, all SNPs with a MAF < 0.02 were discarded. We obtained allele counts for 31 894 340 SNPs. F_{ST} in nonoverlapping sliding window was calculated from allele frequencies with the POPOOLATION2 BIOINFORMATICS software suite (Kofler *et al.*, 2011).

Approximate Bayesian computation analysis

Observed dataset For all subsequent ABC analyses, we randomly selected 50 088 of the 31 million SNPs. For each of these SNPs, we multiplied allele frequencies by the number of set of chromosomes in each pool (26 for *Q. petraea*, 36 for *Q. pubescens* and 40 for both *Q. pyrenaica* and *Q. robur*) to generate the corresponding number of a specific allele in each pool. At this stage, we assumed that any departure from equimolarity due to bias in the mixing of DNA samples would have a negligible effect on our summary statistics calculated for a set of 50 thousand SNPs. Seventeen summary statistics were computed using MSCALC: (1) the number of polymorphic sites specific to each gene pool; (2)

the number of polymorphic sites existing in both gene pools; (3) nucleotide diversity (π) for each gene pool; and, between gene pools, the mean value and standard variation for (4) gross divergence (D_{xy}), (5) net divergence (D_a); (6) F_{ST} ; and (7) Pearson's R^2 correlation coefficient in π (see also Roux *et al.*, 2013, 2016; Leroy *et al.*, 2017). Demographic inferences based on summary statistics of the site frequency spectrum (SFS) are known to be robust to many sources of variation including the number of individuals and loci (Fraïsse *et al.*, 2018). In addition, SFS-based inferences from pool-seq data have been shown to be robust (Christe *et al.*, 2017).

Demographic scenarios We used an ABC procedure similar to that described by Leroy *et al.* (2017). Briefly, we compared two different scenarios: isolation with migration (IM) and secondary contact (SC). Both scenarios assumed the subdivision of an ancestral panmictic population (PopAnc) into two daughter populations (Pop1 and Pop2) at time T_{SPLIT} , with population sizes remaining constant over time (N_{PopAnc} , N_{Pop1} , N_{Pop2}). The IM model assumed uninterrupted gene flow since T_{SPLIT} . The SC model assumed that populations initially evolved in strict isolation, with secondary gene flow beginning at some time before the present (at time T_{SC} , Leroy *et al.*, 2017).

Coalescent simulations For coalescent simulations, we adapted the pipeline described by Leroy *et al.* (2017) for the calculation of summary statistics to large datasets (up to 100 000 SNPs). This pipeline included a modified version of the random prior generator PRIORGEN (Roux *et al.*, 2013, 2016; Leroy *et al.*, 2017), and the MSNSAM (Hudson, 2002; Ross-Ibarra *et al.*, 2008) and MSCALC (Ross-Ibarra *et al.*, 2008; Roux *et al.*, 2016) programs. For each of the two scenarios (IM and SC), 1000 000 random multilocus simulations were performed. Both models made use of genomic heterogeneity in effective migration rates (M) and population sizes (N_e) to take into account the occurrence of genomic barriers to gene flow and the confounding effect of linked selection. For both sources of heterogeneity, we used the strategy described by Leroy *et al.* (2017).

Model selection The 500 best replicate simulations closest to the observed values were selected, and the posterior probabilities for each of the two scenarios (IM or SC) were estimated with a feed-forward neural network, by nonlinear multilocus regression (Leroy *et al.*, 2017 for details). ABC computations were performed with 20 feed-forward trained neural and eight hidden networks.

Parameter estimation Posterior distributions of the parameters were estimated with a two-step hierarchical procedure. We first evaluated the parameters under the best model, to check the consistency of our estimates, particularly concerning our previous support for very recent secondary contacts (Leroy *et al.*, 2017). We then ran an additional set of 1000 000 coalescent simulations under an SC model, assuming that T_{SC} occurred in the last 5% of the divergence time, using a modified version of PRIORGEN. This strategy was used to obtain more precise parameter estimates. For

both rounds of estimation, we used a logit transformation of the parameters on the 500 best simulations providing the smallest Euclidean distance. The posterior probability of parameters was then estimated by the neural network procedure, from the means of weighted nonlinear multivariate regressions of the parameters on the summary statistics for 25 feed-forward trained neural and 10 hidden networks.

Genome scans

Coalescent simulations for outlier detection For each pair of species, we ran 5000 000 coalescent simulations, using parameter values sampled from the 95% confidence interval (CI) of the posterior distribution of all parameters for the pair considered. The simulations assumed genomic homogeneity for effective migration rates (M1 and M2) but heterogeneity for population size. Random values were generated with a modified version of PRIORGEN. For each simulated locus, we then calculated H_e and G_{ST} with a custom-developed script. All scripts and datasets are publicly available for download from a GitHub repository (<https://github.com/ThibaultLeroyFr/GenomeScansByABC>). Extreme quantiles of the distribution of G_{ST} (99.99% of simulated values) relative to the expected heterozygosity of the locus were then calculated, with a strategy similar to that used for Fdist (Beaumont & Nichols, 1996). More specifically, a null envelope was computed from G_{ST} quantiles, with heterozygosity intervals of 2%. This strategy has the advantage of providing a null distribution of G_{ST} under much more complex scenarios (i.e. the best inferred demographic scenario) than other state-of-the-art genome-scan methods.

For each of the 31 million SNPs, we computed the same summary statistics as for simulated neutral markers. The observed G_{ST} values conditioned by heterozygosity at the locus were compared with the previously generated null envelope. Markers with a G_{ST} value above this envelope were considered to be outliers. We then used a nonoverlapping sliding windows approach to estimate the proportion of outliers per 10 kb window. Windows containing < 10 SNPs were discarded.

We generated parameter estimates under the best-fitting SC model for each pair of species. Taking into account the 95% confidence intervals for each $T_{SC} : T_{SPLIT}$ ratio (Tables 1, S2) and

the divergence time between these species (1–10 Myr; Hubert *et al.*, 2014; Hipp *et al.*, 2018), the analysis yielded quite large estimates with SC occurring between 100 and 62 400 yr ago, corresponding to up to 1225 generations, assuming a generation time of 50 yr (Gregorius *et al.*, 2007).

Functional annotations

For the 227 genes found within regions enriched in outliers or in close vicinity to these regions (5 kb on both sides to exclude border effects, see ‘candidate regions’ in Fig. 2), we conducted BLASTP searches in both the SwissProt and nr protein databases. Only BLASTP results with e-values < 10e-5 were considered for protein function annotation. After identification of the protein function by BLASTP analyses, functional annotations were performed using extensive manual literature searches rather than using automatic approaches based on gene ontology (GO)-oriented methods to ensure high-quality gene annotations. We also reported information from a previous identification of orthologous and paralogous genes in 16 plant species, including *Q. robur*, performed with OrthoMCL (Plomion *et al.*, 2018 for details).

Results

Ecological preferences of the four species

We intersected the distribution maps of the four species (Fig. S3) with climatic and soil data derived from a large-scale floristic survey in France. Bivariate density distributions (Fig. 1b) show clear patterns of ecological preferences among the four white oak species. As expected, the two so-called temperate white oaks (*Q. petraea* and *Q. robur*) were more frequently observed under cooler climates than Mediterranean and sub-Mediterranean species (*Q. pubescens* and *Q. pyrenaica*). Mean annual temperatures of the areas occupied by the two latter species extended up to 20°C, while the two former species occupied much cooler climates (mean temperature below 15°C). pH of the soils segregated particularly *Q. pyrenaica* from *Q. pubescens*. The modal value of the former species was close to 5, while the mode of the latter was *c.* 7. Although we could not combine climatic and soil

Table 1 Posterior probabilities of the SC scenario and timing of secondary contacts.

Pair	Post. probability SC	T_{SC}/T_{SPLIT} estimates	T_{SC} (years ago)
<i>Quercus robur</i> – <i>Q. petraea</i>	0.98883 (± 0.01089)	1.487×10^{-3} [0.43 – 4.05] $\times 10^{-3}$	14 870 [4300–40 500]
<i>Q. robur</i> – <i>Q. pyrenaica</i>	0.99249 (± 0.01152)	1.197×10^{-3} [0.31 – 4.95] $\times 10^{-3}$	11 970 [3100–40 500]
<i>Q. robur</i> – <i>Q. pubescens</i>	0.98719 (± 0.01342)	0.245×10^{-3} [0.09 – 0.72] $\times 10^{-3}$	2450 [900–7200]
<i>Q. pubescens</i> – <i>Q. petraea</i>	0.98549 (± 0.02231)	2.176×10^{-3} [0.77 – 6.24] $\times 10^{-3}$	21 760 [7700–62 400]
<i>Q. pubescens</i> – <i>Q. pyrenaica</i>	0.99087 (± 0.01152)	0.865×10^{-3} [0.32 – 2.16] $\times 10^{-3}$	8650 [3200–21 600]
<i>Q. pyrenaica</i> – <i>Q. petraea</i>	0.99378 (± 0.00697)	0.383×10^{-3} [0.10 – 1.14] $\times 10^{-3}$	3830 [1000–11 400]

Mean (bold) relative posterior probability of the secondary contact scenario and standard deviation (round brackets). Median (bold) and 95% confidence intervals (square brackets) for both the inferred ratio between divergence time (T_{SPLIT}) and time of the secondary contact (T_{SC}) and the secondary contact (expressed in number of years) after setting T_{SPLIT} to 10 Myr (the upper bound for the divergence of this species complex; Hipp *et al.*, 2018). More details are given in Supporting Information Tables S2, S3.

data over the whole species' ranges, univariate density distributions for both climate (Fig. S1) and soil pH (Fig. S2) based on continental-scale data showed similar trends.

Divergence and postglacial secondary contact between European white oaks

In total, 31 894 340 SNPs were identified after the filtering of variants with a low minor allele frequency ($MAF < 0.02$) in population samples for the four species (*Q. petraea*, *Q. robur*, *Q. pubescens*, *Q. pyrenaica*), corresponding to one SNP every 23.2 bp, on average. We also used genome-wide data for a *Q. suber* accession described by Leroy *et al.* (2017) to root a phylogenetic tree and investigate relationships between species for 9 084 835 of the 31.9 million SNPs. The best maximum-likelihood tree suggested that *Q. robur* initially diverged from the ancestor of the other three species (Fig. 1c).

We then randomly selected 50 088 SNPs from the entire set of 31.9 million SNPs for ascertainment bias-free demographic ABC inference. We compared two models of divergence with gene flow (Fig. 2) for each of the six possible species pairs: an IM model assuming constant gene flow since the divergence time (T_{SPLIT}), and a model assuming SC with gene flow starting at T_{SC} , a time point after divergence ($T_{SC} < T_{SPLIT}$). For all pairs, we obtained strong statistical support for the SC model ($> 98\%$ posterior probability; Tables 1, S3; Fig. S4), consistent with our previous findings based on individual data for 3524 SNPs (Leroy *et al.*, 2017).

Interspecific genetic differentiation Based on F_{ST} values calculated over 10-kb genome segments, the genetic differentiation between species pairs differed considerably between chromosomes (Fig. S5), and, more interestingly, between segments within chromosomes (Fig. 3):

(1) With rare exceptions, the three most strongly differentiated species pairs, for all oak chromosomes, were *Q. robur*–*Q. pubescens*, *Q. robur*–*Q. petraea* and *Q. pyrenaica*–*Q. petraea*. Lower levels of differentiation were observed for *Q. pubescens*–*Q. pyrenaica* and *Q. pubescens*–*Q. petraea* (Fig. S5a). These results suggested that the phylogenetic history of these species shaped the genomic landscape of differentiation such that the ranking of pairs of species according to mean F_{ST} values is conserved over chromosomes. Conversely, regardless of the pair of species considered, the interchromosomal variation of mean F_{ST} values was considerable (Fig. S5b), with significantly higher mean F_{ST} values for chromosomes 2 and 6 and significantly lower values for chromosome 4. For all pairs of species, the relationship between mean F_{ST} and the rate of recombination was significant at the chromosome scale ($P < 0.05$; Fig. S6), for calculations based on the comparison of the genetic length of each of the 12 linkage groups (Bodénès *et al.*, 2016) and the physical size of the corresponding pseudochromosomes (Plomion *et al.*, 2018), therefore suggesting that the chromosomal recombination rate is a good predictor of the mean chromosomal F_{ST} .

(2) Genome-wide patterns of F_{ST} variation plotted over the entire genome with 10-kb sliding windows (Fig. 3) revealed a

highly heterogeneous differentiation landscape, even for the pair of species displaying the lowest range of differentiation according to F_{ST} values (*Q. pubescens*–*Q. robur*). Indeed 10-kb F_{ST} estimates between *Q. pubescens* and *Q. robur* ranged from 0 to 0.765. For the pairs of species including *Q. petraea*, the values for the window corresponding to the highest level of differentiation were 0.998 for *Q. petraea*–*Q. pyrenaica*, 0.999 for *Q. petraea*–*Q. robur* and 1 for *Q. petraea*–*Q. pubescens*. On closer inspection, very narrow regions of very high F_{ST} were identified on several chromosomes, for most pairs of species (Fig. 3; chromosomes 1, 7, 9 and 11, for example).

Narrow regions of nonneutral evolution

We then took advantage of these demographic inferences to perform differentiation outlier tests. We performed extensive backward simulations (5000 000 independent SNPs) under the best inferred scenario to generate null distributions for each pairwise comparison (Fig. S7; see also Notes S1). The most outlier-enriched windows were retained for the identification of candidate genes underlying species barriers (after excluding SNPs with very low heterozygosities; Figs 2, S8, S9). We identified 281 windows containing the highest proportion of outliers (top 0.1% of window enriched in outliers for at least one pair of species). We then analysed the clustering of these outlier-enriched windows. We defined a candidate genomic region by merging close windows, for example two contiguous sequences of two outlier-enriched windows, with possible interruption by a single undetected window (Fig. 2). The 281 windows were distributed over 215 candidate genomic regions, distributed over all chromosomes (blue lines, Fig. 4).

We listed all the *Quercus* genes located within or flanking these 215 genomic regions. We identified 227 genes distributed over 133 of the 215 regions, with very few candidates per region (mean: 1.71 ± 1.76 genes per region, 1.49 ± 0.82 genes after excluding five regions with chloroplast-like DNA signatures; see also Notes S2). On all these genes, we performed extensive literature searches to generate manually curated gene annotations. Albeit nonexhaustive by definition, manual literature searches represent more than ever a relevant alternative to methods based on automatically extracted information from literature. Improving the accuracy and traceability of the gene annotations is especially important in oaks since genetic-engineering methods, such as forward and reverse genetic approaches, are not yet available for oaks and gene functions cannot be fully validated based on their phenotypic impacts.

In the following sections, we discuss three major functional categories on the basis of their known implications for ecological and intrinsic reproductive isolation (Dataset S1 for all information). These three functional categories contained at least 32 candidate genes (Table 2) among the 227 detected genes (Table S4; Dataset S1 for details). The first category is comprised of genes underlying the ecological preferences of the four species: tolerance of water deficit, cold tolerance and adaptation to alkaline soils. The second category included genes involved in biotic interactions such as immune responses, resistance to biotic stresses

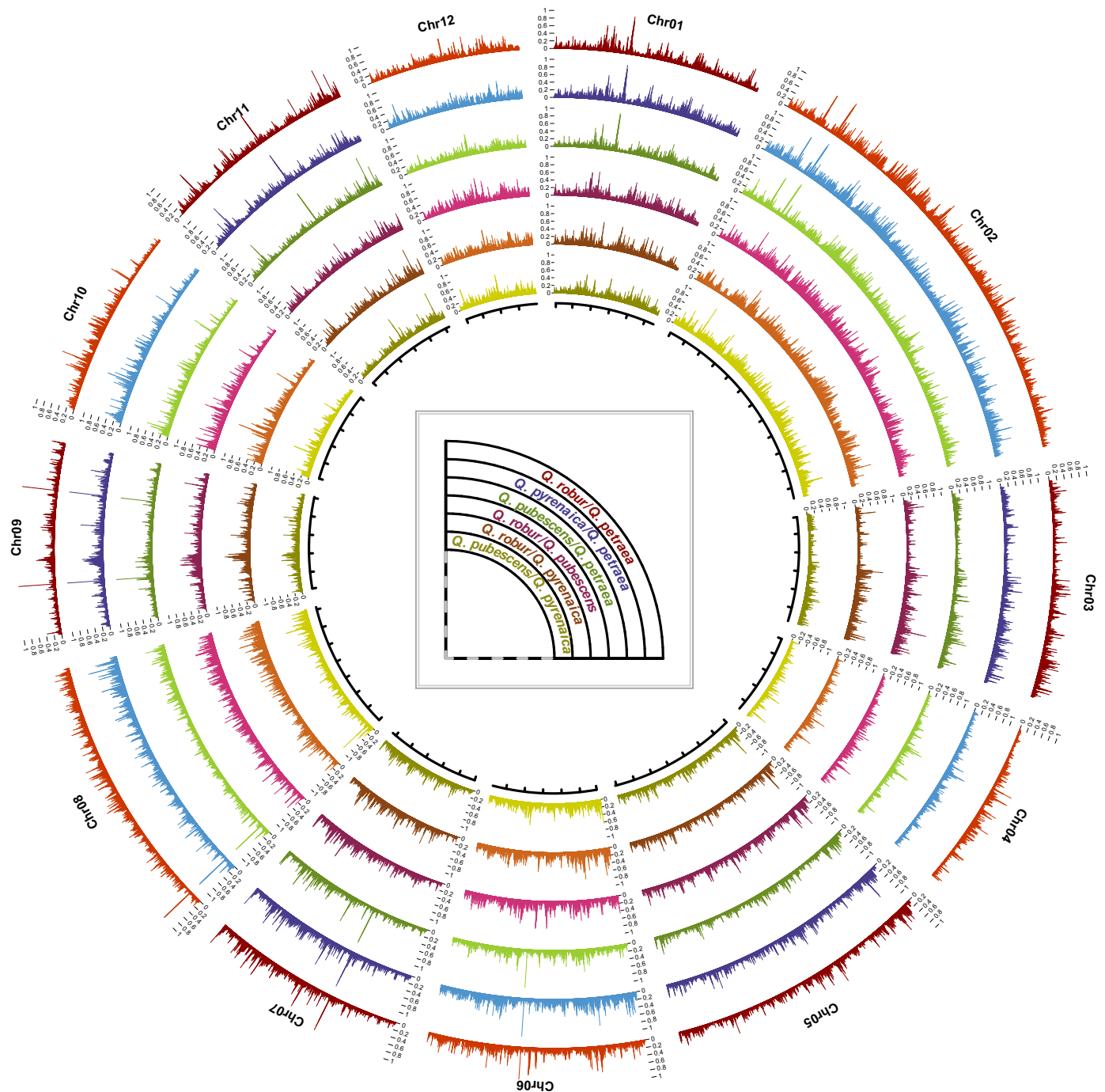


Fig. 3 Circular representation of F_{ST} values along the 12 oak chromosomes. F_{ST} was calculated from allele frequencies, using nonoverlapping 10 kb sliding windows (detailed patterns are accessible from: <https://github.com/ThibaultLeroyFr/GenomeScansByABC/>).

and mycorrhisation. The third category gathered genes probably involved in intrinsic barriers, and includes genes with functions related to flowering time, pollen recognition, pollen growth and embryo development.

Species-specific ecological and nonecological reproductive barriers

Unlike studies aimed at interpreting every region enriched in outliers, our objective was rather to focus on genes displaying

distinct patterns among pairs of species. This situation is especially important as these patterns are unexpected to arise via background selection (see Notes S3 for details). After excluding genes with a 'shared' pattern (see Table 2), several different cases were observed (Table S5): (1) nine regions enriched in outliers for all but one pair of species (including seven regions for all pairs except *Q. robur*–*Q. petraea* and two regions for all pairs except *Q. pubescens*–*Q. pyrenaica* pairs); (2) five regions specific to all pairs sharing the same species (four for *Q. pyrenaica* and one for *Q. pubescens*); and (3) 11 regions with more complex patterns.

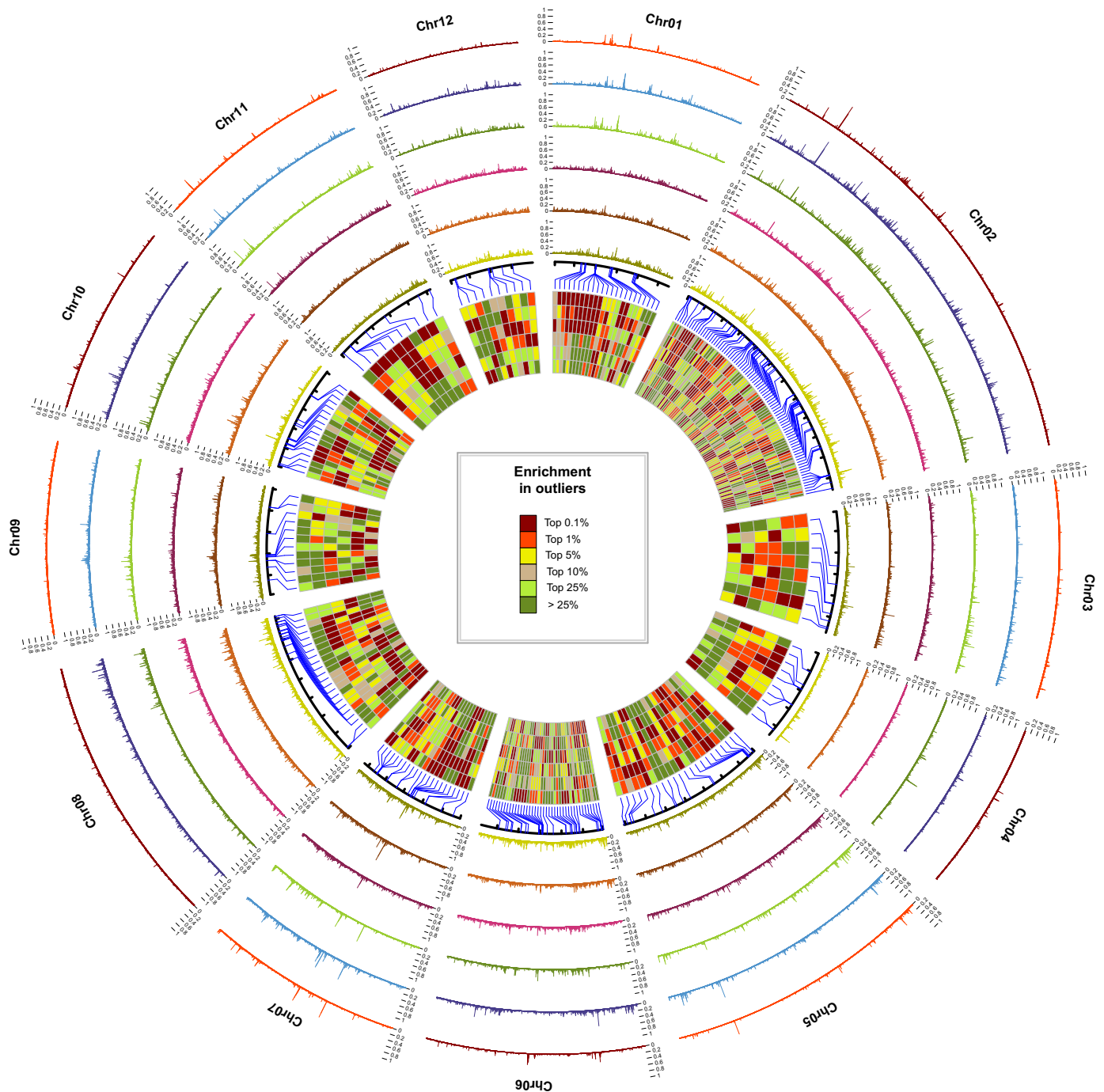


Fig. 4 Local density in outliers per nonoverlapping 10 kb sliding window. From outside to inside, the species pairs are *Quercus. robur*–*Q. petraea*, *Q. pyrenaica*–*Q. petraea*, *Q. pubescens*–*Q. petraea*, *Q. robur*–*Q. pubescens*, *Q. robur*–*Q. pyrenaica* and *Q. pubescens*–*Q. pyrenaica*. An outlier corresponds to a single nucleotide polymorphism (SNP) with a level of differentiation exceeding the expectations assuming the inferred evolutionary history (0.9999 quantile; see Fig. 2 for details). Detailed patterns are accessible from: <https://github.com/ThibaultLeroyFr/GenomeScansByABC/>. Each rectangle in the inner circle represents the level of enrichment in outliers with $H_e \leq 0.2$ for each pair of species at a given position in the genome, assuming the same order of pairs. These rectangles correspond to the 281 most outlier-enriched windows found in at least one of the six pairs (top 0.1%).

Among the nine regions with an ‘all-versus-one-pair’ relationship, seven excluded the *Q. robur*–*Q. petraea* pair and the other two excluded the *Q. pubescens*–*Q. pyrenaica* pair. Four of the seven candidate genes for which a pattern ‘all except *Q. robur*–*Q. petraea* pair’ was observed are known to be involved in

drought tolerance or in lateral root growth (Table 2). This pattern was consistent with the higher drought tolerance of *Q. pyrenaica* and *Q. pubescens* compared with *Q. petraea* or *Q. robur* (Fonti *et al.*, 2013). Reciprocally, we observed an ‘all versus one’ pattern (undetected for the *Q. robur*–*Q. petraea* pair)

Table 2 Genomic positions, gene names, annotations for 32 selected candidate genes for intrinsic and ecological functions driving reproductive isolation.

Chr.	Positions (regions)	Candidate gene	Gene name	Gene functions	#Paralogues	Pattern
Intrinsic barriers						
Flowering						
Chr08	62493250–62513250	Qrob_P0412860.2	Two-component response regulator-like PRR73	Photoperiodic flowering response, circadian clock	1	Complex
Chr08	62673250–62693250	Qrob_P0749650.2	Floral homeotic protein APETALA 2 Transcr. factor RAP2-7	Delay transition to flowering time, biotic/abiotic stresses	0	Complex
Chr07	44592072–44632072	Qrob_P0088630.2	Transcr. activator DEMETER (DME)/protein ROS1-like	Transcriptional activator required for floral development	1	Complex
Pollen development (Cycloartenol synthases)						
Chr06	28660694–28680694	Qrob_P0684330.2	Cycloartenol synthase 2	Sterol or triterpenoid synthesis, pollen development	13	Complex
Chr06	28690694–28710694	Qrob_P0684400.2	Cycloartenol synthase 2	Sterol or triterpenoid synthesis, pollen development	13	Shared
Chr06	28770694–28810694	Qrob_P0684360.2	Cycloartenol synthase 2	Sterol or triterpenoid synthesis, pollen development	13	Shared
Pollen recognition and seed germination (G-type lectin S-receptor-like serine/threonine kinase genes)						
Chr03	26118565–26138565	Qrob_P0538230.2	G-type lectin S-receptor-like STK At2 g19130	Putatively involved in recognition of pollen	0	Complex
Chr05	4001785–4021785	Qrob_P0641650.2	G-type lectin S-receptor-like STK At1 g11300	Putatively involved in recognition of pollen	190	Complex
Chr06	1453464–1473464	Qrob_P0430150.2	G-type lectin S-receptor-like STK LECRK	Regulates expression of immunity genes and seed germination	36	Q. pyrenaica-specific
Chr06	1513464–1563464	Qrob_P0430190.2	G-type lectin S-receptor-like STK LECRK	Regulates expression of immunity genes and seed germination	36	Q. pyrenaica-specific
Chr08	1749735–1769735	Qrob_P0138480.2	G-type lectin S-receptor-like STK LECRK	Regulates expression of immunity genes and seed germination	36	Q. pyrenaica-specific
Embryo development and organogenesis						
Chr12	30514806–30534806	Qrob_P0436820.2	Transcriptional corepressor LEUNIG-like isoform	Leaf, flower (gynoecium) and embryo development	5	All except: Q. pub/Q. pyr
Chr02	31611918–31651918	Qrob_P0297580.2	CHD3-type chromatin-remodelling factor PICKLE	Repressor of LEC1, activator of embryo development	0	Complex
Chr06	43006752–43026752	Qrob_P0309300.2	Probable N-acetyltransferase HLS1-like	Auxin-responsive gene expression, shoot organogenesis	1	All except: Q. rob/Q. pet
Chr08	49805801–49825801	Qrob_P0248780.2	Receptor-like protein 12 (RLP12)	Meristem maintenance control, organogenesis	0	Complex
Photoreceptor and UV-B tolerance						
Chr02	37788250–37808250	Qrob_P0338870.2	Ultraviolet-B receptor UVR8	Photoreceptor/response to UV, circadian clock, stomata	2	Shared
Chr03	34667252–34687252	Qrob_P0500290.2	DNA mismatch repair protein MSH2	UV-B-induced DNA damage response pathway	0	Shared
Chr06	11400887–11420887	Qrob_P0577750.2	Transcription factor MYB12	Positive regulator of flavonoid biosynthesis, UV-B tolerance	0	All except: Q. rob/Q. pet
Ecological barriers – abiotic stresses						
Nramp metal transporters						
Chr09	7739510–7759510	Qrob_P0191830.2	Metal transporter Nramp5	Manganese and cadmium uptake	8	Q. pubescens-specific
Chr06	38764748–38784748	Qrob_P0097150.2	Metal transporter Nramp6	Involved in iron ion homeostasis	1	All except: Q. rob/Q. pet

Table 2. (Continued)

Chr.	Positions (regions)	Candidate gene	Gene name	Gene functions	#Paralogues	Pattern
Dehydration/lateral root growth	Chr08 68418235–68443235	Qrob_P0457680.2	Protein DEHYDRATION-INDUCED 19-like	Stress-induced sensor, interacting with CPK11 and S-RNase	0	All except. <i>Q. rob/Q. pet</i>
	Chr02 27061274–27081274	Qrob_P0304800.2	Transcription factor WER	Controls cell fate specification, for example hairy roots or stomata	0	All except. <i>Q. rob/Q. pet</i>
Chr02	28240136–28260136	Qrob_P0299670.2	Root Primordium Defective 1 (RPD1)	Lateral root morphogenesis; active cell proliferation	0	Complex
Chr02	36392234–36442234	Qrob_P0422470.2	Alkaline/neutral invertase CINV2	Regulator of root growth, sucrose catabolism	7	Shared
Chr09	19506093–19526093	Qrob_P0418880.2	1-Aminocyclopropane-1-carboxylate synthase	Ethylene biosynthetic process, lateral root formation	4	All except. <i>Q. rob/Q. pet</i>
Chr04	19182244–19222244	Qrob_P0652510.2	Phosphatidylinositol-3-phosphatase myotubularin-1 (or 2)	Role in soil–water-deficit stress	2	Shared
Chr02	96008743–96028743	Qrob_P0387640.2	Protein WVD2-like 6	Organ stockiness (periph. root cap, trichomes snf leaf)	0	All except. <i>Q. rob/Q. pet</i>
Freezing/cold adaptation	Chr09 18536093–18556093	Qrob_P0768740.2	Dehydration-responsive element-binding protein 1	Key role in freezing tolerance and cold acclimation	6	Complex
	Chr02 112153196–112173196	Qrob_P0339800.2	B3 domain-containing transcription factor VRN1	Vernalisation responsiveness, repressor of FLC	3	All except. <i>Q. pub/Q. pyr</i>
Ecological barriers – biotic stresses Pathogen resistance/mycorrhisation	Chr10 12310682–12340682	Qrob_P0070130.2	Transportin MOS14	Plant immunity (splicing of resistant genes)	0	<i>Q. pyrenaica</i> -specific
	Chr01 26763631–26783631	Qrob_P0648170.2	Ubiquitin carboxyl-terminal hydrolase 12-like	Protein deubiquitination, regulator of disease resistance	3	Complex
	Chr05 3053435–3073435	Qrob_P0622110.2	Nodulation-signaling pathway 1 (NSP1)	Nodulation and mycorrhisation	0	Complex

All other annotations are available in Table S3 (see Dataset S1 for details, including references). Species patterns were determined from the analysis of the most outlier-enriched windows between pairs, as detailed in Supporting Information Table S5.

for a *VRN1* gene involved in responsiveness to vernalisation and known to play a key role in cold acclimation in many plant species (Levy *et al.*, 2002). Overall, the genomic variation of these nine genes paralleled the northern–southern distribution of the studied species, suggesting that the underlying barriers were driven by climate preferences (Fig. 1a,b).

Among the five candidate genes from genomic regions with branch-specific patterns, that is deviating from neutrality in all pairs containing a given species, four had *Q. pyrenaica*-specific patterns, including three encoding G-type lectin S-receptor-like serine–threonine kinases (LECRKs) and one encoding a transportin (MOS14). The former is known to be essential for proper splicing of several resistance genes in *Arabidopsis* (Xu *et al.*, 2011), and therefore suggests substantial interspecific differences in plant immunity. Along with the four regions containing genes with *Q. pyrenaica*-specific alleles, we identified a fifth species-specific pattern for *Q. pubescens*. The gene encodes a metal transporter (*Nramp5*) involved in the assimilation of manganese and cadmium in rice and barley (Wu *et al.*, 2016).

Among genes with complex patterns, we identified many candidate genes for intrinsic premating and postmating barriers. We identified several genes that are involved in the timing of flowering, including *APETALA2* and *PRR73*. *APETALA2* is a key transcription factor for the establishment of the floral meristem (Irish & Sussex, 1990). Similarly, *PRR73* contributes to flowering time variation in barley and wheat (Higgins *et al.*, 2010 and references therein).

Discussion

The increasing availability of genomic resources for phylogenetically related species has the potential to greatly improve our understanding of their evolutionary trajectories and the molecular basis of their reproductive isolation as shown here for European temperate oaks. Our demographic reconstruction supported the long periods of isolation between these oak species for most of their history and leading to the gradual loss of shared alleles and the accumulation of reproductive barriers.

Systematic shift in the evolutionary trajectories

We found evidence of a systematic shift in the evolutionary trajectories that occurred at the transition between the last glacial maximum and subsequent postglacial period. More precisely, this shift took place while the oak species were migrating northwards as the climate became warmer, and resulted in their residence in central Europe. Even if the results reported here were in line with our previous findings (Leroy *et al.*, 2017), this study is based on ascertainment bias-free analyses and use of 10 times as many SNPs. The genome-wide investigation performed here provided stronger support for the occurrence of SC. Indeed, the previous inferences were drawn from a dataset with a strong ascertainment bias. Such a deviation from the true SFS is known to have a negative impact on the performance of likelihood-free analyses (Albrechtsen *et al.*, 2010). As in our previous report, our ABC inferences were found to be highly robust to alternative models

based on 1000 pseudo-observed datasets, indicating that the probability of an incorrect inference of SC was very low (Fig. S4).

In addition, our use of 10 times as many SNPs and a two-round procedure to generate parameter estimates made it possible to narrow down the time frame over which these contacts took place. After confirming that recent SC had occurred between these four species, we ran additional backward simulations explicitly assuming recent SC ($T_{SC} < 5\% T_{SPLIT}$), to ensure high accuracy for parameter estimates. Assuming that T_{SPLIT} occurred between 1 and 5 Ma (Hubert *et al.*, 2014) or between 7 and 10 Ma (Denk *et al.*, 2017), and taking into account the 95% confidence intervals for each $T_{SC} : T_{SPLIT}$ ratio (Table S2), we estimated that SCs occurred between 100 and 62 400 yr ago, corresponding to up to 1225 generations, assuming a generation time of 50 yr (Gregorius *et al.*, 2007; but see also in this issue Leroy *et al.*, 2020, for a discussion on the uncertainties with regard to the oak generation times), whereas the upper bound for our previous estimate was 11 200 generations (Leroy *et al.*, 2017). Overall, the median estimates of the timing of SC ranged from 2450 to 21 760 yr depending on the species pair considered (49–435 generations), consistent with the general hypothesis of a resumption of secondary gene flow at the start of the current interglacial period.

In line with our previous conclusions (Leroy *et al.*, 2017), our inferences cannot however exclude that a few SC periods had already taken place earlier. As we did not allow for the possibility of multiple periods of contact and isolation in our model, it remains impossible to know if some (few) other periods of contacts had already taken place earlier during the divergence of these species. Indeed, we used summary statistics that were not expected to capture this information well enough to be conclusive. However, it should be noted that a scenario with many SC periods (e.g. once every past postglacial period) remains unlikely, as it would have generated a much higher posterior probability for IM scenarios. In addition, historical variations in effective population sizes are not taken into account. Further work to infer evolutionary history of these species based on summary statistics at the gene scale (i.e. based on summary statistics accounting for linkage disequilibrium) rather than those at the base scale are likely to have more power to capture these more complex scenarios. Lastly, our analyses are only based on pairwise comparisons. Joint inferences of the evolutionary history of the four (or more) European white oak species should provide additional information on the evolutionary history of these species, especially regarding the direction of interspecific gene flow.

Highly heterogeneous genetic differentiation landscape

The mixture of different species and populations in central Europe occurring during the Holocene was so massive that private (or near private alleles) were redistributed among these interfertile species. Indeed, current levels of interspecific differentiation are extremely low along almost all the genome (mean interchromosomal 10-kb estimates of F_{ST} below 0.08 for all pairs) confirming earlier reports by Scotti-Saintagne *et al.*

(2004). Recently, Lang *et al.* (2018) reported a slightly higher mean F_{ST} value for the *Q. robur*–*Q. petraea* pair (mean F_{ST} = 0.13 over *c.* 12 500 SNPs) based on a restricted representation strategy, representing a total sequence data of *c.* 530 kb (0.072% of the oak genome; Plomion *et al.*, 2018). The differences need to be treated with caution because both estimates are potentially slightly biased. Indeed, we do not rule out the possibility that our estimate is negatively impacted by some DNA quantification and pipetting errors or some unfiltered sequencing errors (Gautier *et al.*, 2013; Hivert *et al.*, 2018). Reciprocally, Lang *et al.* (2018) targeted some genes potentially involved in species ecological preferences that may have resulted in an overestimation of the mean F_{ST} value. Notwithstanding the uncertainty in both estimates, these reported values are consistent with an overall low current level of differentiation and are at a level compatible with many reports of within-species population structure in the literature (Roux *et al.*, 2016).

Interestingly, chromosomal F_{ST} estimates differed considerably between pairs of species and chromosomes. For all pairs, interchromosomal F_{ST} variation was significantly correlated with variations of chromosomal recombination rate (Fig. S6). This finding is consistent with the increasing numbers of reports of a correlation between the recombination rate and the rates of introgression, for example in the house mouse (Janoušek *et al.*, 2015), in *Mimulus* monkey flowers (Aeschbacher *et al.*, 2017), in *Heliconius* butterflies (Martin *et al.*, 2019) or in humans (Juric *et al.*, 2016) and expected to be due to the variation of linkage to introgressed deleterious alleles (background selection, Charlesworth *et al.*, 1993). The recent report of a markedly high rate of deleterious mutations relative to 28 other plant species (Plomion *et al.*, 2018) highlighted the putative role of deleterious mutations in shaping the genomic landscape of species differentiation in oaks. The contribution of linkage to the genomic width of reproductive barriers appears to be limited, as no long stretches of extensive differentiation were observed. We identified very restricted, widely distributed ‘islands’ of high differentiation. The high resolution genetic landscape of differentiation observed with scattered microislands of high F_{ST} on otherwise poorly differentiated chromosomes contrasted sharply with many other F_{ST} scan studies reporting either continents of differentiation (e.g. Tine *et al.*, 2014) or islands of high differentiation in highly structured populations (e.g. Renaut *et al.*, 2013).

To summarise so far, extensive secondary gene flow over the last 20 000 yr, together with very high levels of prezygotic and postzygotic selection (Abadie *et al.*, 2012; Lepais *et al.*, 2013), probably eroded interspecific genetic structures other than those at barrier loci, therefore generating a highly heterogeneous differentiation landscape. At some narrow regions distributed throughout the genome, interspecific differentiation, however, reached extremely high levels (10-kb estimates of F_{ST} above 0.8). These peaks are most likely to correspond to narrow regions where selection counteracted the homogenising effect of gene flow, therefore leading to the present-day highly heterogeneous landscape of differentiation.

Candidate genes for drought tolerance and insights for the renewal of oak forests

The highest differentiated SNPs contributing to reproductive barriers mostly set apart southern (*Q. pyrenaica* and *Q. pubescens*) from northern species (*Q. robur* and *Q. petraea*). While this observation is inconsistent with the inferred phylogeny (Fig. 1c; Leroy *et al.*, 2017), it however coincides with the climatic preferences of the four species (Fig. 1a,b). Indeed, all genes with an ‘all-versus-one-pair’ relationship paralleled the northern–southern distribution of the studied species, suggesting that the underlying barriers were driven by ecological preferences. Given that dehydration-associated genes currently act as strong barriers between these species in south-west France, we questioned whether drought tolerance alleles from *Q. pubescens* and *Q. pyrenaica* would introgress easily into the *Q. petraea* or *Q. robur* genomes. In a companion paper (Leroy *et al.*, 2019), we found evidence for adaptive introgression from *Q. robur* to *Q. petraea* populations located at the northern and higher elevational margins. Such introgressed genes were enriched in alleles that exhibited higher *Q. petraea*–*Q. robur* differentiation. These results suggested that introgression may override species barriers between these four white oaks under peculiar ecological contexts, and potentially contribute to adaptation (e.g. Bontrager & Angert, 2019; Suarez-Gonzalez *et al.*, 2018; Taylor & Larson, 2019). As Mediterranean oak species (*Q. pyrenaica* and *Q. pubescens*) are likely to migrate northwards due to climate change, we anticipate that opportunities for hybridisation with temperate species (*Q. petraea*–*Q. robur*) may increase, leading potentially to introgression enhancing adaptation of the temperate species to warmer climates.

In addition, we found genetic support for *Q. pubescens* preference for alkaline soils and found evidence for one key gene, *Nramp5*. Indeed *Nramp5* is involved in the assimilation of manganese and cadmium in rice and barley (Wu *et al.*, 2016). Manganese assimilation is known to be essential for many plant functions, but manganese availability in the soil tends to decrease with increasing pH, and becomes limiting beyond a soil pH of 6.5. This metal transporter gene probably indicates a greater ecological preference of *Q. pubescens* for dolomitic soils (Fig. 1b) in comparison with the other three species.

We also found differences between *Q. pyrenaica* and the three other species for genes involved in plant immunity, in line with previous reports for higher mortality rates in this species due to pathogens (Desprez-Loustau *et al.*, 2011 and references therein). Indeed, *Q. pyrenaica* is known to be extremely sensitive to oak powdery mildew, a pathogen that was introduced into Europe at the start of the 20th century. Soon after the first detection of the fungus in Europe, high mortality rates were reported for *Q. pyrenaica* in the humid warm-temperate forests of southwestern and western France (Desprez-Loustau *et al.*, 2011). *Q. pyrenaica*-specific alleles at these genes may be the signature of the high susceptibility of *Q. pyrenaica* to biotic stresses in moist environments. It however remains to be studied why these genes act as reproductive barriers.

In addition, we identified several candidate genes for intrinsic barriers likely to be involved in pollen or embryo development, suggesting that both premating and postmating intrinsic barriers operate in oaks as suggested by Bodénès *et al.* (2016). Three of these genes encode cycloartenol synthases known to be essential for pollen development in *Arabidopsis* (Babiychuk *et al.*, 2008). Interestingly, some genes can have pleiotropic effects that may act at both ecological and mating levels. *VRN1* is a case of point. In addition to its primary role in vernalisation, *VRN1* is involved in the repression of *FLC* (itself a known repressor of flowering) in *Arabidopsis*, through a vernalisation-independent floral pathway (Levy *et al.*, 2002).

Overall, our results suggested that key selective abiotic and biotic factors triggered by postglacial environmental changes have molded the extant landscape of species reproductive barriers in European temperate oak species. We anticipate that these drivers will operate during ongoing climate changes as Mediterranean oak species (*Q. pyrenaica* and *Q. pubescens*) are migrating northwards getting in contact in more northern latitudes with local temperate species (*Q. petraea* and *Q. robur*).

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Author contributions

TL designed and performed the research, analysed the data and drafted the manuscript; QR, and CBo contributed to data analysis and interpretation. J-LD performed the analysis of the climatic and soil data. CL performed the DNA extractions and equimolarly pooled DNA. CBe, KL and J-MA generated the sequencing data. GLP and CP were involved in the sampling and selection of genotypes. CP and AK contributed to the design of the research, interpretation and drafted the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Gene annotations for all investigated genes.

Fig. S1 Density of probability of presence according to mean annual temperature.

Fig. S2 Density of probability of presence according to soil pH.

Fig. S3 Species distribution maps based on the French National Forest Inventory used to compute main ecological niches.

Fig. S4 ABC model validation.

Fig. S5 Boxplots of pairwise F_{ST} values per chromosome and per pair of species.

Fig. S6 Relationship between recombination rates (estimated at the whole chromosomal level) and F_{ST} values.

Fig. S7 Empirical null distributions of G_{ST} values resulting from the backward simulation of recent secondary contacts.

Fig. S8 Proportion of SNPs detected as outliers within a range of heterozygosity.

Fig. S9 Local density in outliers with $H_e \geq 0.3$ per nonoverlapping 10 kb sliding window.

Notes S1 Additional information concerning our demographically explicit genome scans.

Notes S2 Selection of the best candidate genes for reproductive isolation.

Notes S3 Discarding the alternative hypothesis of background selection.

Table S1 Sampling site locations and number of individuals pooled for each species.

Table S2 Median and 95% confidence intervals of parameter estimates.

Table S3 Relative posterior probabilities of the IM and SC scenarios.

Table S4 Genomic positions, gene names and annotations for the best candidate of each of the 215 detected regions.

Table S5 Density in outlier SNPs in genomic regions of the 32 gene candidates.

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