Tree density and population size affect pollen ﬂow and mating patterns in small fragmented forest stands of pedunculate oak (*Quercus robur* L.)

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# a b s t r a c t

In many parts of the world, once large and continuous forests have been replaced by a mosaic of isolated forest fragments embedded in an agricultural or urban landscape matrix. At the same time, stand char- acteristics such as tree population size and tree density have been strongly modiﬁed as a result of anthro- pogenic activities. Because increased geographical isolation can impede gene ﬂow among forest fragments, and because decreased population size and tree density may reduce the number of local mat- ing partners, this can be expected to lower the opportunity to trees for outcrossing, and to result in increased inbreeding, negatively impacting the viability of fragmented tree populations. In this study, we examined eight isolated stands of the wind-pollinated tree species *Q*. *robur* that strongly differed in population size, tree density and spatial isolation. In the centre of each *Q*. *robur* stand, adult leaves and seeds were collected in a circular plot, in which we evaluated the diversity and differentiation of the local pollen pool, and examined mating patterns. Most forest stands showed high proportions of out-of-plot pollen ﬂow (range: 0.24–0.77), which were positively correlated with the number and density of adult trees within the forest stands. Despite high outcrossing rates (>0.998), seeds within seed families were stronger related than what could be expected under panmixia, which could be attributed to small but signiﬁcant levels of correlated paternity (0.018–0.107) and biparental inbreeding (0.025–0.118) within the study plots. Next to increased coancestry coefﬁcients, deviations from random mating also resulted in signiﬁcant pollen pool differentiation (0.008–0.059) among seed parents. We also found that stand population size and tree density were signiﬁcantly correlated to the relatedness of the seedlings and the degree of pollen differentiation within the study plots. These results suggest that, in small and isolated low density forest stands, reduced mate availability may decrease local pollen pool diversity, increasing the likelihood of consanguineous mating and pollen pool differentiation in the next genera- tions. We conclude that preserving high levels of pollen ﬂow within and between forest fragments may be more important in wind-pollinated tree species than what was previously thought.

1. Introduction

In many parts of the world, once large and continuous forests have been replaced by a mosaic of forest fragments embedded in an agricultural or urban matrix ([Riitters et al., 2000](#_bookmark20)). Stand charac- teristics such as tree population size and tree density can also be strongly modiﬁed following anthropogenic disturbance, which in turn may have important consequences for tree pollen ﬂow patterns and mating ([Ouborg et al., 2006; Eckert et al., 2010](#_bookmark20)). Increased geographical isolation can impede gene ﬂow among for- est fragments, whereas decreased population size and tree density

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reduce the number of local mating partners within forest stands ([Smouse and Sork, 2004; Breed et al., 2013b](#_bookmark21)). Altogether, this can be expected to lower the opportunity for trees to outcrossing and can result in increased inbreeding, which may in the long term negatively impact on the genetic diversity and viability of frag- mented tree populations ([Ellstrand and Elam, 1993; Young et al.,](#_bookmark22) [1996; Vranckx et al., 2012](#_bookmark22)).

Although there is evidence of pollen limitation in tree species ([Sork et al., 2002; Fernández-Manjarrés and Sork, 2005; Jump and](#_bookmark23) [Peñuelas, 2006; Breed et al., 2012](#_bookmark23)), the extent of it remains highly debated in forest conservation genetics, resulting in what is known as the ‘paradox of forest fragmentation genetics’ ([Kramer et al.,](#_bookmark36) [2008](#_bookmark36)). Some studies have indeed not found a reduction of the diver- sity of the pollen pool following forest fragmentation ([O’Connell](#_bookmark47)

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[et al., 2007](#_bookmark47)), or have even demonstrated an increased diversity ([Bacles et al., 2005](#_bookmark13)). The latter can be explained by the smaller size and the lower tree density of forest fragments. When there are fewer nearby trees available as a pollen source, the proportion of pollen from distant (unrelated) trees increases in the local pollen pool, and consequently the average realized pollination distance will be larger ([Robledo-Arnuncio et al., 2004; Bacles and Ennos,](#_bookmark20) [2008; Wang et al., 2010](#_bookmark20)). Moreover, a reduction in tree density and opening up of the landscape may improve air movements within and between forests ([Okubo and Levin, 1989](#_bookmark20)). This can promote pollen ﬂow, both in wind-pollinated tree species, which produce large amounts of small and lightweight pollen ([Young](#_bookmark48) [et al., 1993; Dyer and Sork, 2001; Bacles et al., 2005](#_bookmark48)), as well as in animal pollinated tropical trees ([Dick, 2001; Dick et al., 2008](#_bookmark22)). Such enhanced realized pollen dispersal can ultimately increase the effective population size and thus mitigate the expected negative impacts of forest fragmentation on genetic diversity ([Sork and](#_bookmark24) [Smouse, 2006; Bacles and Jump, 2011](#_bookmark24)).

However, when the number of adult trees within a forest stand decreases too strongly, the larger average effective pollination dis- tances may not totally compensate for the reduction in population size ([Sork et al., 2002; Sork and Smouse, 2006](#_bookmark23)). This may not only affect the ﬁtness of future offspring cohorts through increased sel- ﬁng and biparental inbreeding ([Sork et al., 2002; Breed et al.,](#_bookmark23) [2013b](#_bookmark23)), but smaller pollen loads may also reduce the diversity of the local pollen pool, through which the proportion of recessive deleterious alleles within the paternal mating pool increases ([Goubitz et al., 2002; Breed et al., 2012](#_bookmark25)). In addition, supplementa- tion of the local pollen pool with high rates of out-of-plot pollen ﬂow is not necessarily a safeguard against genetic bottlenecks, as the number and diversity of pollen sources from outside a forest fragment that contribute to the local pollen pool can be small ([Sork and Smouse, 2006](#_bookmark24)). [Fernández-Manjarrés and Sork (2005)](#_bookmark22) found higher pollen immigration rates of *Quercus humboldtii* Bonpl. in small forest fragments, but genetic diversity in the seedling cohort was signiﬁcantly lower, suggesting that only a small effec- tive number of pollen donors contributed to the pollen pool of the fragmented forest stands.

A better understanding of the combined effects of reduced adult numbers and average pollination distances on the local pollen pool diversity is indispensable for understanding the genetic conse- quences of forest fragmentation ([Sork and Smouse, 2006](#_bookmark24)). In this study, we investigated the effects of different stand characteristics on mating patterns and pollen-mediated gene ﬂow in forest stands of the common temperate broadleaved tree species pedunculate oak (*Quercus robur* L.). Studies that have documented the relation- ship between a range of population parameters (population size, tree density and isolation) on the one side and mating and pollen ﬂow patterns on the other in more than 4 forest stands are relatively rare ([Robledo-Arnuncio et al., 2004; Byrne et al., 2007;](#_bookmark20) [Breed et al., 2013b; Ismail et al., 2012; Llorens et al., 2012](#_bookmark20)), and we are unaware of such studies in temperate deciduous tree spe- cies. We selected eight forest stands that strongly differed in population size, tree density and isolation and which were located in a matrix of agricultural land or of stands of different tree species (*Pinus sylvestris* L. or *Fagus sylvatica* L.). The oak stands are representative for the typically strongly fragmented forest land- scape of Northern Belgium (most fragments <5 ha), but also for other densely populated and highly urbanized regions in Europe ([Vandekerkhove, 2013](#_bookmark38)).

More speciﬁcally, we aimed at (1) evaluating the proportion of seeds that originated from fathers within the established study plots compared to pollen-mediated gene ﬂow from outside the study plots, based on a traditional (maximum likelihood) paternity analy- sis ([Marshall et al., 1998](#_bookmark43)); (2) examining local pollen donor diversity and mating system parameters using both a correlated-mating

model ([Ritland, 2002](#_bookmark20)) and a two-generation analysis of pollen ﬂow within forest stands ([Smouse et al., 2001](#_bookmark26)); and (3) clarifying the role of stand characteristics such as population size, tree density, isola- tion and the type of the landscape matrix on local pollen pool diver- sity and mating system parameters.

1. Materials and methods
	1. *Study species*

Pedunculate oak (*Q*. *robur* L.) is a common tree species of many European deciduous lowland forests. It occurs from sub-Mediterra- nean Europe to southern Scandinavia, and eastwards to the Ural Mountains ([Bary-Lenger and Nebout, 1993](#_bookmark14)). Although *Q*. *robur* has a wide ecological range, it grows best on neutral, well-drained soils.

*Q*. *robur* is monoecious and possesses a nearly complete gameto- phytic self-incompatibility system. As a result, selﬁng rates are low (2–5%; [Steinhoff, 1993](#_bookmark31)). Separate staminate and pistillate ﬂow- ers that are carried on the same branches further contribute to the low selﬁng rates. Trees ﬂower generally from April to the end of May in Belgium ([Ducousso et al., 1993](#_bookmark22)). The pollen of pedunculate oak is one of the smallest (26–29 lm, [Rushton, 1976](#_bookmark20)) and lightest among wind-pollinated woody plant species, enhancing the poten- tial for long-distance pollen ﬂow ([Chybicki and Burczyk, 2010](#_bookmark15)). Eight to ten weeks after pollination, fertilization and rapid fruit (acorn) development occur. Acorns of *Q*. *robur* are primarily dis- persed beneath the canopy of the mother tree by gravity ([Streiff](#_bookmark33) [et al., 1998](#_bookmark33)), although dispersal by rodents and especially birds (most notably the European Jay (*Garrulus glandarius* L.)) is known to substantially increase the long-distance potential component of the seed dispersal kernel ([Gómez, 2003; Moran and Clark, 2011](#_bookmark27)).

* 1. *Study sites and sampling*

We sampled eight pedunculate oak stands across the Flanders region of Belgium. *Q*. *robur* was the dominant tree species in these forest stands, and the understory was mainly composed of *Q*. *robur* seedlings, *Rubus* species and small trees of *Acer pseudoplatanus* and *Corylus avellana*. Only the crowns of *Q*. *robur* adult trees reached the upper canopy layer. Stand characteristics were determined from forest registers (Bosprog-Bosdat v. 2.33, ANB 2006), using GIS data layers of the studied forest stands and from measurements with a FieldMap digital forest mapping system (IFER Ltd., Czech Republic). Stands differed in size (range: 32–704 individuals), tree density (range: 52–195 individuals/ha) and degree of isolation (distance from the nearest *Q*. *robur* stand, range: 135–5000 m) ([Table 1](#_bookmark4)). The sampled stands were either located in a matrix of forest com- posed of other tree species, or in a matrix of agricultural land. In the centre of each *Q*. *robur* stand we established a circular plot con- taining ca. 35 adult trees ([Table 1](#_bookmark4)). Trees were mapped to the near- est cm using a FieldMap digital forest mapping system and from each sampled tree a leaf was sampled for microsatellite analysis. Within each circular plot, ﬁve seed traps (1.5 m2 each) were ran- domly placed, with each seed trap positioned under the canopy of a single mother tree. From each seed trap ten acorns were randomly collected in the Autumn of 2011. Since 2011 was a mast year for pedunculate oak in Flanders ([Sioen and Roskams, 2012](#_bookmark20)), acorn pro- duction was high and most seed traps contained plenty of acorns. The collected acorns were stored at 5 °C for 4 weeks to increase the percentage and synchronization of seed germination ([Manzanera et al., 1993](#_bookmark42)). After cold storage the acorns were sown in seed trays containing commercial soil (20% organic matter, pH = 6, EC = 750 lS cm-1, N:P:K 14:16:18 1 kg m-3), and grown in a greenhouse under controlled environmental conditions (12/ 12 h day/night light regime, 20 °C). One month after seedlings

Table 1

Characteristics of the eight studied pedunculate oak stands.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Site | Latitude (N) | Longitude (E) | Area (ha) | Population size | Density (trees/ha) | Isolation (m) | Landscape matrix | Plot size (ha) | Sampled adults | Sampled seedlings | Number of families[a](#_bookmark4) |
| Keffers | 50°5002600 | 4°4200000 | 3.04 | 328 | 118 | 400 | Forest | 0.49 | 35 | 50 | 4 |
| Vos | 50°4902700 | 4°3903300 | 3.97 | 682 | 195 | 175 | Forest | 0.24 | 35 | 50 | 5 |
| Hornebos | 50°4300200 | 5°1503400 | 4.50 | 242 | 65 | 1000 | Agriculture | 0.78 | 37 | 50 | 4 |
| Chartreuse | 50°5404500 | 4°4602500 | 0.43 | 32 | 74 | 135 | Forest | 0.43 | 32 | 49 | 5 |
| Overheide | 51°2501700 | 5°0301600 | 1.05 | 139 | 132 | 360 | Forest | 0.28 | 36 | 48 | 5 |
| Hoge Vijvers | 51°2102800 | 5°0602700 | 3.80 | 704 | 186 | 240 | Forest | 0.19 | 36 | 49 | 5 |
| Meikensbos | 50°5804000 | 3°2205200 | 1.64 | 171 | 104 | >2 km | Agriculture | 0.33 | 35 | 50 | 5 |
| Egemse | 51°0200200 | 3°1605900 | 6.24 | 324 | 52 | >5 km | Agriculture | 0.88 | 35 | 50 | 5 |

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a Number of families used for the estimation of gene ﬂow and mating system parameters in the 8 stands.

emerged, leaves were taken from each individual for DNA extraction.

* 1. *Microsatellite analyses*

Leaf samples from both adult trees and seedlings were stored on silica gel prior to DNA extraction. 200 mg dried leaf tissue was homogenized to a ﬁne powder followed by DNA extraction with the Nucleospin Plant II kit (Macherey–Nagel). For the micro- satellite analysis ten microsatellites were used, which were iso- lated from *Q*. *robur*: QrZAG112 ([Kampfer et al., 1998](#_bookmark35)), *Quercus petraea*: QpZAG9, QpZAG108, QpZAG46, QpZAG15, QpZAG110,

QpZAG104 ([Steinkellner et al., 1997](#_bookmark32)) and *Quercus macrocarpa*: MSQ4, MSQ13, MSQ16 ([Dow et al., 1995; Dow and Ashley, 1996](#_bookmark22)). Microsatellites were ampliﬁed in three multiplex PCRs using the Multiplex PCR Master Mix kit (QIAGEN). PCR cycling was carried out in a thermocycler programmed as follows: 30 rounds of 45 s 94 °C, 45 s 50 °C and 45 s 72 °C and a ﬁnal extension step of 10 min at 72 °C. The PCR products were analyzed on an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Micro- satellite alleles were visualized and scored using GeneMapper v.

4.1. We checked the microsatellites for possible genotyping errors such as null alleles, stutter peaks and large allele drop-out, using the software Micro-Checker ([Van Oosterhout et al., 2004](#_bookmark37)). The fre- quencies of null alleles were estimated using GENEPOP 4.0 ([Rousset, 2008](#_bookmark20)). One locus (MSQ16) showed consistently high null allele frequencies (0.05–0.15), and was therefore removed from further analyses. In most forest stands, the 9 remaining microsat- ellites showed low null allele frequencies (<0.05) (Supplementary Table 1).

* 1. *Data analysis*

First, we veriﬁed whether the adult trees that covered the seed traps with their canopies were seed parents by performing a sim- ple exclusion analysis based on the multilocus genotypes of seed- lings and mother trees ([Nakanishi et al., 2005](#_bookmark44)). Seedlings with genotypes incompatible with those of their putative mothers were eliminated from the seed families that were used in further analy- ses. To investigate the effect of stand characteristics on pollen ﬂow from distant and nearby trees, we estimated the proportion of pol- len that originated from fathers within the study plot in each forest stand. Therefore, we performed a maximum likelihood paternity analysis using CERVUS 3.0.3. ([Marshall et al., 1998](#_bookmark43)) to assign pater- nity to the grown seedlings. The used set of microsatellite markers yielded high exclusion probabilities for paternal assignment (>0.995). The most likely father trees were detected using LOD (log-likelihood ratio) scores ([Marshall et al., 1998](#_bookmark43)), which were based on the following simulation parameters: 10,000 simulated offspring, 0.01 (default settings) as the proportion of mistyped loci, and the number of candidate fathers ranging between 32 and 704

individuals (all mature trees in the studied forest stands). Father- offspring matches were conducted using the CERVUS 80% thresh- old level, and were veriﬁed by a straightforward exclusion analysis based on the genotypes of seedlings and adult trees ([Nakanishi](#_bookmark44) [et al., 2005](#_bookmark44)). When the threshold LOD-score for paternity was exceeded, the adult tree with the highest LOD-score, was desig- nated as the father tree of a seedling. Based on the Euclidean dis- tance between pollen donors and maternal trees (distance from the stem), we calculated the average realized pollination distances within the study plots.

Since forest fragmentation may lower the opportunity to trees for outcrossing and potentially result in increased inbreeding, we characterized in each study plot a range of mating system param- eters. This was done by using the mixed and correlated mating model implemented in the software package MLTR version 3.2 ([Ritland, 2002](#_bookmark20)). MLTR was only applied to seed families composed of more than 5 seedlings per mother tree. Based on maximum likelihood procedures, we estimated single-locus (*ts*) and multilo-

cus (*tm*) outcrossing rates, biparental inbreeding (*tm*–*ts*), and mul-

tilocus correlated paternity (*rp*). Standard errors and conﬁdence intervals (95%) were estimated for the mating system parameters using 1000 bootstrap replicates, re-sampling families within forest stands. Deviations from random mating through biparental inbreeding and correlated paternity may possibly increase the relatedness of the seedlings within seed families. This was examined by using the results of the mating system analysis for

the calculation of the average coancestry coefﬁcient within fami- lies (H*xy*), which was estimated following [Sousa et al. (2005)](#_bookmark29): H*xy* ¼ 0:125ð1 þ *FP* Þð1 - *rp* Þ, with *FP* the inbreeding coefﬁcient in the parental generation. Additionally, we investigated the genetic structure within progeny by estimating the variance effective size within seed families: *Ne*ð*v*Þ ¼ 0:5=H*xy* ([Cockerham, 1969](#_bookmark22)). In an ide- alized panmictic population the maximum value of *Ne*(*v*) within families corresponds to four unrelated individuals. However, devi- ations from random mating may reduce *Ne*(*v*). Also the microsatel- lite data of the 35 adult trees within the 8 study plots was examined for possible signiﬁcant spatial autocorrelation, by calcu- lating the Nason’s kinship coefﬁcient *Fij* ([Loiselle et al., 1995](#_bookmark39)) and their conﬁdence intervals (95%; 10,000 permutations) using SPA- GeDi 1.3 ([Hardy and Vekemans, 2002](#_bookmark28)) (Supplementary Fig. 2).

The diversity of the male gametes that contribute to the off- spring of each mother tree may also shape the differentiation of the pollen gene pool among seed trees. To quantify the divergence in sampled pollen pools among the mother trees, the pollen pool differentiation (U*FT*) among maternal plants was estimated with the TWOGENER method ([Smouse et al., 2001](#_bookmark26)) as implemented in the software package POLDISP 1.0c ([Robledo-Arnuncio et al.,](#_bookmark20)

[2007](#_bookmark20)). This analysis uses an AMOVA (analysis of molecular vari- ance) approach, and is based on the genotypes of the seed families and their seed trees combined with spatial data of the mother trees ([Excofﬁer et al., 1992](#_bookmark22)). Conﬁdence intervals (95%) around U*FT*

estimates were computed using 10,000 permutations among prog- eny. Finally, we also calculated the total genetic differentiation between families within the eight studied forest stands. This was done based on Nei’s unbiased genetic distance (D) between seed families using GENALEX (version 6.2; [Peakall and Smouse, 2006](#_bookmark20)).

* 1. *Correlations of pollen ﬂow and mating system parameters with forest stand characteristics*

A Spearman’s rank correlation coefﬁcient (*rs*) was used to inves- tigate the relationships between stand characteristics (population size, tree density and isolation) and measures of pollen ﬂow and mating system parameters. We applied a nonparametric test, since the number of data points was limited. The stand characteristics

were not signiﬁcantly correlated to each other (*rs*(size vs. isolation) = 0;

signiﬁcantly higher genetically realized pollen ﬂow distances within the study plot (*rs* = -0.98, *P* < 0.001, [Fig. 1](#_bookmark7)D). Furthermore, these effective pollination distances were signiﬁcantly higher in forest stands surrounded by agricultural land (*Z* = -2.24, *P* = 0.036). Reductions in stand size or conspeciﬁc density did not alter the opportunity for outcrossing in the studied oak plots, as all forest stands showed high multilocus outcrossing rates (*tm* > 0.998), indi- cating that selﬁng was almost completely lacking (*s* 6 0.002, [Table 3](#_bookmark8)). Moreover, the level of biparental inbreeding (*tm*–*ts*) was also low (0.025–0.118), although values were signiﬁcantly differ- ent from zero in all study plots. The average biparental inbreeding per study plot was signiﬁcantly and positively related to the esti- mated mean genetic differentiation (Nei’s D) between seed fami- lies within study plots (*rs* = 0.83, *P* = 0.010, [Fig. 2](#_bookmark10)A). This suggests that mating between relatives could lead to stronger genetic differ-

*s*(size vs. density)

*r*

= 0.48 and *rs*(density vs. isolation)

= -0.59, all *P* > 0.05).

ences among the progenies of different mother trees. Next to bipa-

rental inbreeding, most study plots showed a signiﬁcant (*P* < 0.05)

The effect of the landscape matrix (forest vs. agricultural land) on

the estimated pollen ﬂow and mating system parameters was examined using a Mann–Whitney U test. The forest stands that were located within agriculture land were characterized by a higher degree of isolation and tended to have lower tree densities. All statistical analyses were performed using SPSS (SPSS 20.0; SPSS Inc., Chicago, IL).

1. Results

A high percentage (>90%) of seedlings was successfully grown from the acorns collected in the seed traps. In total, we sampled leaves of 281 adult trees and 396 grown seedlings. The nine micro- satellites were highly variable, with the number of alleles per locus ranging from 11 (QpZAG9) to 36 (QpZAG104), with an average of

22.9 (±2.8) (means are followed by SE in parentheses). Most of the 396 grown seedlings (96%) showed genotypes that were con- sistent with the genotypes of their putative mother trees. In total, we detected 38 seed families that were composed of more than 5 seedlings per mother tree, and which were used for the estimation of gene ﬂow and mating system parameters in the 8 stands ([Table 1](#_bookmark4)).

When we compared the proportion of pollen that originated from nearby trees (within the study plot) with that of more distant trees, we found extensive pollen ﬂow (0.50–0.77) from outside the study plot in most forest stands. Only in the stands Chartreuse and Hornebos a larger proportion of seeds originated from fathers within the study plot ([Table 2](#_bookmark5)). Stand characteristics signiﬁcantly inﬂuenced the distribution of the pollen pools, as the proportion of pollen donors found within the study plots was signiﬁcantly related to the size of the stands (*rs* = -0.74, *P* = 0.037, [Fig. 1](#_bookmark7)A) and

tree density (*rs* = -0.74, *P* = 0.037, [Fig. 1](#_bookmark7)C). Besides decreased

out-of-plot pollen ﬂow, low density forest stands also showed

presence of full sibs within the seed families, only in the stands Keffers and Hoge Vijvers the 95% conﬁdence intervals of correlated paternity (*rp*) included zero ([Table 3](#_bookmark8)). The above deviations from random mating also increased the relatedness of the seedlings within seed families. The average coancestry coefﬁcient (H*xy*) was higher (0.132–0.142) than the value expected in half-sib fam- ilies (H*xy* = 0.125), whereas the variance effective size within seed families (*Ne*(*v*)) was lower than 4 (3.52–3.80), indicating the pres- ence of an increased genetic structure within the progeny cohort. Contrary to the grown seedlings, no signiﬁcant genetic structure within the adult generation was found in the 8 study plots, as the mean kinship coefﬁcient *Fij* fell within the 95% conﬁdence interval for all distance classes (Supplementary Fig. 2). Although the range in coancestry coefﬁcients was restricted, stand charac- teristics inﬂuenced the relatedness of the seedlings, as H*xy* was sig- niﬁcantly negatively related to tree density (*rs* = -0.76, *P* = 0.028, [Fig. 1](#_bookmark7)E) and increased with increasing pollen ﬂow distances within the study plots (*rs* = 0.83, *P* = 0.010). Furthermore, signiﬁcantly higher estimates of H*xy* were found in forest stands that were sur- rounded by a matrix of agricultural land (*Z* = -2.26, *P* = 0.036). Mating system parameters were also affected by the diversity of the male gametes that contributed to the pollen pool. Both *rp* and H*xy* estimates signiﬁcantly decreased with increasing pollen ﬂow from outside the study plot (*rs* = -0.81, *P* < 0.015, [Fig. 2](#_bookmark10)B and *rs* = -0.79, *P* < 0.021 respectively).

Finally, the estimates of the TWOGENER analysis showed signif-

icant pollen pool differentiation among maternal plants (U*FT*) in most of the forest stands (95% CI excludes zero) and were signiﬁ- cantly negatively correlated to stand size (*rs* = -0.79, *P* = 0.021, [Fig. 1](#_bookmark7)B). Pollen pool differentiation among mother trees was also affected by the relatedness of the seedlings, as we found that U*FT* estimates increased with increasing values of H*xy* (*rs* = 0.74, *P* = 0.037, [Fig. 2](#_bookmark10)C).

Table 2

The results of the maximum likelihood paternity analysis conducted with CERVUS 3.0.3.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Forest stand | *N*seeds | *N*local fathers | EPp | Proportion local fathers | Proportion of the seed cohort with different | Mean pollen dispersal |
|  |  |  |  |  | father trees | distance (m) [a](#_bookmark6) |
| Keffers | 45 | 13 | 0.998 | 0.289 | 0.86 (0.07) | 18.8 (2.9) |
| Vos | 45 | 14 | 0.999 | 0.311 | 0.66 (0.22) | 13.4 (1.9) |
| Hornebos | 46 | 35 | 0.999 | 0.761 | 0.63 (0.14) | 32.9 (2.5) |
| Chartreuse | 49 | 29 | 0.999 | 0.592 | 0.64 (0.09) | 20.0 (2.8) |
| Overheide | 46 | 15 | 0.998 | 0.326 | 0.75 (0.19) | 17.2 (2.1) |
| Hoge Vijvers | 48 | 11 | 0.995 | 0.229 | 0.73 (0.18) | 17.0 (2.6) |
| Meikensbos | 50 | 25 | 0.998 | 0.500 | 0.74 (0.12) | 22.4 (3.1) |
| Egemse Veldekens | 48 | 21 | 0.998 | 0.438 | 0.89 (0.07) | 47.5 (8.5) |

*N*seeds, the number of seeds examined in the paternity analysis; *N*local fathers, the number of seeds assigned to a father tree from within the study plot; EPp, exclusion probabilities for paternal assignment; proportion local fathers, *N*local fathers/*N*seeds.

a Within the study plot.

Fig. 1. Spearman rank correlations (*rs*) between stand characteristics and mating system and pollen dispersal parameters for eight forest stands of *Quercus robur*. (A) Percentage pollen donors from within the study plot (based on paternity assignment, CERVUS) vs. population size, (B) pollen pool differentiation (U*FT*, based on TWOGENER method) vs. population size, (C) percentage pollen donors from within the study plot vs. tree density, (D) mean pollen dispersal distance within study plots (based on paternity assignment) vs. tree density, (E) coancestry coefﬁcient within families (H*xy*, based on estimates obtained with MLTR) vs. tree density.

Table 3

Mating system estimates and pollen pool differentiation (with standard errors in parentheses) for the eight studied *Q*. *robur* stands.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Forest stand | *tm* | *ts* | *tm*–*ts* | *rp* | 1/*rp* | H*xy* | *Ne*(*v*) | U*FT* |
| Keffers | 0.998 (0.000) | 0.949 (0.009)[\*](#_bookmark9) | 0.049 (0.009)[\*](#_bookmark9) | 0.033 (0.021) | 30.3 | 0.134 | 3.73 | 0.009 (0.007) |
| Vos | 0.998 (0.001) | 0.948 (0.010)[\*](#_bookmark9) | 0.050 (0.009)[\*](#_bookmark9) | 0.057 (0.020)[\*](#_bookmark9) | 17.5 | 0.132 | 3.80 | 0.008 (0.007) |
| Hornebos | 0.998 (0.000) | 0.973 (0.006)[\*](#_bookmark9) | 0.025 (0.006)[\*](#_bookmark9) | 0.083 (0.017)[\*](#_bookmark9) | 12.1 | 0.140 | 3.57 | 0.034 (0.008)[\*](#_bookmark9) |
| Chartreuse | 0.998 (0.000) | 0.937 (0.012)[\*](#_bookmark9) | 0.061 (0.012)[\*](#_bookmark9) | 0.078 (0.026)[\*](#_bookmark9) | 12.8 | 0.135 | 3.70 | 0.059 (0.007)[\*](#_bookmark9) |
| Overheide | 0.999 (0.001) | 0.936 (0.008)[\*](#_bookmark9) | 0.062 (0.007)[\*](#_bookmark9) | 0.028 (0.005)[\*](#_bookmark9) | 35.7 | 0.135 | 3.71 | 0.026 (0.005)[\*](#_bookmark9) |
| Hoge Vijvers | 0.999 (0.000) | 0.881 (0.010)[\*](#_bookmark9) | 0.118 (0.010)[\*](#_bookmark9) | 0.018 (0.011) | 55.6 | 0.134 | 3.73 | 0.025 (0.005)[\*](#_bookmark9) |
| Meikensbos | 0.998 (0.000) | 0.956 (0.008)[\*](#_bookmark9) | 0.042 (0.008)[\*](#_bookmark9) | 0.107 (0.042)[\*](#_bookmark9) | 9.4 | 0.142 | 3.52 | 0.038 (0.006)[\*](#_bookmark9) |
| Egemse Veldekens | 0.999 (0.000) | 0.939 (0.011)[\*](#_bookmark9) | 0.060 (0.011)[\*](#_bookmark9) | 0.039 (0.008)[\*](#_bookmark9) | 25.6 | 0.136 | 3.68 | 0.012 (0.006)[\*](#_bookmark9) |

*tm*, Multilocus outcrossing rate; *ts*, singlelocus outcrossing rate; *tm*–*ts*, biparental inbreeding; *rp*, correlated paternity estimated with Ritland’s mixed mating model (2002); 1/ *rp*, effective number of pollen donors; H*xy*, coancestry coefﬁcient within families; *Ne*(*v*), variance effective population size within families; U*FT*, Pollen pool differentiation among maternal plants estimated with the TWOGENER method ([Smouse et al., 2001](#_bookmark26)).

\* Signiﬁcantly less than 1 for *tm* and *ts*, and signiﬁcantly greater than zero for *tm*–*ts*, *rp* and U*FT* (*P* < 0.05).

Fig. 2. Spearman rank correlations (*rs*) between mating system and pollen dispersal parameters for eight forest stands of *Quercus robur*. (A) Unbiased genetic differentiation between seed families (Nei’s D) vs. biparental inbreeding (*tm*–*ts*), (B) correlated paternity (*rp*) vs. the percentage pollen donors from within the study plot, (C) pollen pool differentiation (U*FT*) vs. the coancestry coefﬁcient within families (H*xy*).

1. Discussion
	1. *Rates of pollen dispersal into and within the study plots*

The high proportions of out-of-plot pollen-mediated gene ﬂow (0.24–0.77) that were found in our study, are consistent with those detected in previous gene ﬂow studies in *Quercus* species (*mp* = 0.35–0.70: [Streiff et al., 1999; Nakanishi et al., 2005;](#_bookmark34) [Valbuena-Carabana et al., 2005; Chybicki and Burczyk, 2010;](#_bookmark34) [Vranckx et al., 2014](#_bookmark34)). Similar to our study, the plots in these studies were part of larger forest stands and were thus characterized by a relatively low level of geographic isolation from neighboring oak trees (<1000 m). It can be expected that when the scale of isolation between neighboring and distant trees exceeds the scale of pollen dispersal, pollen exchange will be much more restricted, even in

wind-pollinated tree species ([Ellstrand, 1992; Sork and Smouse,](#_bookmark22) [2006](#_bookmark22)). This was demonstrated by [Schuster and Mitton (2000)](#_bookmark20) and [Bittencourt and Sebbenn (2007)](#_bookmark16), who obtained lower rates of out of plot pollen ﬂow (0.065 and 0.1 respectively) in stronger iso- lated (>2000 m to the nearest adult tree) study plots of *Pinus ﬂexilis* and *Araucaria angustifolia*. Since our study plots (35 genotyped adult trees) were surrounded by a large proportion of ungenotyped father trees, one would expect high rates of pollen ﬂow into all of our study plots. However, the proportion of seeds that originated from fathers from outside our study plots varied strongly between forest stands. For example out-of-plot pollen ﬂow was three times higher in the forest stand Hoge Vijver (0.77) compared to the plot located in Hornebos (0.24). Contrary to the ﬁndings of [Robledo-](#_bookmark20) [Arnuncio et al. (2004)](#_bookmark20), who observed increased pollen ﬂow rates after conspeciﬁc tree density decreased, we found signiﬁcant posi- tive correlations between out-of-plot pollen ﬂow and the number and density of adult trees within the forest stands. Reduced adult numbers may decrease the proportion of ungenotyped adult trees, relative to the total forest stand, whereas low conspeciﬁc density will increase the pollination distances between the 35 genotyped adults and the ungenotyped trees from outside the study plot ([Smouse and Sork, 2004; Bianchi and Cunningham, 2012; Breed](#_bookmark21) [et al., 2012](#_bookmark21)). As a result, a larger proportion of pollen in the local pollen pool may originate from adult trees within the study plot.

Based on the paternity analysis performed in *Cervus*, we also demonstrated that the Euclidean distances between pollen donors and mother trees were strongly negatively correlated with the tree density of the forest stands, and were signiﬁcantly higher in forest stands surrounded by agricultural land. It has been shown before in *Fraxinus excelsior*, *Q*. *robur* and *Q*. *petraea* that low conspeciﬁc tree density increased the average distance between mates ([Robledo-Arnuncio et al., 2004; Bacles and Ennos, 2008; Wang](#_bookmark20) [et al., 2010](#_bookmark20)). The most likely explanation for this observation is that pollen competition between distant and nearby trees decreases when there are fewer nearby trees available as a pollen donor, through which the average realized pollination distance will be greater. Furthermore, in more open landscapes, airborne pollen movement will be facilitated, as winds in open landscapes are typ- ically stronger than winds in a canopy closed forest matrix ([Nathan](#_bookmark45) [et al., 2002; Bacles et al., 2005](#_bookmark45)).

* 1. *Mating patterns and local pollen diversity*

Our results suggest that not only population size, but also tree density has a major impact on local pollen ﬂow patterns, and may ultimately inﬂuence mating patterns and local pollen diversity in small scale forestry systems of *Q*. *robur* ([Stacy et al., 1996; Kamm](#_bookmark30) [et al., 2009](#_bookmark30)). Although high outcrossing rates (*tm >* 0.998) were observed in all stands, the estimates of both the average coancestry coefﬁcient (H*xy >* 0.125) and variance effective size within families

(*Ne*(*v*) < 4) suggested that seeds within seed families were stronger related than what was expected under panmixia ([Sousa et al.,](#_bookmark29) [2005; Bittencourt and Sebbenn, 2008](#_bookmark29)). Such deviations from ran- dom mating can be attributed to several factors such as selﬁng, cor- related paternity and mating among relatives ([Robledo-Arnuncio](#_bookmark20) [et al., 2004; Bittencourt and Sebbenn, 2008](#_bookmark20)). Since oak species pos-

sess a nearly complete gametophytic self-incompatibility system ([Ducousso et al., 1993; Steinhoff, 1993](#_bookmark22)), only negligible selﬁng rates could be detected (*s* = 0.1–0.2%). In contrast, low but signiﬁcant levels of correlated paternity and biparental inbreeding were obtained, which may both have contributed to the observed genetic structure within the studied seed families. The non-signiﬁcant spatial genetic structure that was observed in the adult generation can be attributed to several factors, such as: tree planting in the past, the restricted spatial scale of the adult plots (only 32–35 trees) and the limited number of samples used in the SGS analyses.

Although the range in coancestry coefﬁcients was restricted, the relatedness of the seeds within families decreased with increasing tree density. Reduced local tree density may alter mate availability, such that the number of nearby pollen sources surrounding a mother tree decreases and the pollination distance between mates increases ([Sork et al., 2002; Eckert et al., 2010; Breed et al., 2013b](#_bookmark23)). However, this larger average effective pollination distance did not totally compensate for the reduction in conspeciﬁc tree density, through which the diversity of the pollen pool contributing to each seed tree was lower than under high tree density, which ultimately resulted in slightly more related progeny ([Robledo-Arnuncio et al.,](#_bookmark20) [2004; Sork and Smouse, 2006](#_bookmark20)). Next to tree density, the landscape matrix signiﬁcantly inﬂuenced the relatedness of the seeds within seed families. Although we found some evidence for higher pollen ﬂow distances in more open landscapes, higher coancestry coefﬁ- cients for the seedling cohort were found, indicating less diverse pollen pools compared to forest stands that were located in a matrix of other forests. This is in contrast with what was expected based on the study of [Bacles and Ennos (2008)](#_bookmark11), who found higher pollen pool diversities in forest remnants that were located in open landscapes. The effects of the landscape matrix could, however, be confounded by stand isolation and tree density in our study, as the forest stands that were surrounded by a matrix of agricultural land, were much more isolated from the nearest *Q*. *robur* stand than the stands located in a forest matrix and tended to have lower tree densities. This could have lowered the number of pollen sources contributing to the local pollen pool, both as a result of increased pollination distances between mates and through reduced gene inﬂow from outside the forest stands when isolation between stands became too high ([Sork and Smouse, 2006](#_bookmark24)). Reduced mate availability may ultimately have led to less diverse local pollen pools and stronger biparental inbreeding, through which the pro- portion of outcrossed half- and full-sibs in the progeny cohort increases ([Lowe et al., 2005; Sork et al., 2002](#_bookmark40)). High levels of pollen exchange may possibly mitigate the negative effects of small and low density forest stands on mating patterns, through enlarging the diversity of pollen sources within the local pollen pool ([Sork](#_bookmark24) [and Smouse, 2006](#_bookmark24)). Such positive effect of strong pollen ﬂow has been demonstrated in previous studies in wind-pollinated tree species ([O’Connell et al., 2006; Bittencourt and Sebbenn, 2007;](#_bookmark49) [Wang et al., 2010](#_bookmark49)), and was also conﬁrmed in our study, as we found a signiﬁcant negative correlation between pollen ﬂow from outside the study plots and the estimates of both the correlated paternity and coancestry coefﬁcient.

Next to the slightly higher coancestry coefﬁcients, the occur- rence of correlated paternity and biparental inbreeding may also produce pollen pool differentiation among seed parents ([Austerlitz and Smouse, 2001; Hardy et al., 2004; Bittencourt and](#_bookmark12) [Sebbenn, 2007](#_bookmark12)). Our study supports this, as the measures that were used to quantify genetic differentiation between pollen pools and

seed families (U*FT* and Nei’s D respectively) showed positive, signiﬁcant relationships with biparental inbreeding and correlated paternity. The estimates of pollen pool heterogeneity obtained with the TWOGENER approach (U*FT* = 0.008–0.059) were signiﬁcant in 6 out of 8 stands, and were consistent with U*FT*-values reported in previous oak studies (U*FT* = 0.011–0.087) ([Smouse et al., 2001;](#_bookmark26) [Nakanishi et al., 2005; Fernández-Manjarrés et al., 2006; Pakkad](#_bookmark26) [et al., 2008](#_bookmark26)). Furthermore, U*FT*-estimates signiﬁcantly increased with decreasing population size, suggesting less diverse local pollen pools, and consequently, an increased likelihood of consanguineous matings in small forest stands ([Robledo-Arnuncio et al., 2004](#_bookmark20)). In general, any factor (tree density, stand size, isolation) that may dis- rupt mating patterns and genetic structure within forest stands, will increase the differentiation among maternal pollen pools ([Austerlitz](#_bookmark12) [and Smouse, 2001; Gram and Sork, 2001](#_bookmark12)).

* 1. *Implications for current and future forest management*

The presence of signiﬁcant levels of correlated mating and reduced local pollen diversity may have implications for forest management aiming at maximizing genetic diversity in frag- mented *Q*. *robur* stands. First, compared to random mating condi- tions, our results suggest that a larger number of seed trees have to contribute to the offspring cohort to retain minimum effective population sizes within forest stands ([Bittencourt and Sebbenn,](#_bookmark17) [2008](#_bookmark17)). This is especially important when forest reproductive mate- rial used for artiﬁcial regeneration is harvested in one of the stud- ied forest stands. Preferably seeds should be harvested in one or several large, continuous and higher-density forest stands, to avoid the collection of inbred seed material ([Breed et al., 2013a](#_bookmark18)). Second, our data showed signiﬁcant relationships between stand charac- teristics (population size, tree density and landscape matrix) and several mating and pollen ﬂow parameters, emphasizing the role of forest management in shaping mating patterns and pollen-med- iated gene ﬂow within *Q*. *robur* stands. Removal of adult trees and decreasing tree density directly increased the genetic structure within the studied offspring cohort. This may not only affect future tree ﬁtness through increasing the likelihood of consanguineous matings (among half and full-sibs) in subsequent generations ([Young and Brown, 1999](#_bookmark46)), but the signiﬁcant levels of pollen pool differentiation and reduced local pollen diversity may directly reduce pollen competition within the paternal mating pool, through which the proportion of recessive deleterious alleles increases in the progeny cohort ([Breed et al., 2012](#_bookmark19)).

Our results should be interpreted with some caution, as the studied seedlings were grown under controlled greenhouse condi- tions, whereas natural selection in the ﬁeld may possibly shape pat- terns of relatedness across generations. A gradual decrease of less ﬁt homozygotes from the seedling cohort to the adult generation will potentially reduce the number of individuals that originated from biparental inbreeding in the adult generation. Furthermore, we collected seeds from one masting year only, whereas adult trees may reproduce during many decades. Nonetheless, light-demand- ing tree species such as *Q*. *robur* are characterized by a narrow recruitment window, restricted in time (mast years) and space (light requirement) and therefore the number of reproduction events contributing to the future adult generation are limited ([Vera et al., 2006; Bary-Lenger and Nebout, 1993](#_bookmark41)). Finally, part of the shortcomings in this study resulted from the limited number of populations and individuals that were examined. Consequently, some of the stand characteristics (isolation and density) were con- founded with the landscape matrix. To disentangle these confound- ing effects and to allow rigorous statistical testing of the effects of stand characteristics on mating and pollen ﬂow patterns, more forest stands differing in tree density, isolation and matrix type were necessary. The analyses of multiple forest stands in our study

also had a price: sample sizes in all stands were restricted. The examination of more seed trees, adults and seedlings per site would have made the estimations of gene ﬂow and mating system param- eters much more robust.

Since small and low density forest stands are common in the extremely fragmented forest landscapes of Northern Belgium and other parts of Europe ([Vandekerkhove, 2013](#_bookmark38)), high levels of pollen ﬂow within and between forest fragments should be maintained to counteract the negative genetic processes associated with forest fragmentation. However, increasing the genetic connectivity of natural regenerated forest stands is not always realistic in highly fragmented forest landscapes, through which more customized management practices are needed to maintain or increase *Ne*. Sup-

plementation of natural regeneration through large scale sowing

and planting may be the best option to maintain and increase genetic diversity in small and low-density forest stands. Selecting the best regeneration method (natural or artiﬁcial regeneration, or both) will be determined by its likelihood to maintain a high genetic diversity on the long term, and will consequently depend on the characteristics of the forest stand.

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