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Unusually limited pollen dispersal and connectivity of Pedunculate oak (*Quercus robur*) refugial populations at the species' southern range margin

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Abstract

Low-latitudinal range margins of temperate and boreal plant species typically consist of scattered populations that persist locally in microrefugia. It remains poorly understood how their refugial habitats affect patterns of gene flow and connectivity, key components for their long-term viability and evolution. We examine landscape-scale patterns of historical and contemporary gene flow in refugial populations of the widespread European forest tree Pedunculate oak (*Quercus robur*) at the species' southwestern range margin. We sampled all adult trees (n = 135) growing in a 20 km long valley and genotyped 724 acorns from 72 mother trees at 17 microsatellite loci. The ten oak stands that we identified were highly differentiated and formed four distinct genetic clusters, despite sporadic historical dispersal being detectable. By far most contemporary pollination occurred within stands, either between local mates (85.6%) or through selfing (6.8%). Pollen exchange between stands (2.6%) was remarkably rare given their relative proximity and was complemented by long-distance pollen immigration (4.4%) and hybridization with the locally abundant *Quercus pyrenaica* (0.6%). The frequency of between-stand mating events decreased with increasing size and spatial isolation of stands. Overall, our results reveal outstandingly little long-distance gene flow for a wind-pollinated tree species. We argue that the distinct landscape characteristics of oaks' refugial habitats, with a combination of a rugged topography, dense vegetation and humid microclimate, are likely to increase plant survival but to hamper effective long-distance pollen dispersal. Moreover, local mating might be favoured by high tree compatibility resulting from genetic purging in these long-term relict populations.

Keywords: genetic differentiation, genetic diversity, landscape scale, marginal populations, paternity analysis, rear edge

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Introduction

Many temperate and boreal plant species have populations along the low-latitudinal limit of their current distribution range that have persisted roughly in situ through the numerous major climate transitions of the Quaternary (Gavin *et al.* 2014). These so-called rear-edge populations are important targets for the preservation of the genetic diversity, phylogenetic history and evolutionary potential of species (Hampe & Petit 2005). They are, moreover, excellent models for studying local adaptation under natural conditions and for understanding the processes that help tree populations to successfully persist in an adverse abiotic environment (Woolbright *et al.* 2014). Rear-edge populations typically survive locally in microrefugia: areas with climatic conditions that have existed for an extended period of time but are currently rare within their surroundings ('relict climates' sensu Dobrowski 2010).

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Hence, populations are typically small, highly scattered and restricted to particularly favourable habitats within heterogeneous landscapes. It remains poorly understood which ecological mechanisms have enabled them to thrive under the constraints of their climate-driven long-term fragmentation (Hampe & Jump 2011). In particular, we know little about how their particular relictual distribution affects patterns of gene flow and population connectivity, a key component for their genetic diversity, long-term resilience and evolution.

Trees share life history characteristics that render them particularly resistant to the erosion of population genetic diversity, including a long lifespan, a prodigious fecundity and a great propensity for long-distance gene dispersal (Petit & Hampe 2006). A rapidly accumulating body of evidence indicates that pollen-mediated gene flow between tree populations regularly spans various kilometres (reviewed in Kremer *et al.* 2012). In particular, in wind-pollinated species, small and geographically isolated tree populations commonly experience non-negligible amounts of mating events with immigrant pollen arriving over great distances (e.g. Buschbom *et al.* 2011; Robledo-Arnuncio 2011; Lesser & Jackson 2013). This pollen-mediated gene immigration can be further exacerbated through the purging of inbred individuals during the subsequent recruitment process (Hampe *et al.* 2013). On the other hand, tree populations also are more dependent on efficient gene exchange than other plants because they are mostly self-incompatible and disproportionately susceptible to inbreeding depression (Petit & Hampe 2006). In addition, the effective population size of trees is often much smaller than apparent in the field because of great inequality in fecundity, with a few individuals contributing very many offspring and the great majority little or nothing (Oddou-Muratorio *et al.* 2005). Hence, a minimum size and connectivity of relict populations seem crucial for their fitness and long-term persistence in a changing environment (Sexton *et al.* 2011).

Relict tree populations at the rear edge of species ranges share many features with other small and isolated tree populations. However, they also assemble some specific characteristics that could render them particularly prone to experiencing lower levels of effective pollen flow. First, they typically grow far away (i.e. tens to hundreds of kilometres) from large pollen sources such as extensive populations belonging to the continuous distribution range. Second, their continued persistence at relatively low population size implies that they probably have undergone extensive genetic purging and may be suffering less from incompatibility and inbreeding depression than more recently isolated populations (such as pioneer stands at the leading edge or those resulting from recent anthropogenic fragmentation). Third, their particular relictual habitats usually are located in topographic settings that help maintain a constant minimum humidity (Hampe & Jump 2011), such as shady valleys, gorges or ravines; these habitats often occur in rugged terrain and contain a dense vegetation that tends to represent an obstacle to long-distance pollen flow (Damschen *et al.* 2014; Shohami & Nathan 2014). Hence, relict populations residing at the rear edge of species ranges could experience less long-distance gene flow than many other tree populations that have experienced fragmentation and isolation as a consequence of human activity (Kramer *et al.* 2008). Yet we still have a limited understanding of how landscape complexity combines with individual tree traits to result in the distinct fecundity patterns of relictual scenarios (Bacles & Jump 2011).

One of the most direct and powerful methods for studying the movement of genes within and among small, isolated populations consists in performing population-specific paternity tests. The precise detection of male parents and their spatial position allows to retrace pollen movements, providing information about the spatial patterns of mating across complex landscapes, and the potential effects of the landscape context on spatial genetic structure and contemporary gene flow (Klein *et al.* 2011). Documenting these patterns across networks of tree stands connected at a landscape scale can unveil demographic and environmental correlates of geneflow patterns (Dyer & Nason 2004; Cheptou & Schoen 2007; DiLeo *et al.* 2014) and ultimately mechanisms of isolation associated to their relictual situation. Finally, complementing these analyses with an in-depth assessment of historical geneflow patterns as reflected by the adult tree populations can reveal how consistent through time and hence biologically relevant observed patterns of pollen flow are.

Here, we use a particularly suited model system to examine landscape-scale patterns of historical and contemporary gene flow and connectivity in relict tree populations residing at the southwestern range limit of the species. This particular setting provides a rare opportunity for comprehensively assessing landscape-scale mating patterns in a major European forest tree. Specifically, we (i) describe the genetic structure of adult trees and patterns of historical gene flow based on an exhaustive sampling of individuals, (ii) test hypotheses about contemporary pollen flow limitation within and among stands as well as limited pollen immigration (either through long-distance pollen dispersal or through hybridization with the locally abundant sister species *Quercus pyrenaica*), and (iii) test the effects of stand characteristics (size, geographic isolation and phenology) on patterns of within and among-stand gene exchange.

Materials and methods

Study species

Pedunculate oak (*Quercus robur* L.) is one of the most prominent and widely distributed European forest tree species. Its southwestern range margin is located in the mountain ranges of central Spain, where scattered populations occur along water courses and in other environments that mitigate the summer drought characteristic of the regional Mediterranean climate.

Quercus robur is an almost exclusively outcrossing, wind-pollinated species. Extensive long-distance pollen flow into highly disjunct stands has been documented in this and other oak species (e.g. Buschbom *et al.* 2011; Hampe *et al.* 2013; Gerber *et al.* 2014). In the study area, it flowers from early to late April and acorns ripen in late September and early October. Hybridization with the sister species Pyrenean oak (*Quercus pyrenaica*) is possible due to partial overlap of flowering periods (Lepais *et al.* 2009). *Quercus robur* shows alternate fruit bearing in the area (G. Moreno, unpublished) as it does in many other parts of its range.

Study area and field sampling

The study was conducted in the Jerte valley (40° 13′ N, 5° 44′ W; Cáceres province), ca. 25 km northeast of the town of Plasencia in western Spain. The valley stretches almost linearly over ca. 30 km descending from an elevation of 1275 m a.s.l. at the header (Fig. 1a,b). Its relatively temperate and humid local climate has favoured the development of an extensive fruticulture in the smoother, more accessible slopes while steeper and higher slopes are covered by broadleaved forests dominated by *Q. pyrenaica*. Streamsides and gorges harbour mesic and riparian tree species including *Q. robur*, *Celtis australis*, *Castanea sativa*, *Fraxinus angustifolia* and *Alnus glutinosa* among others.

Our study benefited from a comprehensive survey of adult and close to adult *Q. robur* trees throughout the Jerte valley conducted between 2002 and 2003 (Pulido *et al.* 2007). The species' earlier budburst and later shedding of leaves allows, during certain periods of the year, spotting and distinguishing individuals over hundreds of metres from the far more abundant sister species *Q. pyrenaica*. The survey detected a total of ten small *Q. robur* stands (n = 2–35 adult trees) spread over ca. 20 km of the Jerte valley (Fig. 1b). Somewhat larger populations (i.e. with a few hundred trees) are located at ca. 10–15 km, although outside the valley and therefore separated by a mountain chain with an altitudinal differential of up to 1000 m.

We mapped all adult and close to adult *Q. robur* trees known to occur in the Jerte valley (n = 135) and collected several leaves from each tree that were stored in silica gel until genetic analyses. We collected up to 20 acorns (mean: 19.0) in October 2009 from or, if impossible, beneath the canopy of 72 trees from all ten stands (i.e. virtually all individuals reproducing this year in the Jerte valley). Acorns were weighted, frozen and stored until being processed. We extracted the embryo from each acorn and dried it for genotyping. A pilot analysis of acorns from 10 randomly chosen mother trees using genetic diversity accumulation curves of multilocus genotypes indicated that a sample of 8–11 acorns per mother adequately describes the genetic diversity of seed families (see Fig. S1, Supporting information). We hence decided to analyse a minimum of 10 acorns per mother tree, resulting in a total of 724 acorns from 72 seed families. Finally, we characterized the flowering phenology of all adult trees in the Jerte valley on two dates somewhat before and after the peak of the flowering period, respectively (28 March 2015 and 4 April 2015). Four phenological stages of male flowers were distinguished during each survey: (i) swelling buds (score 0); (ii) emerging and immature catkins (score 1); (iii) mature catkins (score 2); and (iv) old, dried catkins (score 3). The phenological stage of the upper and the lower part of each tree was separately recorded and subsequently averaged. A similar survey in the previous year (2014) allowed us to corroborate that the phenological sequence of trees within stands remains reasonably consistent from year to year (Spearman rank correlation, r_s = 0.38, P = 0.004 (see also Bacilieri *et al.* 1995).

Microsatellite genotyping

All adult trees and acorns were genotyped using the 20 nuclear microsatellite markers described in Guichoux *et al.* (2011), integrated in two multiplex kits of twelve and eight markers each. DNA extraction, PCR amplifications and genotype scoring with an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) were performed following the protocols described by these authors. Electropherograms were independently scored by two persons to minimize genotyping error. We actually quantified the error rate using 44 randomly chosen acorns as blind samples. Three independent extractions, amplifications and analyses of each blind sample, respectively, resulted in an estimated overall genotyping error rate of 0.85% (n = 880 scorings), thus confirming the high marker quality pointed out by Guichoux *et al.* (2011).

Two loci (S19 and AB) did not fully amplify across all acorns and another one (Qr20) was found to strongly deviate from Hardy–Weinberg equilibrium due to allele

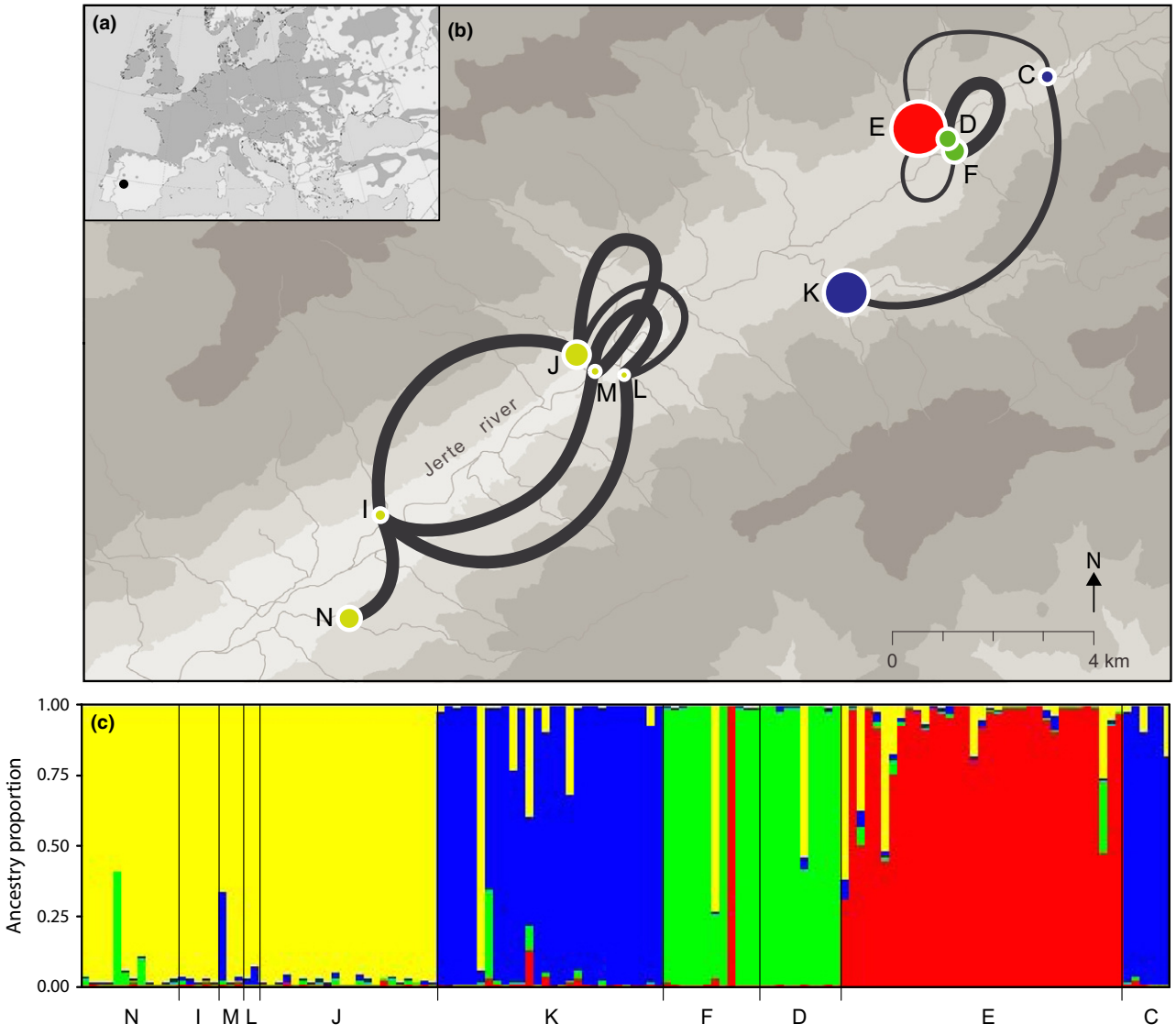

Fig. 1 (a) Distribution area of *Quercus robur* according to EUFORGEN with a black dot indicating the study area. (b) Map of target stands. Shading indicates altitudinal ranges in 500 m steps from <500 m asl (white) to >2000 m asl (dark grey). Study stands are indicated by circles with circle size indicating the number of adult trees and circle colour the dominant genetic cluster as identified by STRUCTURE. Dark grey lines indicate significant genetic covariance ($0.01 \leq \alpha \leq 0.1$) between stands according to Population Graph analysis, and their thickness is proportional to the conditional graph distance value, that is the genetic similarity of stands. (c) Bar plots showing STRUCTURE ancestry proportions for $K = 4$ clusters. Each individual is represented as a line segment, which is vertically partitioned with different colours representing the individual's estimated proportions of ancestry in each cluster (cluster 1 in yellow; 2 in red; 3 in green and 4 in blue). Letters below the graph refer to stands as shown in plate b.

dropout. All three loci were discarded, leaving 17 loci for the paternity analyses (while we used all 20 loci for the analyses of adult trees).

Data analysis

Adult genetic diversity and relatedness. The following statistics were calculated overall and for each stand using the GSTUDIO package in R version 3.1.0 (R Development Core Team 2014): mean number of alleles (N_a), effective number of alleles (N_{ae}), observed and expected heterozygosity (H_o and H_e) and fixation index (F_{IS}). We tested the null hypothesis H_0: $F_{IS} = 0$ using 10 000 randomizations of alleles among individuals. The effective number of alleles was estimated according to Nielsen *et al.* (2003) to account for the unequal sample sizes among stands. Gene diversity (H_e) was also corrected for sample size (Nei 1978).

We estimated genetic relatedness (Queller and Goodnight 1989) among all within-stand adult tree pairs with

SPAGEDI 1.4 (Hardy & Vekemans 2002). The expected relatedness values (R) are 0.5 among full-sibs, 0.25 among half-sibs and 0.0 for unrelated individuals. To calculate within-stand pairwise relatedness we used the allele frequency of the total, pooled 10 stands in the valley.

Adult genetic differentiation and historical migration. We assessed overall and pairwise genetic differentiation among stands computing F_{ST} (Weir & Cockerham 1984) in SPAGEDI 1.4 (Hardy & Vekemans 2002). F_{ST} was tested against the null expectation of absence of population structure based on 10 000 permutations. We also tested for isolation by distance between stands by means of a Mantel test with 9999 permutations in GENALEX 6.5 (Peakall & Smouse 2012). For this purpose, we calculated the geographic distance among stands (taking their altitude into account).

Bayesian clustering of the genetic data was performed using STRUCTURE v.2.3.4 (Pritchard *et al.* 2000). We ran STRUCTURE with K ranging from 1 to 10, and with 10 runs for each K value. A burn-in period of 50 000 iterations was followed by 200 000 MCMC repetitions assuming allele frequencies to be correlated among populations and an admixture model of population structure. No prior information was used to assist clustering in the first 10 × 10 runs, whereas we included stand identity as prior information in a second series of 10 × 10 runs. Results were highly consistent between both approaches, and we report the results including stand identity. We selected the K value that best described the data from the change in likelihood (delta K) as proposed by Evanno *et al.* (2005) and the highest posterior probability (LnP(D|K)).

We further explored patterns of historical gene exchange between stands using Population Graphs (Dyer & Nason 2004) as implemented in the popgraph library in R version 3.1.0. (R Development Core Team 2014). This graph theoretic approach analyses how genetic variation is distributed across the investigated landscape by plotting historical migration. The advantage of the approach is to explicitly account for differences in genetic covariation associated with both direct and indirect connectivity (gene flow) among populations, making it potentially better suited for use in landscape genetic modelling than more conventional measures of pairwise genetic distance such as F_{ST} and D_c (Dyer *et al.* 2010). Within a graph, populations are represented as nodes and the genetic covariation among populations determines the topology. To achieve this, the genetic variance–covariance matrix among populations is inverted and standardized to obtain a partial correlation matrix, with the significance of individual partial correlations determined. If the partial correlation between populations i and j is significantly greater than expected by chance, then an edge is placed between nodes i and j (Dyer & Nason 2004). By setting the significance level from 0.1 to 0.01, we assure robustness of the topology. The Population Graph is constructed by applying this procedure to all possible population pairs, resulting in a graphical model of population genetic structure. The pattern of connections among populations is thus estimated conditional on the entire data set, improving the way genetic covariance is estimated and quantified in landscape models. The shortest path connecting pairs of populations across the entire graph is defined as the conditional graph distance (cGD; Dyer *et al.* 2010). It can be used as a metric of genetic distance that, contrary to traditional F_{ST} approaches, considers the entire population network when comparing pairs of populations (Dyer 2015).

Paternity analysis of acorns. Before starting the paternity assignment, we checked and removed all offspring whose multilocus genotypes did not match their putative mother tree (indicating sampling errors committed in the field). The final progeny array consisted of 684 acorns, with 9.4 ± 1.3 (mean ± SD) acorns per mother tree. We used categorical paternity assignments to identify candidate father trees. This approach was facilitated by the high exclusion power of our molecular markers (0.99995, see Table S1, Supporting information) and the exhaustive sampling of candidate fathers within the Jerte valley. The paternity of each offspring was determined by likelihood ratios with CERVUS 3.0 (Kalinowski *et al.* 2007) assuming the strict confidence criterion (95%) for assignments. Preliminary tests showed that an assumed error rate of 0.5% provided the most reasonable balance between considering genotyping errors and getting a biologically meaningful set of assignments. We performed simulations with the following parameters: number of offspring genotypes = 50 000, number of candidate fathers = 150, proportion of candidate fathers sampled = 0.9, mistyping rate = 0.005 and proportion of loci typed = 0.9936. These parameters correspond to the adult population size of the valley plus an incoming gene flow of 10%, which seems reasonable given our study context (e.g. Gerber *et al.* 2014). Most acorns were assigned a single pollen parent (650 of 684 seeds analysed) and no acorns showed multiple paternity assignments. A posterior check confirmed that all unassigned acorns (n = 34) showed mismatches with the most likely candidate father at a minimum of two loci.

Pollen dispersal. Following paternity assignment, we used three different approaches to characterize pollen dispersal within and among stands. First, we

constructed a frequency distribution of observed intermate distances from all acorns for which we had identified a single candidate father. This allowed us to infer a global frequency distribution of pollen dispersal distances for the study populations. Second, we quantified the proportions of different types of pollination events by assigning each acorn to one of four classes: (i) *selfing*: the proportion fertilized by pollen from the same mother tree; (ii) *local pollination*: the proportion fertilized by any pollen donor candidates from within the oak stand of the mother tree; (iii) *between-stand pollination*: the proportion fertilized by any pollen donor candidates from stands other than the stand of the mother tree; and (iv) *unassigned*: the proportion fertilized by pollen from an unsampled *Q. robur* donor (i.e. a long-distance immigration event from outside the Jerte valley) or from a *Q. pyrenaica* donor (i.e. a hybridization event). This classification ignores cryptic gene flow (i.e. local pollinations that cannot be distinguished from immigration events), which we consider unlikely given the high exclusion probability of our markers and the exhaustive sampling of candidate trees. We computed, for each individual mother tree, the proportions of pollination events belonging to each of the four classes. Third, we tested if mating within stands (corresponding to our local pollination class) occurred at random or primarily between nearby trees. For this purpose, we computed and compared, for each stand, the median distance of observed within-stand pollination events and the median distance between all trees.

An additional analysis was performed to infer whether our nonassigned acorns stemmed from long-distance pollen immigration or from hybridization with *Q. pyrenaica*. For this purpose, we conducted an assignment test using STRUCTURE and a data set that included all unassigned acorns, all 135 *Q. robur* trees and a sample of 109 adult *Q. pyrenaica* trees collected next to our focal trees and analysed with the same SSR loci. We ran STRUCTURE with the same configuration as described above and, unsurprisingly, identified $K = 2$ as the by far most likely scenario (Fig. S2, Supporting information).

Stand-level correlates of pollen dispersal. We hypothesized that stand-level mating parameters should be governed by the size, the location and the flowering phenology of stands. We constructed three variables to characterize these components (see Table 1): (i) stand size measured as the number of trees. (ii) isolation measured as the mean distance (controlling for altitude) from the focal stand to every other stand within the valley. Note that this measure integrates the 'centrality' of stands within the Jerte valley: those near the valley ends are on average more distant from all others than those situated in the centre. (iii) Flowering phenology measured as the average of the phenological stages (scored between 0 and 3) of all trees in a given stand. This averaging of the individual phenological scores resulted in a numerical value where early-flowering stands score near three and late-flowering stands score near zero. We used generalized linear mixed models (GLMM) with a binomial distribution to test for effects of stand size, isolation and phenology on the proportions of the four pollination types described above (selfing, local pollination, between-stand pollination and unassigned). The proportion of each pollination type on each mother tree was used as response variable, stand size, isolation and phenology as fixed effects and the stand identity of the mother tree as random effect. GLMM analyses were performed using the LME4 package in R version 3.1.0 (R Development Core Team 2014). Previous tests showed that collinearity among predictor variables and correlations among response variables (i.e. the proportions) were moderate (predictor variables: Spearman $r_s \leq 0.63$; response variables: Spearman $r_s \leq 0.54$).

We also tested whether large or dense stands showed a stronger tendency for mating between nearby trees than small or sparse stands. For this purpose, we calculated the ratio between the median distance of observed within-stand mating events and the median distance between all trees (see Table 1). This ratio was then regressed against stand size and density.

Results

Adult genetic diversity and relatedness

We detected a relatively high total number of alleles per locus (mean 11.1, range 5–19) and a markedly lower effective number of alleles (mean 4.4, range 1.2–9.1; see Table S1, Supporting information). Allelic composition varied greatly among stands and private alleles occurred in all but one (M, Table S2, Supporting information). Observed and expected heterozygosities were overall moderate although again with great variation among stands (Table 1). Significant heterozygote deficit was only detected in stands J and L.

Mean relatedness among adult trees in the Jerte valley was 0.167 (CI: 0.159–0.176). Mean values for stands ranged from −0.046 in L to 0.430 (0.36–0.50) in M (Table 1). Except for stand L, all stands with <10 trees had R values above 0.25 (the value expected for half-sib pairs). When excluding the outlier stand L (with only two adult trees), R showed a significant tendency to decrease with increasing stand size (linear regression: $F = 8.69$, df = 7, $P = 0.02$).

Table 1 Stand features, genetic diversity estimates for adult trees (H_E, H_O and F_{IS}), R, adult relatedness and mean rates of pollination types according to paternity analysis (selfing, local pollination, between-stand pollination and unassigned) for each *Quercus robur* stand

Stand	No. of trees	Sample size (trees/acorns)	Altitude	Phenology	Isolation	H_E	H_O	F_{IS}	Selfing	R (CI)	Local pollination	Between-stand pollination	Unassigned	m_{obs}/m_{all}
C	6	5/46	757.9	2.5 (0.11)	0.80	0.54	0.54	−0.01	0.16	0.35 (0.24—0.47)	0.82	0.00	0.03	0.61
D	10	7/66	628.8	1.6 (0.42)	0.61	0.61	0.60	−0.11	0.04	0.27 (0.21—0.33)	0.86	0.10	0.00	0.24
E	35	17/162	742.3	2.0 (0.41)	0.62	0.65	0.65	−0.07	0.03	0.15 (0.13—0.16)	0.86	0.01	0.10	0.34
F	12	7/66	629.9	1.8 (1.10)	0.62	0.64	0.64	−0.03	0.04	0.19 (0.14—0.24)	0.87	0.02	0.07	1.16
I	5	4/41	467.4	2.5 (0.18)	0.85	0.63	0.62	−0.12	0.00	0.28 (0.18—0.38)	0.85	0.00	0.15	0.98
J	22	14/129	622.2	2.4 (0.24)	0.57	0.63	0.63	0.06*	0.08	0.17 (0.15—0.18)	0.89	0.00	0.02	0.62
K	28	11/107	699.4	1.9 (0.44)	0.55	0.61	0.61	0.04	0.13	0.19 (0.18—0.21)	0.86	0.01	0.00	0.27
L	2	1/10	523.1	1.7 (0.18)	0.54	0.75	0.80	0.19*	0.10	−0.05	0.40	0.10	0.40	—
M	3	3/29	484.7	1.7 (0.18)	0.55	0.61	0.58	−0.28	0.10	0.43 (0.36—0.50)	0.69	0.21	0.00	0.12
N	12	3/28	577.7	2.5 (0.14)	1.00	0.72	0.72	−0.05	0.00	0.04 (0.01—0.08)	1.00	0.00	0.00	0.06
Overall	135	73/684		—	—	0.71	0.65	0.08*	0.07	0.17 (0.16—0.18)	0.86	0.03	0.05	—

H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, fixation index; *$P < 0.05$, R, adult relatedness. Phenology indicates the mean phenological state (SD) for each stand from individual tree scorings, where 0, 1, 2 and 3 scores represent swelling buds, emerging and immature catkins, mature catkins and old dried catkins, respectively, at the peak of the flowering period. Isolation is computed as the mean distance to the centroid of every stand in the valley (expressed in 0—1 range). The ratio between the median distance of observed within-stand mating events and the median distance between all trees (m_{obs}/m_{all}) is also presented.

Genetic differentiation of and historical gene flow among stands

Overall differentiation among stands was high ($F_{ST} = 0.12$; $P < 0.001$; see also Table S3 (Supporting information) for pairwise F_{ST} values). We observed weak signs of isolation by distance (Mantel test: $R^2 = 0.06$; $P = 0.08$). The STRUCTURE analysis indicated the existence of four distinct genetic clusters (Fig. 1b, c; see also Fig. S3, Supporting information): Cluster 1 comprised the five stands located in the middle to lower parts of the valley (J, M, L, I and N); cluster 2 dominated stand E; cluster 3 assembled the nearby stands D and F; and cluster 4 included the relatively distant stands C and K. The majority of trees had high probabilities of ancestry in a single cluster. Some individuals were assigned to two different clusters with ancestry probabilities around 50%, indicating that they stemmed from between-cluster pollination events. This phenomenon involved primarily trees located in the stands dominated by clusters 2–4 that were partly assigned to cluster 1. We also observed two trees strongly assigned to one cluster that were situated within populations dominated by another cluster (suggesting that they stemmed from between-cluster acorn dispersal events; see stands F and K in Fig. 1c).

The Population Graph analysis produced an invariant network topology (Fig. 1b) across the range of α values examined (0.01–0.1). The most salient feature of the network was the disconnection between stands of the upper and the lower valley. The lower valley stands were on average markedly less differentiated among them than the upper stands (average pairwise cGD = 0.75 vs. 1.61; t test: $t = 4.86$, df = 10.8, $P < 0.001$; see Table S3, Supporting information). The Population Graph analysis also revealed some links between nearby pairs of stands that belonged to different STRUCTURE clusters (C and E as well as E and D).

Contemporary pollen dispersal

The frequency distribution of mating events and its associated pollen dispersal kernel were robustly estimated based on the high proportion of paternity assignments (95%). The shape of the pollen dispersal curve was leptokurtic (Fig. 2), with the median and the 95th percentile pollination distances being 30.5 m and 390.1 m, respectively. A small fraction of mating events (1.8%) spanned distances between 1000 and 8500 m, the maximum pollen dispersal distance observed. Within stands, the median distance between mates was significantly smaller than the median distance among trees (22.6 vs. 60.6 m; paired t test: $t = -2.61$, df = 8, $P = 0.03$). This trend was unrelated with the size or

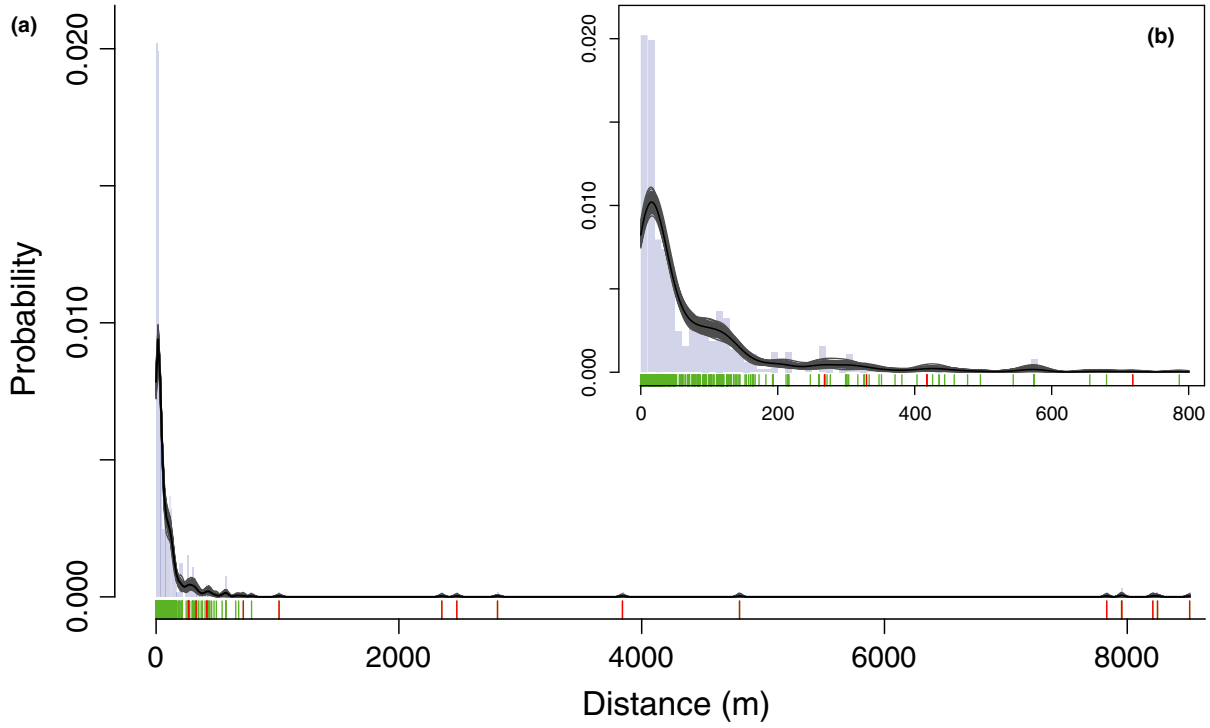

Fig. 2 (a) Frequency distributions of pollen dispersal distances for *Quercus robur*, estimated via paternity analysis. The blue line indicates the nonparametric smoothing spline fit to the empirical distance distribution together with bootstrapped estimates (n = 100 randomizations). Individual pollination events are indicated by vertical lines under the plot, with light green and red lines indicating within-stand and interstand pollinations, respectively. (b) Zoom spanning 800 m.

density of stands (linear regressions: F = 0.21, df = 7, P = 0.66; F = 0.83, df = 7, P = 0.39, respectively).

Observed proportions of different pollination types varied extensively among stands (Table 1). By far most pollination events (85.6%) involved fathers from the stand of the mother. This fraction was even higher when adding the percentage of selfing events (6.8%). On the contrary, few events (5.0%) involved fathers located outside the Jerte valley or *Quercus pyrenaica* trees, and even fewer between-stand pollination events (2.6%) were observed. Our STRUCTURE analysis indicated that only four of the acorns without identified fathers (i.e. 0.6% of the overall acorn sample) are likely to stem in first generation from a hybridization event with *Q. pyrenaica* (i.e. probability of assignment to cluster '*Q. pyrenaica*' around 0.5, see Table S4, Supporting information). On the other hand, a few of our adult *Quercus robur* trees show signs of being hybrids in

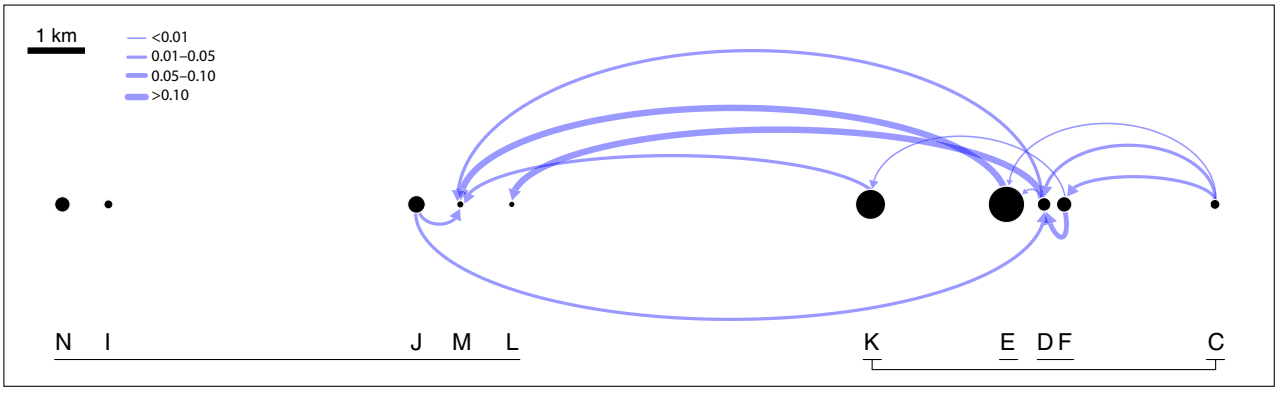

Fig. 3 Contemporary pollen-mediated connectivity of the investigated *Quercus robur* stands. Stands are drawn as nodes with circle size being proportional to stand size. Arrows represent pollen dispersal probability from source to sink according to the proportion of between-stand pollen dispersion inferred by paternity analysis. Letters beneath circles indicate the identity of stands while horizontal lines below stand codes indicate those stands that belong to the same genetic cluster.

Table 2 Effect of stand characteristics on the proportion of each pollination type

	Selfing			Local pollination			Between-stand pollination			Unassigned		
	Estimate	SE	*P*	Estimate	SE	*P*	Estimate	SE	*P*	Estimate	SE	*P*
Fixed effects												
Intercept	−0.23	2.11	ns	−1.20	1.28	ns	6.58	0.01	***	−7.81	5.88	ns
Size	−0.02	0.03	ns	0.03	0.02	—	−0.06	0.01	***	−0.09	0.08	ns
Isolation	−0.57	0.43	ns	0.34	0.24	ns	−0.21	0.01	***	−1.11	1.05	ns
Phenology	0.68	1.55	ns	0.11	0.85	ns	−3.90	0.01	***	5.75	4.80	ns
Random effects (SD)												
Stand	0.54			0.23			0.42			1.76		

Results of generalized linear mixed-effects models with binomial errors are shown. $P < 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

first or second generation (see Fig. S2, Supporting information).

We observed a marked directionality of pollen movements between stands (Fig. 3): Of the 18 events detected overall, 11 (61%) were from upstream to downstream stands, six (33%) involved stands growing nearby (<1 km) across the valley and only one long-distance pollination event (5%) occurred in upstream direction.

Correlates of variation in pollen dispersal among stands

As revealed by the GLMM model fit, stand characteristics influenced the proportions of between-stand mating events and slightly with local pollinations (see Fig. 3), whereas no effect on the proportions of selfed and unassigned acorns was evident (Table 2). The frequency of local pollinations tended to increase with stand size, whereas that of between-stand pollinations decreased with increasing stand size and in particular isolation. We also found a marked effect of phenology on the proportion of between-stand pollinations, with late-flowering stands receiving more immigrant pollen than early-flowering stands (Table 2).

Discussion

Adult population structure and historical gene flow

Our study stands showed a relatively high level of genetic diversity given their geographical isolation and relatively small population size [global $H_E = 0.71$, compared to 0.75–0.85 (Buschbom *et al.* 2011), 0.79 (Hampe *et al.* 2010), 0.79 (Muir & Schlötterer 2005;), or 0.87 (Streiff *et al.* 1998) for SSR-based estimates from other *Quercus robur* populations]. This diversity was strongly structured, indicating that gene flow and population connectivity along the Jerte valley have been weak through an extended period of time. We observed no <4 well-supported genetic clusters and an overall F_{ST} (0.12) that clearly exceeds those of far more geographically separated oak populations from other ecological settings (e.g. Bruschi *et al.* 2003; Muir *et al.* 2004; Craft & Ashley 2007, 2010; Cavender-Bares & Pahlich 2009). Even though the F_{ST} estimate might be somewhat inflated due to the small size of some stands, our study system clearly is at odds with the widespread notion that disjunct populations of wind-pollinated temperate trees tend to experience extensive long-distance gene exchange (Kramer *et al.* 2008; Kremer *et al.* 2012). Stands of the upper and those of the lower valley actually were largely disconnected, although no apparent topographic or other landscape-related barriers to gene flow exist between the upstream and the downstream stands (except for population K, which is located in a lateral valley; see Fig. 1b). The observed disconnection should hence largely be driven by the mere spatial distance between the two groups (ca. 8 km). Note also, however, that the STRUCTURE analysis identified a certain number of trees (ca. 10%) that stem from between-cluster pollination or acorn dispersal events.

At a finer spatial scale, the genetic differentiation of stands was not homogeneous throughout the valley but much more pronounced in the upper than in the lower part. This difference is remarkable because the two valley parts do not appreciably differ in the spatial distribution of stands, the topography or greater landscape structure. The strong genetic divergence of stands in the upper part of the valley suggests that the species must have persisted more or less independently at different sites in the valley. Such circumstances and the limited size of these stands probably have led to their rather low genetic diversity ($H_E = 0.61$). Note that high population differentiation combined with low within-population diversity is a key characteristic of stable rear-edge populations (Hampe & Petit 2005); thus, our study stands are likely to be real long-term relicts with a history of persistence in situ through many generations.

On the other hand, the relatively weak differentiation of the lower valley stands compared to their upper valley counterparts could reflect that they are remainders of a once larger and more continuous stand that has undergone shrinking and fragmentation in relatively recent time. Such a process would explain that the lower valley stands show higher levels of diversity than their upper valley counterparts (0.67 vs. 0.61; paired *t* test: $t = -2.42$, df = 19, $P = 0.03$), and that most historical long-distance pollination events occurred from lower towards upper parts of the valley (i.e. from cluster 1 towards the other clusters, see Fig. 1c). It is well documented that human land use in the Jerte valley steadily increased in intensity through the 20th century (Cruz 1983), although the remotest horticultural areas are now being abandoned and recolonized by scrub and forest (Ezquerra & Gil 2008). A concomitant size reduction and fragmentation of *Q. robur* stands, especially in the more populated and readily transformable lower part of the valley, appears hence realistic.

Contemporary pollen dispersal

By far most mating events involved trees from the same stand (>90% when adding local pollinations and selfing). Larger stands tended to experience somewhat more local pollinations than smaller ones, although the tendency was weak. The dispersal kernel (Fig. 2) and the estimated median pollen dispersal distance of only 30 m underpin moreover that mating occurred primarily between neighbouring trees within each stand. We also detected a remarkably high number of selfing events for a strongly outcrossing species such as *Q. robur* (Bacilieri *et al.* 1996). These stemmed mostly from a few heavily selfing trees (i.e. five individuals accounted for more than half of all selfed offspring), suggesting that breakdown of self-incompatibility has occurred several times in our study system (see Hampe *et al.* 2013 for a similar case in a marginal oak population).

Overall, the probability to receive pollen from other stands within the valley decreased with increasing size of stands and especially with their geographical isolation, whereas it increased from early- to late-flowering stands. The two lowermost stands I and N did not participate at all in the observed pollen flow, whereas the central stand D exchanged pollen with no <6 other stands. The small and late-flowering stands L and M served as sinks for 39% of all observed between-stand pollination events, whereas the more isolated, early-flowering stands J and C served exclusively as pollen sources (see Fig. 3). Overall, these patterns point to great spatial heterogeneity in the network of pollen gene flow. The detected phenology effect indicates that between-stand mating tends to increase with decreasing pollen abundance, suggesting that competition for access to ovules could to some extent trigger the observed mating patterns (see also Lagache *et al.* 2013).

Interestingly, patterns of contemporary pollen flow showed two major inconsistencies with those of historical gene flow as reflected by the adult trees. First, we detected several instances of ongoing pollen dispersal across the major genetic disconnection between upper valley and lower valley stands (see Figs 1b and 3). Second, contemporary pollen movements occurred markedly more often downstream than upstream, whereas the STRUCTURE analysis of the adult trees indicated that historical pollen flow has primarily occurred in the opposite direction (from cluster 1 towards the other clusters). The directionality of contemporary pollen flow is in agreement with the dominant wind direction during the flowering season of our study year (*Sistema de Información Agroclimática para el Regadío*, http://eportal.magrama.gob.es/websiar/Inicio.aspx). We do not know whether our study year was an exception and upstream winds have been historically prevailing in the Jerte valley. But the dominance of upstream pollen dispersal in the past would be in line with our hypothesis that *Q. robur* could have been more abundant in the lower Jerte valley during past decades than it is today.

Pollen inflow from other sources than our target stands (i.e. either *Q. robur* populations outside the Jerte valley or *Quercus pyrenaica*) was also infrequent. Even the smallest and most isolated stands did not show any signs of being particularly susceptible to experiencing long-distance gene inflow or hybridization. Our STRUCTURE analysis indicated that only a tiny fraction of all mating events (0.6%) involves hybridization with *Q. pyrenaica*. Given the great abundance of this species in the Jerte valley, our finding implies that interspecific gene flow played a very minor role. We observed, however, signs of hybridization in some of our adult *Q. robur* trees, which are roughly in line with the hybridization rate of 5.9% that Lepais *et al.* (2009) reported from a mixed oak stand with *Q. robur* and *Q. pyrenaica*. This discrepancy suggests that hybridization might be more frequent in our study system in years of less abundant flowering and concomitant stronger pollen limitation (cf. Lepais *et al.* 2009; Lagache *et al.* 2013).

Mechanisms behind restricted gene flow in relict Quercus robur stands

We observed only marginal effects of isolation by distance in explaining genetic differentiation; thus, other processes must be triggering levels of gene flow in addition to simple geographic distance effects. *Quercus robur* stands in the Jerte valley exchanged little pollen,

and the marked genetic structure of the adult population clearly indicates that this weak connectivity is not only a short-term phenomenon. It is particularly remarkable since oaks and other wind-pollinated forest tree species are known for their great ability to disseminate pollen over great distances (Schueler & Schlünzen 2006; Kremer *et al.* 2012). For instance, Hampe *et al.* (2013) observed 6% of pollen inflow into a small, marginal holm oak (*Quercus ilex*) population 30 km ahead of the nearest larger stands. Buschbom *et al.* (2011) reported that no <35% of all mating events involved immigrant pollen in a small *Q. robur* stand whose closest conspecifics grow >80 km away. And Robledo-Arnuncio & Gil (2005) detected 4.3% of pollen immigration in a *Pinus sylvestris* stand 30 km apart from other populations. So why is gene flow so much more restricted in our study system?

At least two mechanisms arising from the relictual character of our stands could help explain the phenomenon. The first is related to their refugial environment: almost all stands grow within dense riparian forests along water courses that are surrounded by rugged terrain (typically gorges). This setting provides the humidity required by *Q. robur* to withstand the dry Mediterranean summers typical of the region. But it also represents a significant obstacle to both the departure and the arrival of oak pollen. The effect of this refugial habitat is probably twofold. First, by creating abundant small-scale turbulences and weakening thermal air uplift, fine-scale topography and the dense canopy reduce the probability of pollen grains to enter free atmosphere layers where directed air flows could transport them over longer distances (cf. Dupont & Brunet 2008; Dobrowski 2010). Second, the humid environment of these riparian forests renders floating pollen likely to be 'captured' by sticky surfaces such as leaves or branches. This phenomenon is known from tropical rain forests and has been used to explain the scarcity of wind-pollinated species in this biome (e.g. Turner 2001). Our study suggests that it could also constrain gene flow when wind-pollinated species are confined to other humid habitats. Relict trees that persist in these refugial microenvironments may hence result environmentally 'trapped' in terms of dispersal limitation and realized gene flow.

The second mechanism combines environmental and genetic components and refers to the observed high rates of mating between neighbours. Our study stands have apparently persisted through extended periods of time in isolation and at small population size under the constraints of their scattered refugial habitat. This situation has provided opportunities for genetic purging, while the great ability of trees to maintain within-population diversity (Petit & Hampe 2006) would have limited negative genetic effects on population regeneration and viability. This suggests that incompatibility and inbreeding depression do not profoundly hinder extensive mating within refugial populations. An abundant local pollen production, such as in the year of our study, can hence easily saturate stigmas and thwart the arrival of foreign pollen. This so-called pollen 'swamping' from nearby trees has been described from closed oak forests (Lagache *et al.* 2013) and should be even much stronger in the highly disjunct stands of the Jerte valley. On the contrary, cases of extensive pollen inflow into geographically isolated populations typically come from genetically strongly impoverished, little stands where adult trees are likely to experience compatibility issues (e.g. Buschbom *et al.* 2011; Hampe *et al.* 2013). Our findings of moderate genetic diversity and relatively low extent of family structures give support to this mechanism as a plausible cause of the extensive within-stand mating in these relict populations. In addition, two lines of evidence indicate that inbreeding depression is not a major issue in our study system: (i) the size of acorns is well known to trigger seedling establishment and is hence used as a proxy for fitness (e.g. Hampe *et al.* 2013); yet the size and the progeny inbreeding of our acorns were uncorrelated (Pearson $r = 0.16$, $t = 1.38$, df = 70, $P = 0.17$); (ii) a concomitant greenhouse experiment with >1500 seedlings revealed no differences in the performance of individuals from small and from large stands (G. Moreno, unpublished). Our peculiar case study and its unusual results hence suggest that compatibility issues and inbreeding depression could be a key driver shaping long-distance dispersal patterns of small populations of wind-pollinated trees (see also Kremer *et al.* 2012).

Insights into organism–environment relationships within microrefugia

The functioning of microrefugia and their role for the survival of species in a rapidly changing climate are the object of rapidly growing attention (e.g. Hampe & Jump 2011; Gavin *et al.* 2014; Woolbright *et al.* 2014). Refugia offer pockets of suitable climate space where climatic conditions in the surroundings do not permit the existence of the species (Dobrowski 2010). Long-term persistence in strong isolation and resulting microevolutionary differentiation has converted many refugial populations in conservation targets of high priority (Hampe & Petit 2005). Their isolation is usually attributed to their highly scattered distribution within a climatically adverse landscape matrix. Our study suggests that not only the climatic conditions around refugia but also those within them can constrain the connectivity of the populations that they harbour. This notion adds an important piece to

our understanding of organism–environment relationships and population dynamics at species' low-latitudinal range margins.

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A.H. conceived the research. G.M., A.H. and E.M. performed the field sampling and E.M. performed the laboratory analyses. E.M. analysed the data with help from P.J. and A.H. E.M., A.H. and P.J. wrote the study. All four authors reviewed the complete manuscript.

Data accessibility

Sample locations, microsatellite data and phenological data: Files genotypes.xlsx and phenology_data.xlsx are available in the DRYAD repository: Moracho E., Moreno G., Jordano P. and Hampe A. (2015) Data from: Unusually limited pollen dispersal and connectivity of Pedunculate oak (*Quercus robur*) refugial populations at the species' southern range margin. Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.j3s5f.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Nuclear microsatellite marker diversity across all ten *Quercus robur* stands.

Table S2. Genetic diversity of adult trees for each stand.

Table S3. Pairwise differentiation (F_{ST}) and pairwise conditional genetic distances (cGD) among stands.

Table S4. Probability of non-assigned acorns to stem in first generation from a hybridization event with *Quercus pyrenaica*.

Fig. S1. Genetic diversity accumulation curves for the acorn progenies of some illustrative mother trees.

Fig. S2. STRUCTURE bar plots of ancestry proportions for K = 2 clusters using as sample unassigned acorns (n = 35), *Quercus pyrenaica* (n = 109) and *Quercus robur* (n = 135) trees.

Fig. S3. Posterior probability and change in likelihood used for detecting the K value that best described the adult genotypes.