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ORIGINAL ARTICLE

Paternity analysis of pollen-mediated gene flow for *Fraxinus excelsior* L. in a chronically fragmented landscape

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Paternity analysis based on microsatellite marker genotyping was used to infer contemporary genetic connectivity by pollen of three population remnants of the wind-pollinated, wind-dispersed tree *Fraxinus excelsior,* in a deforested Scottish landscape. By deterministically accounting for genotyping error and comparing a range of assignment methods, individual-based paternity assignments were used to derive population-level estimates of gene flow. Pollen immigration into a 300ha landscape represents between 43% and 68% of effective pollination, mostly depending on assignment method. Individual male reproductive success is unequal, with 31 of 48 trees fertilising one seed or more, but only three trees fertilising more than ten seeds. Spatial analysis suggests a fat-tailed pollen dispersal curve with 85% of detected pollination occurring within 100m, and 15% spreading between 300m and 1900m from the source. Identification of immigrating pollen sourced from two neighbouring remnants indicates further effective dispersal at 2900m. Pollen exchange among remnants is driven by population size rather than geographic distance, with larger remnants acting predominantly as pollen donors, and smaller remnants as pollen recipients. Enhanced wind dispersal of pollen in a barren landscape ensures that the seed produced within the catchment includes genetic material from a wide geographic area. However, gene flow estimates based on analysis of non-dispersed seeds were shown to underestimate realised gene immigration into the remnants by a factor of two suggesting that predictive landscape conservation requires integrated estimates of post-recruitment gene flow occurring via both pollen and seed.

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Introduction

The accuracy of our prediction of the response of forest trees to deforestation and population fragmentation relies on an understanding of how pollen and seed movement is modified as a consequence of changes in the landscape (Sork and Smouse, 2006). Trees, which are characterised by their individual longevity, high intra-population genetic diversity, and often substantial potential for gene flow via pollen and seed, may be particularly well-equipped to withstand habitat disturbance (Hamrick, 2004). Although theoretical predictions of reduced genetic diversity and elevated inbreeding following habitat fragmentation (Young *et al.*, 1996) are upheld for a number of wind-pollinated temperate tree species (Sork e*t al*., 2002; Jump and Penuelas, 2006), a recent review of empirical studies conducted in neotropical tree species suggests that fragmentation generally has more complex effects (Lowe *et al.*, 2005).

An emerging picture is of an increase in pollen and seed-mediated gene flow across deforested landscapes (Aldrich and Hamrick, 1998; Dick, 2001; White *et al.*, 2002; Bittencourt and Stebbenn, 2007) However this enhanced gene flow does not necessarily lead to an increase in genetic diversity or reduction in inbreeding if a limited number of pollen and seed sources contribute to the gene pool (Aldrich and Hamrick, 1998; O'Connell *et al.*, 2006; Sork *et al.*, 2002). Moreover the effect of fragmentation is often not uniform over the landscape. Smaller fragments tend to receive proportionally more pollen immigration than larger fragments because of a paucity of local pollen donors (Sork and Smouse, 2006). It is clear from these considerations that to understand the genetic connectivity of tree species living in fragmented habitats requires an appreciation of the contemporary processes of dispersal and establishment and an analysis of how they are affected by the spatial

scale of fragmentation and the heterogeneity of the landscape in which the fragmentation occurs (Sork and Smouse, 2006).

The combined development of highly polymorphic microsatellite markers and statistical analysis of parentage assignment (reviewed in Jones and Ardren, 2003) have made it possible to gather empirical evidence of contemporary gene movement within various landscapes for wild animals (e.g. Hazlitt *et al.*, 2006) and plants (e.g. Bittencourt and Stebbenn, 2007). For measuring contemporary gene flow these methods have many advantages over previous approaches that relied on inferences from genetic structure and yielded estimates of historical, average values of effective migration rate (Sork *et al.*, 1999; Whitlock and McCauley, 1999). However a number of problems are beginning to emerge with adopting parentage assignment approaches for measuring gene flow in practice.

The first issue is that the microsatellite methodology widely used for genotyping has significant assay limitations that may call the accuracy of the pedigree inference into question (Dakin and Avise, 2004; Hoffman and Amos, 2005; Slavov *et al.*, 2005). Two main types of genotyping error can be distinguished, allele dropouts (Dakin and Avise, 2004) and erroneous calling of allele size (Amos *et al*., 2007) both of which can be either of a systematic or stochastic nature. While some studies suggest that it is best to discard affected loci from parentage analyses where occurrence of non-amplifying (null) alleles is suspected (Dakin and Avise, 2004), Wagner *et al.* (2006) argue that when the number of loci is low, discriminatory power may decrease dramatically as a result. They suggest that a better alternative to either removing loci or ignoring the presence of null alleles is to accommodate them within the analyses. Indeed the use of many, even moderately variable, loci rather than fewer hypervariable ones, reduces the impact of error at

any particular locus on parentage assignment (Hoffman and Amos, 2005; Slavov *et al.*, 2005). Despite the profusion of recent publications establishing that even a low genotyping error rate had non-trivial consequences for parentage and relatedness studies, quantification and publication of error rates are not yet routinely performed (Hoffman and Amos, 2005).

The second issue is that conclusions drawn from the analysis depend on the method of paternity assignment adopted and assumptions about the size and genetic composition of the population of potential paternal parents that have not been sampled (Oddou-Muratorio *et al.*, 2003; Burczyck and Chybicki, 2004). While simple exclusion is a useful starting point for paternity inference, refined statistical approaches are necessary to assess the confidence in paternity assignment (Marshall *et al.,* 1998). For example in natural tree populations it is virtually impossible to sample all trees contributing to the reproductive pollen pool. It is therefore necessary to assess the risk of excluding a candidate pollen parent on the sole grounds that it has not been sampled. Recent methods based on either Likelihood or Bayesian approximation allow us to estimate the statistical precision of a paternity assignment for a given sample of a reproductive population (Marshall *et al.*, 1998; Nielsen *et al.*, 2001; Gerber *et al.*, 2003; Araki and Blouin, 2005; Hadfield *et al.*, 2006). Overall, it is preferable to use more than one of these approaches to estimate genetic exchange among populations (Oddou-Muratorio *et al.*, 2003).

In plants a further complication with measuring interpopulation gene flow using parentage assignment arises because gene flow is effected by two asynchronous dispersal processes, the first involving pollen and the second involving seeds. The genetic material transferred between populations via pollen and incorporated into

seed present on a mother tree only brings about gene flow if that seed is recruited into the local population. Parentage assignment of recruited seedlings is difficult to track with current analytical tools (Sork and Smouse, 2006). Therefore contemporary gene flow among fragmented tree populations has often been estimated by measuring pollen movement to pre-recruitment seeds. These estimates of gene flow are reasonable if seed-mediated gene flow among existing populations is rare, and seed dispersal is primarily important for recolonisation and range expansion. However in tree species with a high potential for long-distance seed dispersal and subsequent recruitment this assumption may be invalid (Smouse and Sork, 2004) and realised gene flow following seed dispersal may differ significantly from gene flow measured in pre-recruitment seed within a population. The extent of the discrepancy between gene flow measured from parentage analysis before and after seed recruitment has still to be properly documented.

The overall objective of the current study is to describe the genetic connectivity among population fragments of common ash (*Fraxinus excelsior*) in a chronically deforested landscape in the Southern Uplands of Scotland. Previous work on these fragments has shown that they maintain high levels of genetic diversity and weak inter-fragment differentiation (Θ = 0.080), indicating that historical gene flow has not been limited (*Nm* = 3.48). We also found from an analysis of seed families, using a mixed-mating model approach (Ritland 2002), that contemporary matings are, on average, predominantly outcrossed (t_m = 0.971 ± 0.028) and using a neighbourhood model approach (Burczyk *et al.*, 2002) that contemporary effective pollen dispersal distance within the landscape averages 328 m (Bacles *et al.*, 2005). Both seeds collected from forest fragments and newly recruited seedlings were found to harbour high levels of genetic diversity comparable to that of the

adult population suggesting an essential contribution of long distance dispersal to genetic diversity in this wind-pollinated wind-dispersed species (Bacles *et al.*, 2005; 2006). The present paper complements these studies by quantifying the genetic exchange among the individual fragments brought about by pollen flow and relates this to the size and landscape context of the fragments.

To achieve this we estimate pollen-mediated gene flow and male reproductive success of local *F. excelsior* trees from a paternity analysis of non-dispersed seeds genotyped at hypervariable microsatellite markers. This is the best methodological approach currently available to address this question. Nonetheless, in full awareness of the potential limitations of the methodology, we take a number of steps in order to ensure that our estimation describes true biological phenomena. Firstly, we quantify genotyping error at marker loci and set out to minimise error due to possible mis-scoring or null alleles using a simple deterministic approach. Secondly, we use a range of paternity assignment methods to obtain a confidence interval rather than a point estimate of pollen-mediated gene flow. Lastly, we compare such estimates derived from non-dispersed seeds with those derived from seedlings establishing in the same *F. excelsior* remnants estimated by means of parent-pair analysis (Bacles *et al.*, 2006), to assess how they relate to absolute levels of genetic exchange among remnant populations.

Material and methods

Study species

Fraxinus excelsior, common ash, is a post-pioneer tree species widespread in temperate Europe and native throughout the British Isles. The phylogeography of the species is now well described (Ferrazzini *et al.*, 2007; Heuertz *et al.*, 2004; Morand *et al.*, 2002). *F. excelsior* displays a complex, polygamous sexual system (FRAXIGEN, 2005) in which individuals may be classified phenotypically across a continuum from purely male to purely female with a whole range of hermaphroditic intermediates. Hermaphrodite individuals are self-fertile and levels of seed sets are similar in hermaphrodite and female trees, but in natural populations, *F. excelsior* is preferentially outcrossed and male fertility of hermaphrodite trees appears to be much lower than that of male trees (FRAXIGEN, 2005). Fruits are dry and winged, adapted to wind-dispersal. Regular fruit bearing begins around 20 years of age but fruiting phenology will vary depending on latitude, altitude, temperature and between years with great variation from no seeding to masting (FRAXIGEN, 2005).

Sampling and data collection

The study site is a highly deforested catchment of 900ha (Moffat Dale) located 80km south of Edinburgh, Scotland (N 55° 23' 51" W 3° 19' 50"), which forms part of a glacially derived landscape in which steep sided valleys have been carved by ice. Many native tree species including *F. excelsior* are confined to steep and narrow stream sides situated at the bottom of steep valleys inaccessible to grazing herbivores. Populations of *F. excelsior* tend to be very small, comprising ten to 30 mature individuals, with no natural regeneration in grazed areas. Remnant stands are typically separated by hundreds of metres although some can be isolated by more than one kilometre. In this catchment, *F. excelsior* is present in only five

Figure 1

forest remnants, two of them within the Carrifran Burn and three others in its immediate surroundings (Figure 1, see also Bacles *et al.* 2005).

Two remnants in the bare open landscape of the Carrifran Burn, CDa and CMa, and one remnant confined to a higher altitude dense conifer plantation upstream of Carrifran in Swine Cleuchs, SCa, chosen for their heterogeneity in size, density and landscape features, were exhaustively sampled for adult trees and family arrays. Two neighbouring remnants, in Spoon Burn (SBa), the adjacent valley to Moffat Dale nearest to Carrifran, downstream and in Whitewells (Wa) located at the bottom of the Moffat Dale where the Carrifran streams drain into Moffat Water (Figure 1), were partially sampled for adult trees to gather an indication of potential pollen immigration because they are the only two other known local pollen sources within ten km.

In 2000 and 2001, leaf material was collected from all mature trees in CDa, CMa and SCa (comprising 30, four and 12 individuals respectively, Figure 1). Leaf material was also collected from two trees (A, B) isolated from the nearest remnants by a distance of 250 m and from a sample of 20 mature trees in SBa and Wa (Figure 1). Such sampling represents no less than 40% of the composition of these two remnants. 30 fruits, or all fruits if the seed crop was less, were collected from all 19 trees producing fruits in 2000, a non-masting year, in each of remnants CDa, CMa and SCa. In total, we sampled 88 trees and 483 seeds from 19 families.

The complete sample of 88 trees and 483 seeds were genotyped for five microsatellite markers previously developed for *F. excelsior*, namely, M2-30B, 1.19 and 3.1 (Brachet *et al.*, 1999), and FEMSATL2 and FEMSATL5 (Lefort *et al.*, 1999). DNA isolation, amplification by polymerase chain reaction (PCR) and

electrophoretic separation of PCR products were carried out as described elsewhere (Bacles *et al.*, 2005).

Evaluating microsatellite scoring and accounting for mistyping

Out of 483 seeds genotyped, 61 presented a mismatch with their mother at one or more loci. Furthermore, genotypes observed at loci 3.1 and FEMSATL 5 suggest departure from Mendelian segregation (Bacles *et al*., 2005) and occurrence of null alleles which has also been discussed in other *F. excelsior* studies (e.g. Heuertz *et al.*, 2001; Morand *et al.*, 2002).

In the rare instances where correction for genotyping error is applied in empirical studies, it is generally introduced as a global stochastic error rate (Gerber *et al.*, 2000; Marshall *et al.*, 1998). A major drawback of such practice is that the benefits of accounting for error are often outweighed by costs in precision of paternity assignment which becomes uninformative (Oddou-Muratorio *et al.*, 2003; Morissey and Wilson, 2005). Therefore, here we chose to deterministically account for allele dropouts and size miscalls by performing two successive transformations to the raw multilocus genotypes (referred to hereafter as RAW).

Erroneous allele sizing is most likely to occur between alleles of similar size and when alleles are rare. Therefore, we applied an initial transformation to account for size miscalls in the form of a binning procedure. At each locus, rare alleles were binned with common alleles of the nearest size. Alleles were deemed rare when they occurred at a frequency of less than 0.01 for the entire dataset. The procedure was applied strictly to loci M2.30B and FEMSATL2. For loci 1.19, 3.1 and FEMSATL5, more common alleles were also binned in order to reflect difficulties in gel scoring for 1.19 and difficulties in respect of Mendelian segregation for 3.1 and FEMSATL5

(Bacles *et al*. 2005). The procedure reduced the number of alleles observed from 54 to 29, from 35 to 20, from 10 to 7, from 46 to 12 and from 17 to 8 at loci FEMSATL2, M2-30B, 1.19, FEMSATL5 and 3.1 respectively in the binned dataset (referred to hereafter as BIN).

A second transformation was then performed to account for the possible occurrence of allele dropouts. A one-allele dropout model was applied to each locus by introducing a new (i.e. non-observed) allele, by rescoring every individual with a non-amplifying genotype as homozygote null, and every observed homozygote as heterozygote null in the transformed dataset (hereafter referred to as BINNULL).

For each dataset, genotyping error rates were quantified by means of direct comparison of offspring-mother genotype at each locus and averaged over loci in CERVUS 2.0 (Marshall *et al.*, 1998). Loci were retained for subsequent analyses if the estimated genotyping error was less than 5%. In order to assess the discriminatory power of each dataset, a paternity exclusion probability (PEP) was computed for each locus and accumulated over loci in FAMOZ (version released on 17.04.2007, Gerber *et al.*, 2003).

Estimating contemporary pollen-mediated gene flow at the landscape scale

Paternity analyses were undertaken to identify the pollen parent of the 422 seeds that shared a compatible multilocus genotype with their putative mother only and excluding the 61 seeds presenting at least one mismatching allele with their mother. Pollen parents were considered either among the 48 trees sampled in CDa, CMa and SCa, including the possibility for mother trees to self, or as pollen-

mediated gene flow from outside the landscape covered by the three completely censed remnants (Figure 1).

For each of the RAW, BIN and BINNULL datasets, paternity was assigned using both a simple exclusion (SE) and a maximum-likelihood (ML) approach in FAMOZ (Gerber *et al.*, 2003). In each case, outcomes of paternity assignment may be, for each individual seed, either that one unique individual among the 48 trees of Carrifran and Swine Cleuchs is assigned as its pollen parent, or that its paternity is unresolved with more than one possible pollen parent among the 48 trees, or finally that all 48 trees are excluded as potential pollen parents and its paternity is assigned to immigrant pollen. A range of values for apparent pollen-mediated gene flow into the landscape is subsequently obtained as the percentage of seeds in the sample for which paternity was assigned to immigrant pollen.

In FAMOZ, confidence in paternity assignment is estimated using a simulation procedure for hypothesis testing (Gerber *et al.*, 2000). The paternity of the 422 seeds sampled from 19 mother trees in three *F. excelsior* remnants of Moffat Dale was assigned to the most-likely fathers detected by means of 'log of the odds' ratios (LOD scores) based on pollen pool gene frequencies estimated from progeny arrays in MLTR (Ritland, 2002). We chose to approximate the (non-observed) allele frequencies of the reproductive population by using the observed pollen pool frequencies instead of the frequencies observed for the small sample of 88 mature trees because the latter, which is sampled *a priori* based on spatial location, may be a poor estimate of the actual reproductive population if gene flow is extensive. No significant genotypic association was detected among any pair of loci (Bacles *et al.*, 2005). LOD scores over all loci were therefore obtained by adding LOD scores calculated for each locus.

Confidence in paternity assignment was then determined in FAMOZ by comparing the distribution of the LOD scores of the most-likely fathers of 50 000 randomly generated seeds with their father randomly chosen among the 48 trees to the distribution of LOD scores of the most-likely fathers of 50 000 seeds whose paternal genotype was randomly generated according to pollen pool allele frequencies. The test threshold for rejecting a candidate as a true father (TF), was chosen at the intersection of the two distributions of LOD scores to minimise both type I error, wrongly considering as resulting from pollen immigration a seed sired by a sampled father, and type II error, wrongly assigning true pollen immigration to a sampled father (Gerber *et al.*, 2000). For paternity assignment by simple exclusion (SE), all candidate males with a positive LOD score (i.e. test threshold TRUE FATHER=0) were not excluded from being the true father.

Global results of paternity assignment obtained for each of RAW, BIN and BINNULL datasets with both SE and ML methods are discussed in respect of estimated error rates, confidence levels in assignments and estimated pollen-mediated gene flow at the landscape scale. The dataset/method combination found to minimise genotyping error rates while maximising confidence in paternity assignments was retained for subsequent detailed description of individual male reproductive success. In particular, results were summarised in order to identify the number of sires among the sample trees and the number of seeds they sired among the sampled seeds. The pollen dispersal curve was estimated by plotting the distance between mother trees and pollen donors for each most-likely assignment. When more than one likely father was identified (unresolved assignment), a fraction of the seed was assigned to all likely fathers evenly and proportionally to the number of likely fathers found.

Estimating the fractional pollen contribution of forest remnants and identifying local sources of pollen immigration

In order to estimate landscape connectivity and pollen-mediated genetic exchange among forest remnants, it may be most relevant to assess the relative pollen contribution of forest remnants to the seed crop rather than to define individual paternity *per se.* It has been argued that such (meta)population-scale phenomena may be better addressed with fractional-likelihood assignment methodology that will assign a fraction of the paternity of a given seed to all male candidates with a positive LOD score in proportion to their likelihood probability (Nielsen *et al.*, 2001). In order to estimate potential pollen immigration into CDa, CMa, and SCa from other known *F. excelsior* remnants of Moffat Dale, SBa and Wa (Figure 1), we estimated the posterior expectation of the number of sampled offspring in each of five remnants by means of fractional-likelihood assignment in PATRI (Signorovitch and Nielsen, 2002).

The approach in PATRI also allows us to make prior assumptions about the proportion of the pollen parents that have not been sampled (Nielsen *et al.*, 2001). While the actual effective male population size is unknown, we do have some expectations of the number of trees occurring in the landscape and likely to contribute to the pollen pool. Therefore, we tested the sensitivity of the fractional-likelihood assignment to assumptions made on the population size (*N*) by successively repeating analyses for an *N* of 88, the number of trees sampled; an *N* of 150, the approximate number of *F. excelsior* trees occurring in the catchment and *N* modeled as a uniform function varying between 100 and 500. We compared results with those of a maximum-likelihood assignment performed in FAMOZ when considering all 88 trees sampled in Moffat Dale.

Comparing potential to realised pollen-mediated gene flow among forest remnants.

To assess whether estimates of pollen-mediated gene flow from seeds that have not yet dispersed reflect estimates of pollen-mediated gene flow seen after seed dispersal and establishment, for each of remnant CDa, CMa and SCa, we used most-likely fathers to attribute the origin of the pollen grain to either local pollen, foreign pollen of known source (in other identified remnants) or of unknown source. We compared these figures with previously published results derived from a ML parent-pair analysis performed on seedlings establishing in the same three remnants (Bacles *et al.*, 2006).

In addition we estimated total gene flow into fragments using genotypic data generated both from progeny arrays (T_p) and from established seedlings (T'_s). Note that pollen grains only carry one gene copy while diploid seeds carry two. Let *A* and *A'* represents the number of local seeds fertilised by immigrant pollen in progeny arrays and establishing seedlings respectively. Let B' be the number of immigrating seeds, and C and C' the total number of seeds sampled in progeny arrays and establishing seedlings respectively, then:

$$Tp = (A / 2C) \times 100 \qquad \textbf{Equation 1}$$

and

$$T's = (A' + 2B') / 2C' \times 100 \qquad \textbf{Equation 2}$$

In equation 1, seeds are sampled on known mother trees and are all local. *Tp* therefore assumes that pollen is the main vector of gene flow among populations and that seed dispersal is mostly local. Results are discussed in terms of

comparison of potential (i.e.*ante* dispersal) and realised (i.e.*post* dispersal and establishment) gene flow in the three heterogeneous forest remnants.

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Results

Genotyping error and choice of dataset

Genotyping error rates estimated by means of offspring-mother genotype comparison are reported for each locus and each genotype transformation in Table 1. They were found to be highest for loci 3.1 (error (RAW) = 0.2847) and FEMSATL 5 (error (RAW) = 0.2237). Drastically reducing the number of alleles, from 17 to 8 and from 46 to 12 at locus 3.1 and FEMSATL5 respectively, decreases the error rate only slightly (error (3.1, BIN) = 0.2679; error (FEMSATL5, BIN) = 0.2133) but additional inclusion of a null allele decreases the error significantly (error (3.1, BINNULL) = 0.1499; error (FEMSATL5, BINNULL) = 0.0762) suggesting that null alleles may be responsible for the non-Mendelian segregation observed in progeny arrays at these loci. In contrast, genotyping error rates estimated at loci 1.19, M2.30B and FEMSATL2 were under 5% (Table 1), and lowest at loci M2.30B and FEMSATL2 after binning of alleles (error (M2.30B, BIN) = 0.0347; error (FEMSATL2, BIN) = 0.0443) and with inclusion of a null allele at locus 1.19 (error (1.19, BINNULL) = 0.0289). Overall, inclusion of loci 3.1 and FEMSATL5 in estimates increases mean error across loci dramatically, up to 3 times (Table 1). When these loci were excluded, estimates of mean error across loci were consistently under 5%.

Table 1

Paternity exclusion probabilities (PEP) estimated per locus for RAW vary between 0.594 at locus 1.19 and 0.864 at locus FEMSATL2 (Table 1) reflecting variation in level and evenness of polymorphism among loci (Bacles *et al.*, 2005). As expected, reducing the number of alleles at each locus systematically lowers discriminatory power among genotypes at each locus, albeit moderately, with PEP estimated per locus from BIN varying from 0.504 at locus 1.19 to 0.857 at locus FEMSATL2. Conversely, additional inclusion of a null allele results in contrasting effects on single locus estimates of PEP (Table 1). However, cumulative estimates of PEP,

including all five loci, are consistently very high across datasets, reaching values upward of 99.9%. Excluding loci 3.1 and FEMSATL5 decreased cumulative PEP only slightly (Table 1).

On the basis both that including loci 3.1 and FEMSATL5 increases genotyping error to rates well above 5% and that excluding them hardly affects multilocus genotype discrimination of individuals, they were not retained for subsequent analyses. Meanwhile, considering loci 1.19, M2.30B and FEMSATL2 only, mean genotyping error rate is lowest when multilocus genotypes are transformed with binning at loci M2.30B and FEMSATL2 and with binning and inclusion of a null allele at locus 1.19 (dataset hereafter referred to as BIN3NULL1; error (MEAN, BIN3NULL1) = 0.0360). No identical multilocus genotype was found among the 88 trees sampled in Moffat Dale and cumulative PEP is estimated at 0.991 which is nearly as high as for RAW data (Table 1).

Contemporary pollen-mediated gene flow at the landscape scale

Results of SE and ML paternity analysis for RAW, BIN, BIN3NULL1 datasets and for assignment of pollen parents to the 422 seeds that do not show any mismatch with their mother on raw data, are given in table 2. 43% to 68% of the 422 seeds analysed were found to have been fertilized with pollen dispersed from trees located outside the landscape covered by three *F. excelsior* remnants of Carrifran and Swine Cleuchs. Highest estimates were obtained using a ML method (Table 2). Differences between SE and ML estimates of apparent pollen flow are due to a number of seeds that were not assigned a father among the 48 sampled trees with the ML method because their LOD was positive but below the given threshold for assignment. However they were assigned one (or several potential) fathers with the SE method, most frequently for the bin dataset ($N_{assigned}$ (BIN, SE) = 239, Table

2). False rejection of true sampled fathers was lowest (Type I error < 0.05 for TF = 2.90) for fewer seeds ($N_{assigned}$ = 141) with the BIN3NULL1 transformation (Table 2).

Individual male reproductive success and distance of pollen dispersal

On the basis that the BIN3NULL1 dataset is characterised by the lowest estimates of genotyping error and type I error in ML assignment, the most-likely pollen parents identified for 141 seeds under these conditions were retained for subsequent description of male reproductive success and spatial patterns of pollen dispersal at the landscape scale.

In total, 31 of the 48 trees sampled in CDa, CMa and SCa were found to sire one or more of the sampled seeds (Supplementary Table S1). Of these, 14 trees fertilised three seeds or less, while three trees fertilised more than ten seeds each. In the latter case, all fertilized seeds were sampled from neighbouring trees within the same remnant as the sire.

This pattern in individual male reproductive success is reflected in the L-shape of the pollen dispersal curve constructed by plotting the distribution of spatial distances between the 141 assigned seeds (i.e. based on spatial location of mother trees) and their most-likely pollen-parent (Figure 2a). Over 80% of effective pollen dispersal is confined to less than 100m from the source, corresponding to local pollen movement within remnant. The observed proportion of these local pollinations is significantly higher than expected under random dispersal (48%, Wilcoxon two-sided signed rank test *p*-value<0.05, Figure 2a). However, a number of rarer events, each representing less than 10% of total effective pollen dispersal (which is up to 25% less than expected from random dispersal), were identified

Figure 2

between 200m and 600 m, and between 1600m and 1800m corresponding to inter-remnant long-distance dispersal.

Further analysis that included trees in partially sampled remnants SBa and Wa allowed us to refine the shape of the pollen dispersal distribution (Figure 2b). ML paternity assignment of 163 seeds (TF=2.92, Type I error <0.05; Type II error<0.28) attributed their paternity either to trees sampled in CDa, CMa and SCa or to trees sampled in SBa and Wa (Supplementary Table S2). The inclusion of these nearby pollen sources allowed the detection of a small number of effective pollination events at distances between 1100m and 1600m, between 1800m and 200m and as great as 2900m (Figure 2b).

Pollen-mediated genetic connectivity of forest remnants

Pollen contribution of the five *F. excelsior* population remnants sampled for adult trees was estimated from fractional likelihood (FL) paternity analysis of a subset of 404 seeds and 83 male candidates with no missing value in their multilocus genotype at 1.19, M2.30B and FEMSATL2 because PATRI computes complete genotypes only (Signorovitch and Nielsen, 2002). Estimates of the relative contribution of the five *F. excelsior* remnants of Moffat Dale to effective pollination in remnants CDa, CMa and SCa are similar when a FL or ML approach is applied to all 88 genotyped trees (Table 3). However, the absolute fractional contribution of remnants to paternity of sampled seeds is highly sensitive to the sampled fraction of reproductive adults (Nielsen *et al.* 2001) as illustrated by decreasing posterior expectation of the number of sampled offspring fathered in each remnant with increasing prior *N* (Table 3). Overall however, the relative pollen contribution of the five forest remnants remains unchanged. CDa contributes most to paternity of the sampled seeds. SCa, SBa and Wa also contribute in decreasing proportion (Table

3), while remnant CMa is a poor contributor (Table 3). The analysis clearly demonstrates that neighbouring remnants may act as sources of immigrant pollen.

Estimates of pollen-mediated genetic exchange among remnants CDa, CMa and SCa derived from ML paternity analysis confirm that the largest remnant CDa acts as a pollen donor, siring 26% of the seeds sampled in remnant CMa, located 600m away, and 7% of the seeds sampled in the most spatially isolated remnant, SCa, located 1700m away (Figure 3). Conversely, CMa, the smallest remnant (N_{trees} = 4), only sired 3% of its local seeds, and 1% the seeds within CDa (Figure 3).

Figure 3

Potential and realised pollen-mediated gene flow

How such pollen-mediated genetic exchange will impact on genetic structure depends on dispersal and establishment of the seeds. Estimates of potential pollen-mediated gene flow into remnants CDa, CMa, and SCa from ML paternity analysis of 422 seeds collected on mother trees before their dispersal described above (65% to 94%, Table 4) are comparable to those of potential pollen-mediated gene flow from a ML parent-pair analysis of 60 seedlings that were establishing in the same three remnants the following year (70% to 100%, Table 4). However, such comparison also show that pollen-mediated gene flow realised after seed dispersal and seedling establishment is much lower, ranging from 12.5% in remnant CMa to 17.5% in remnants CDa and SCa. Total gene flow estimates from progeny arrays are much lower (T_p ranging between 32.5% and 47%) than from establishing seedlings (*T's* ranging between 67.5% and 87.5%, Table 4).

Table 4

Discussion

Despite a number of significant concerns over genotyping error and uncertainties associated with statistical modeling, the application of paternity assignment analysis in the fragmented populations of *F. excelsior* has significantly enhanced our understanding of their genetic behaviour. It is clear that the population fragments within a single valley receive about half their pollen from outside the valley. Remnants within the valley are genetically connected via pollen flow, but the patterns of pollen flow among fragments are not symmetrical; pollen is preferentially transferred from large to small fragments. The analysis has also demonstrated that the effective pollen dispersal curve is fat-tailed. While the majority of detected pollen movement occurs over short distances (within 100m), there is still substantial pollen flow occurring over distances greater than 1km. Although these general conclusions are important for guiding the management of fragmented tree populations, this study has also highlighted the practical difficulties associated with obtaining quantitative assessments of gene flow from large scale studies that rely on parentage analysis.

A predictive understanding of the genetic connectivity of fragmented populations requires reliable estimation of contemporary gene dispersal across heterogeneous landscapes (Sork and Smouse, 2006). While development of both molecular techniques and statistical tools has greatly improved prospects for accuracy, the application of parentage analyses to natural populations remains an evolving area of research leading to regular reanalysis of empirical data within new statistical frameworks (e.g. Hadfield *et al.*, 2006; Slate *et al.*, 2000). At the centre of the debate, lies the question of sensitivity of parentage analyses to partial sampling of the reproductive population and to genotyping error at marker loci (Nielsen *et al.*, 2001; Oddou-Muratorio *et al.*, 2003; Slavov *et al.*, 2005). In order to obtain reliable

546 population-level inference of gene flow from a collection of individual-level paternity
547 assignments, we chose to address these concerns by applying a range of paternity
548 analysis methods to *F. excelsior* population remnants of the chronically deforested
549 catchment of Moffat Dale (Table 2 and 3).
550
551 Critically, application of parentage analyses to estimating gene movement in
552 natural populations relies on a conundrum: accuracy in estimation of the proportion
553 of the reproductive population that has not been sampled (i.e. immigrant gene flow)
554 strongly increases as the proportion of the -yet unknown- reproductive population
555 that has not been sampled decreases (Oddou-Muratorio *et al.*, 2003). Approaching
556 true reproductive population size seems particularly important to analyses
557 performed when using a fractional likelihood approach in PATRI because estimating
558 the absolute contribution of *F. excelsior* trees to paternity of sampled seeds is
559 sensitive to input prior information on the effective male population size (Table 3).
560 The advantage of the hypothesis testing-based simulation approach to determining
561 assignment confidence in FAMOZ is that it does not require any assumption on the
562 size of the true reproductive population. Comparison of SE and ML methods for a
563 range of transformed multilocus genotypes accounting for genotyping error *sensu*
564 *lato* at microsatellite markers suggest an immigration of at least 43% (SE, BIN) and
565 up to 68% (ML, RAW) of the pollen fertilising seeds from 19 trees of three forest
566 remnants of the Moffat Dale catchment.
567
568 The range in gene flow rates seems mostly affected by the choice of paternity
569 assignment method rather than by dataset transformation. Indeed, while
570 transformation of raw data allowed us both to reduce mean genotyping error (for
571 BIN3NULL1 at 3.60%), and to minimise false rejection of true fathers that were
572 sampled (for BIN3NULL1 Type I< 5%) to acceptable levels, estimates of gene flow

between RAW, BIN and BIN3NULL1 vary by up to 15% or a given paternity assignment method. Variation in gene flow estimates between the SE and ML methods can be attributed to the fact that, under ML, between 10% and 16% of seeds are not assigned a father among the sampled trees. This is because the LOD score of candidates is positive but below the given threshold for assignment (TF=2.90, Table 2). Although paternity of these seeds cannot be assigned at the chosen confidence threshold, it is arguable that their paternity should necessarily be attributed to immigrant pollen. Indeed, it has been demonstrated that assignment error may be much higher than random on unobserved (i.e. immigrant) events (Slate *et al.*, 2000) which suggests that estimates of pollen-mediated gene flow from ML method (here inclusive of seeds that were not assigned a father because genotypically compatible candidates had a low LOD score) should be seen as upper limits. Conversely, ML analysis suggests that even for a strict LOD score threshold, Type II error of wrongly assigning immigrant pollen to an unrelated sampled tree is high (up to 27% for BIN and TF=2.50, Table 2) indicating substantial cryptic gene flow. Therefore, apparent pollen flow estimated with relaxed assignment (equivalent to TF>0) by SE method, and those obtained with BIN data because allele binning results in lower discrimination of multilocus genotypes, are most conservative, with increased risk of cryptic gene flow and therefore represent lower limits of effective pollen immigration into forest remnants of Moffat Dale.

We justify our deterministic transformation of genotypic data not as substitution of raw datasets for transformed ones that may be more biased but rather as a simple way of minimising genotyping error and its possible influence on paternity assignment. The transformed dataset still includes a mean genotyping error rate of about 3.6% per locus which may still have an impact on the conclusions drawn from this study. Nonetheless, we deliberately chose not to include this global rate in

paternity analyses because there is evidence that including global genotyping error rates inflates errors in paternity assignments (Oddou-Muratorio *et al*., 2003; Slavov *et al.,* 2005). Given the limitations of the dataset, which are here clearly quantified, a range of estimates from several paternity analyses provides an ecologically meaningful interpretation of pollen-mediated gene flow at the landscape scale.

Extensive contemporary pollen-mediated gene flow averaging 60% has already been reported in plots located within continuous stands of *F. excelsior* (Hebel *et al.*, 2007) and of other wind-pollinated temperate tree species, for instance for *Quercus* (Dow and Ashley, 1998) covering only small areas. Contemporary pollen-mediated gene flow estimates of 43% to 68% for *F. excelsior* in the fragmented landscape of Moffat Dale are comparatively higher because all *F. excelsior* trees were sampled in an area of 300ha suggesting that *F. excelsior* maintains extensive pollen exchange across a landscape heavily deforested not only locally but also at the wider regional scale of the Southern Uplands of Scotland (>50km). Although trees standing solitarily in grazed pastures, spatially isolated from congeners following deforestation, were once described as *living-dead* (Janzen, 1986), there is now plethora of evidence of reproductive activity of isolated pasture trees, mainly in tropical species (Aldrich and Hamrick, 1998; Dick, 2001; White *et al.*, 2002) corroborating our findings of enhanced pollen-mediated gene flow following anthropogenic disturbance. Nonetheless, of the two *F. excelsior* trees of Moffat Dale that are isolated from others by a distance of at least 250m (Figure 1), neither produced a seed crop nor did they contribute to effective pollination of seeding trees for the sampled reproductive season. On the basis of this observation, we cannot reject the hypothesis that such isolated pasture trees are *living-dead*. Similarly, only three of the 48 trees sampled locally have a high male reproductive success and 26 of them contribute to effective pollination of fewer than two seeds

to none (Supplementary Table S1). However, whereas the evidence suggests that most sampled trees have a low individual male reproductive success locally, we cannot reject either the hypothesis that pollen from such trees effectively emigrated to other forest remnants outside the sample area, as the presence of a large component of immigrant pollination of either unknown origin or originated from identified neighbouring sources would suggest (Figure 3).

Several ecological factors may have contributed to confer such an advantage to long-distance pollination. Firstly, comparison of temporal variation of effective pollen movement between mast seeding and non-masting years showed that in a non-masting year, as is the case in the present study pollen-mediated gene flow was favoured in a *F. excelsior* stand in southern England (FRAXIGEN 2005). Furthermore, in such a situation, the small seed crops that were produced by a number of trees (in particular, trees SCa34 and SCa38 displayed only one seed branch with fewer than 10 seeds, Supplementary Table S1) may create a sampling effect. Secondly, temporal variation in individual flowering phenology may greatly affect mate availability. Indeed, Gerard *et al.* (2006) not only found that co-flowering individuals were patchily distributed in space in a *F. angustifolia* and *F. excelsior* hybrid zone, they also detected an asymmetry in male reproductive success with early flowering trees participating more as pollen donors than late flowering ones. A scenario where immigrant pollen would be preferentially available during the period of stigma receptivity of seeding trees of Moffat Dale would also favour long-distance pollination.

However, high levels of gene immigration are not necessarily sufficient to prevent assortative mating and selfing (Gerard *et al.*, 2006). For gene flow to become an efficient force counteracting the deleterious genetic effects of habitat

654 fragmentation, gene flow must not only be sustained at high levels across seasons,
655 but must also be qualitatively diverse. Here we find that efficient pollen immigration
656 allows for new and diverse genetic material to establish in the seed generation
657 (Bacles *et al.*, 2005). Such genetically diverse pollen pool composition may be
658 explained by the type of decay of pollen dispersal. Indeed, Klein *et al.* (2006)
659 demonstrated by means of simulation that fat-tailed dispersal kernels lead
660 asymptotically to a diverse propagule pool containing a balance of mixing of the
661 propagules of two sources and therefore that the diversity of the pollen pool of a
662 mother plant should increase with increased spatial isolation. Pollen dispersal
663 patterns observed for *F. excelsior* in Moffat Dale seem to corroborate such
664 theoretical findings. The majority of detected pollen dispersal was found between
665 near-neighbours at distances under 100m (Figure 2). However, not only were a
666 number of rare events detected among forest remnants at distances up to 2900m,
667 in proportions departing from random dispersal (Wilcoxon two-sided signed ranked
668 test, $p<0.05$), but undetected events were also in the majority and may have
669 originated at much greater distances, suggesting a L-shaped pollen dispersal
670 kernel with a tail spreading over several kilometres and underlining the difficulty of
671 detecting long distance dispersal (Nathan, 2006).
672
673 Such pollen dispersal effective over long distances can be linked to landscape
674 features resulting from habitat disturbance. Indeed, in the Southern Uplands of
675 Scotland, deforestation and land use for pasture have greatly opened the barren
676 landscape that is regularly battered by strong winds. It is therefore likely that wind-
677 mediated pollen movement for *F. excelsior* has been facilitated by the modification
678 of the landscape in Moffat Dale. In particular, genetic connectivity of forest
679 remnants seems favoured by landscape openess and remnant size rather than by
680 geographic proximity. Indeed, although no seeding trees were sampled in two of

five remnants (SBa and Wa), fractional-likelihood paternity assignment shows that their contribution to effective pollination of seeding trees of the other three extant remnants (CDa, CMa and SCa) is higher than that of remnant CMa which is a much smaller remnant of only 4 trees located in the bed of the river running through an exposed and barren pasture in the Carrifran valley. Within remnant pollination for CMa is indeed much lower than for other remnants (Figure 3), highlighting the fact that remnants are smaller, but are spatially well connected to neighbouring forest remnants tend to receive proportionally higher gene flow simply because there are fewer potential local pollen donors (Sork and Smouse, 2006).

An important practical consequence of the high rate of pollination by immigrant pollen is that the locally produced seed in Carrifran will contain genes sampled from a wide geographic area around the valley How the high rate of success of immigrant pollen in the production of local seeds will ultimately affect the genetic structure of *F. excelsior* remnants depends on how much of the pollen pool genetic diversity is effectively carried into successive generations by established seedlings that reach maturity. Natural regeneration in Moffat Dale has been severely limited by continuous grazing pressure. Colonisation of mountain grasslands by *F. excelsior* seedlings has been found to be connected to grazing activities, with seedlings found preferentially in high layers of vegetation, in shaded and ungrazed areas (Julien *et al.*, 2006). Pasture habitats in Moffat Dale may therefore be unfavourable to seedling establishment. Thus, actual gene flow may be recruitment limited rather than dispersal limited (Imbert and Lefèvre, 2003). In fact, comparison of total gene flow estimated here from non-dispersed seeds with total gene flow estimated from newly established seedlings in three *F. excelsior* remnants shows that actual recruitment of genes carried by immigrant pollen is limited (Table 4). Note that the ratio of potential to realised pollen-mediated gene flow is low, not

because there seems to be an advantage conferred to recruitment of local seeds fertilised with local pollen but because the majority of establishing seedlings have immigrated into the remnants (Bacles *et al.*, 2006). This indicates that, in cases when seed dispersal is an important vector of long-distance dispersal, estimating seed-mediated gene flow is essential to predicting landscape connectivity (Sork and Smouse 2006).

In the Southern Uplands of Scotland and in other severely deforested landscapes, conservation management aimed at sustainable forest restoration without human intervention must move away from *conservation gardening* (Hobbs, 2007). Predictive conservation necessitate better understanding of the evolution of dispersal in a changing environment (Kokko and Lopez-Sepulcre, 2006) and an appreciation of how population genetic processes operate in ecological space and time. Bringing knowledge of contemporary gene flow among population remnants generated from this study and others into conservation will ensure that the evolutionary processes maintaining genetic connectivity and evolutionary potential are restored at the landscape scale (Meagher, 2007).

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Titles and legends of figures

Figure 1 Distribution of *Fraxinus excelsior* mature trees in Moffat Dale remnants. Each dot represents a tree. A) Mature trees grow in small forest remnants, confined to steep slopes (elevations are given in meters) along streams (highlighted in dark lines). An exhaustive sampling and mapping was performed in remnants CMa (*N*=4), CDa (*N*=30) and SCa (*N*=12) while in two larger remnants SBa and Wa which include approximately 50 mature trees each, 20 individuals were sampled throughout each of them as potential sources of immigrant pollen flowing into remnants CMa, CDa and SCa. Two lone trees of the Carrifran Burn (Labelled A and B) were also sampled. B to F) Close up of spatial distribution of individuals sampled in remnant CDa, CMa, SCa, SBa and Wa respectively. In CDa, CMa, and Sca, all individuals producing fruits in 2000 are represented by a star: 30 seeds were collected throughout the tree canopy from 11, 2 and 6 trees in CDa, CMa and SCa respectively. The background map is a section of Ordnance Survey product Land-line.Plus-nt11 © Crown copyright Ordnance Survey. An EDINA digimap / JISC supplied service. © Figure 1 was originally published in Bacles *et al.* (2005) reprinted with kind permission from *Evolution* (Blackwell Publishing)..

Figure 2 Comparison of the frequency distribution of possible (white) and detected (black) effective pollen dispersal events for *Fraxinus excelsior* in relation to the location of pollen donors within the fragmented landscape of Moffat Dale. 2a) Detection within three censed remnants. Possible dispersal distances were estimated from Euclidian distances between 141 assignable seeds, spatially located on 19 mother trees, and all 48 *F. excelsior* candidate pollen parents sampled in remnants CDa, CMa and SCa of Moffat Dale (Figure 1). Detected

903 pollen dispersal distances were estimated from Euclidian distances between the
904 141 assignable seeds and their likely father when identified by means of maximum-
905 likelihood paternity analysis in FAMOZ (Gerber et al. 2003, Table 2). 2b) Detection
906 including neighbouring sources of immigrant pollen. Possible dispersal distances
907 were estimated from Euclidian distances between 163 assignable seeds, spatially
908 located on 19 mother trees, and all 88 *F. excelsior* candidate pollen parents
909 sampled in all five remnants of Moffat Dale (Figure 1). Detected pollen dispersal
910 distances were estimated from Euclidian distances between the 163 assignable
911 seeds and their likely father when identified by means of maximum-likelihood
912 paternity analysis in FAMOZ (Gerber et al. 2003, Table 3). In both situations, when
913 more than one likely father was identified (unresolved assignment), a fraction of the
914 seed was assigned to all likely fathers evenly and proportionally to the number of
915 likely fathers (Supplementary Table S1 and S2). Distance distributions of detected
916 pollen dispersal were found to differ significantly from random dispersal (Wilcoxon
917 two-sided signed rank test, p-value <0.05 for both n =141 and n =163).
918
919 **Figure 3** Schematic map of pollen-mediated genetic exchange among three
920 *Fraxinus excelsior* forest remnants varying in their population size, density and
921 degree of spatial isolation to other forest remnants in the mosaic landscape of
922 Moffat Dale. Estimates of gene movement within remnants (continuous white
923 arrows), of gene flow among remnants (continuous black arrows) and of gene
924 immigration from external sources (dashed white arrows) are based on results of
925 ML paternity analysis performed in FAMOZ (Gerber et al. 2003) 422 seeds sampled
926 from all 19 seeding trees in remnants CDa (N_{trees}=30), CMa (N_{trees}=4) and SCa
927 (N_{trees}=12), considering all 48 trees occurring within the landscape, including two
928 isolated trees (A and B, Figure 1) as potential pollen donors (Table 2). Relevant
929 potential geographic barriers to gene flow among remnants are highlighted:

930 Remnants CDa and CMa are located in close proximity (600 m) at the bottom of
931 the bare and open valley while remnant SCa is most isolated over a ridge (dashed
932 white rectangle) located about 1700 m away and surrounded by a dense closed
933 conifer plantation (continuous black square).

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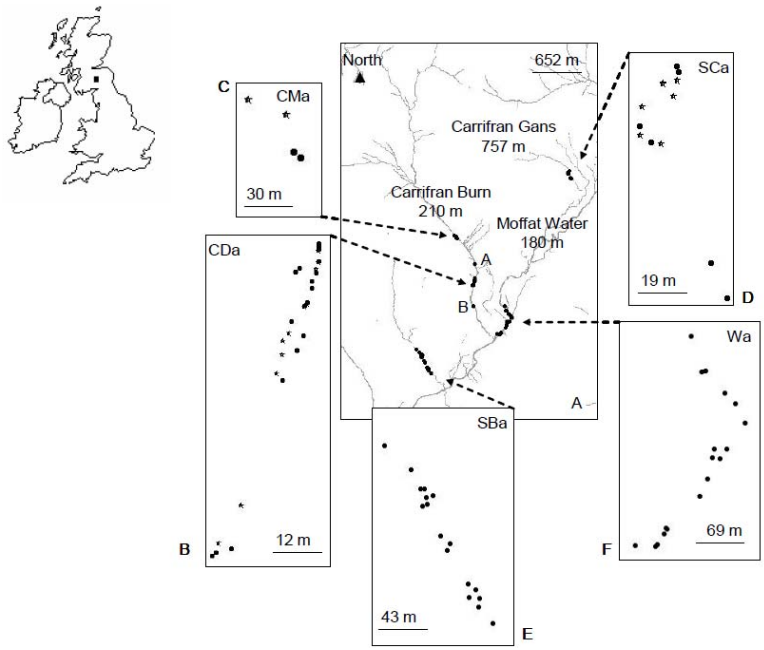
934 FIGURE 1



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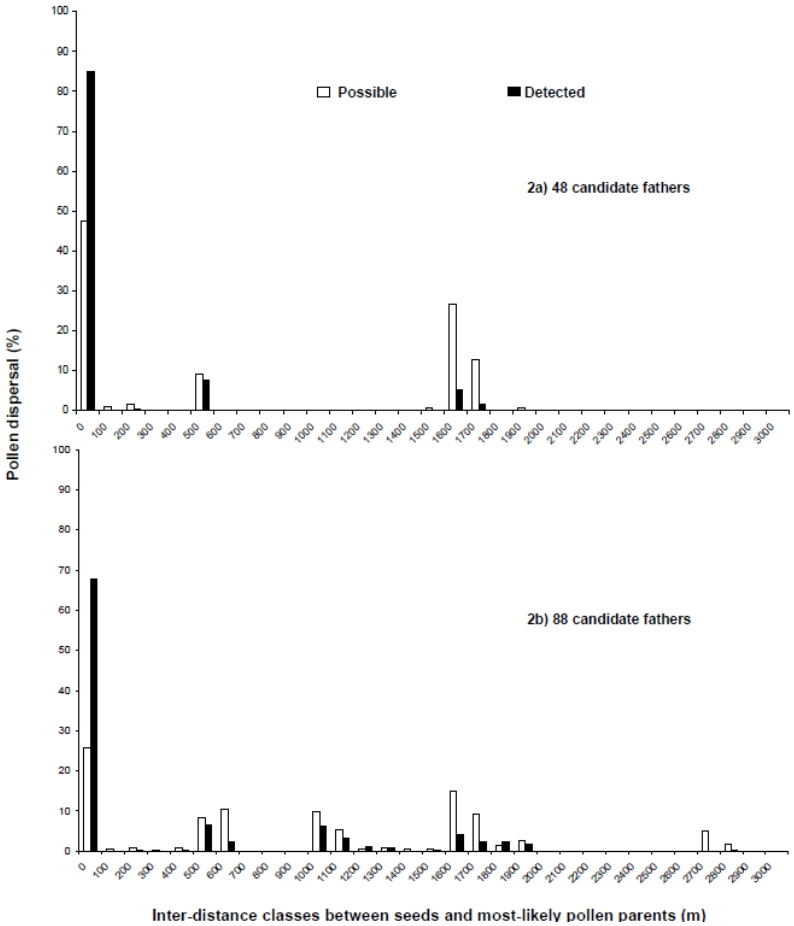
936 FIGURE 2



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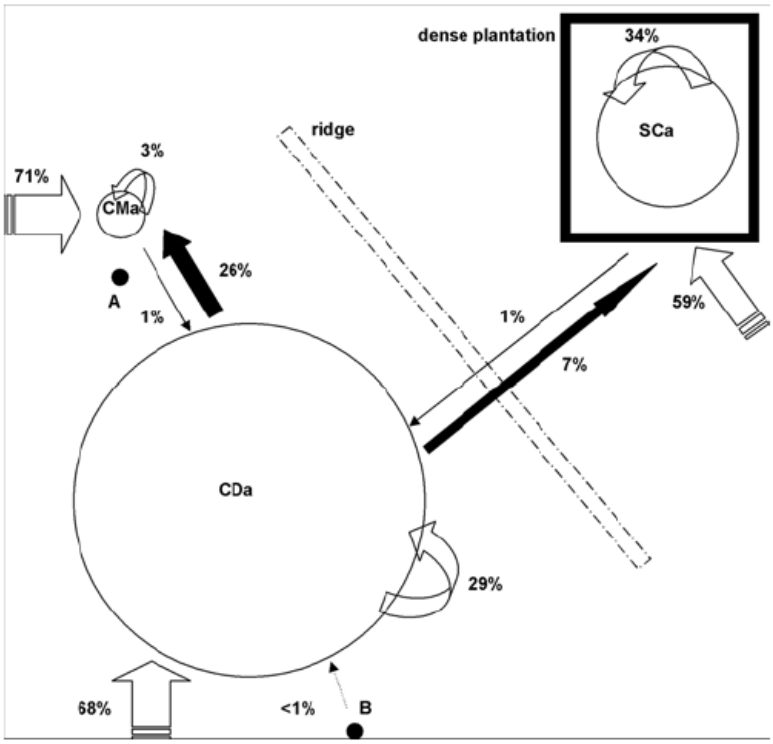
938 FIGURE3



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940 **Table 1.** Estimates of genotyping error and paternity exclusion probabilities (PEP) at each of five *Fraxinus excelsior* microsatellite loci and overall,
941 computed in CERVUS 2.0 (Marshall et al. 1998) and based on genotyping of 483 seeds sampled from 19 mothers and of 88 trees with no transformation
942 of genotypes (RAW), with binning of rare alleles (BIN), with binning of rare alleles and inclusion of a generalised null allele (NULL). Detailed procedures for
943 allele binning and null allele inclusion are given in material and methods section. Genotyping error estimates based on comparison of the genotype of
944 the 483 seeds with that of their mother. The number of mismatching seed genotypes is given ($N_{mismatch}$) but the number of comparisons may be under
945 483 when values are missing ($N_{comparison}$). Lowest estimates of genotyping error for each locus are highlighted in bold.

Dataset	**RAW**			**BIN**			**BINNULL**		
Locus	$N_{mismatch}/N_{comparison}$	**error**	**PEP**	$N_{mismatch}/N_{comparison}$	**error**	**PEP**	$N_{mismatch}/N_{comparison}$	**error**	**PEP**
3.1	129/431	0.2847	0.692	118/431	0.2679	0.680	69/483	**0.1499**	0.653
FEMSATL5	132/434	0.2237	0.808	102/434	0.2133	0.713	39/483	**0.0762**	0.685
1.19	13/469	0.0332	0.594	9/469	0.0292	0.504	12/483	**0.0289**	0.606
M2.30B	26/460	0.0378	0.855	23/460	**0.0347**	0.837	45/483	0.0641	0.841
FEMSATL2	34/481	0.0463	0.864	32/481	**0.0443**	0.857	34/483	0.0457	0.870
Overall (excl. 3.1 and 5)	-	0.0391	0.992	-	0.0361	0.988	-	0.0462	0.992
Overall (all)	-	0.1251	0.999	-	0.1179	0.999	-	0.0732	0.999

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Table 2. Comparison of global results of paternity analysis of 422 *Fraxinus excelsior* seeds sampled from 19 mother trees and 48 candidate fathers and of their translation into percentage of apparent pollen-mediated gene flow into three forest remnants of the Moffat Dale catchment for a range of paternity assignment methods and of microsatellite genotype transformations. Simple exclusion (SE) and maximum-likelihood (ML) paternity analyses were conducted in FAMOZ (Gerber *et al.,* 2003). Lod of the odds score (LOD) thresholds (TF) for ML paternity assignment and Type I error of false rejection and Type II error of false assignment were determined by means of 50 000 simulations in FAMOZ. Both SE and ML methods were applied to seeds genotyped at microsatellites FEMSATL2 (Brachet *et al*., 1999), M2.30B and 1.19 (Lefort *et al*., 1999) that displayed a multilocus genotype compatible with the multilocus genotype of their mother, with no further transformation (RAW), after allele binning at all three loci (BIN), after additional inclusion of a generalised null allele at locus 1.19 (BIN3NULL1). Results highlighted in bold were retained for detailed analysis of genetic connectivity among remnants.

Dataset	Paternity assignment method	TF	Type I error	Type II error	N_{seed} Excluded	N_{seed} Not assigned	N_{seed} Assigned, resolved	N_{seed} Assigned, unresolved	Apparent pollen flow
					LOD=0	0<LOD<TF	LOD>TF, unique	LOD>TF, multiple	LOD≤TF
RAW		0	-	-	219	-	120	83	52%
BIN	SE	0	-	-	183	-	125	114	43%
BIN3NULL1		0	-	-	224	-	119	79	53%
RAW		3.20	<0.10	<0.13	219	68	102	33	68%
BIN	ML	2.50	<0.07	<0.28	183	41	135	63	53%
BIN3NULL1		**2.90**	**<0.05**	**<0.22**	**224**	**57**	**92**	**49**	**67%**

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Table 3. Comparison of fractional and maximum likelihood estimation of the contribution of the five *Fraxinus excelsior* forest remnants of Moffat Dale to effective pollination of seeds sampled in three of them. Results are based on the 422 seeds sampled in remnants CDa, CMa and SCa which displayed a genotype compatible with that of their mother, transformed to minimise genotyping error. †20 trees were genotyped in each of SBa and Wa as an indication of potential local sources of immigrant pollen. The most-likely number of seeds sired in each remnant was determined by means of maximum-likelihood paternity analysis in FAMOZ (Gerber *et al.,* 2003) considering all 88 trees genotyped and assigning paternity at a threshold TF of 2.92 (Type I error <0.05, Type II error <0..28). The fractional-likelihood paternity analysis was computed in PATRI (Signorovitch and Nielsen, 2002). The posterior expectation of the number of seeds fertilised by trees from each of five forest remnants was estimated for a given population size (*N*) of 88 (the number of trees genotyped), of 150 (approximating the total number of trees occurring in Moffat Dale) and modelled as a uniform function of 100-500 individuals.

		Fractional likelihood assignment in PATRI			**Maximum likelihood assignment in FAMOZ**
		Posterior expectations of the number of sampled seeds			**Most likely number of seed sired**
	Prior N	**88**	**150**	**Uniform [100-500]**	**88**
Remnant	CDa	88	56	23	91
	CMa	3	2	1	4
	SCa	32	28	19	35
Gene flow†	SBa	15	11	6	18
	Wa	8	7	4	15
All		146	104	53	163

964 **Table 4.** Comparison of pollen and total gene flow estimates from maximum-likelihood (ML) paternity analysis of non-dispersed seeds with estimates
965 from ML parent-pair analysis of established seedlings in three *Fraxinus excelsior* forest remnants of Moffat Dale. Forest remnants CDa, CMa and SCa
966 were exhaustively sampled for 48 adult trees which were all considered as potential pollen donors and seeds were sampled from all trees producing
967 fruits, respectively, 11, 2 and 6 trees in CDa, CMa and SCa. [#]sample size given as number of seed families/total number of seeds. [*]Results of ML
968 parent-pair analysis of seedlings establishing in *F. excelsior* remnants CDa, CMa and SCa were previously reported in Bacles *et al.* (2006). [§]See
969 Equation 1 estimating total gene flow from progeny arrays (T_p) and where A is the number of local seeds fertilised by immigrant pollen and C is the total
970 number of seeds sampled [†]See Equation 2 estimating total gene flow from established seedlings (T'_s) and where A' is the number of seeds fertilised by
971 immigrant pollen, B' is the number of immigrating seeds and C' is the total number of established seedlings sampled.

	progeny arrays: ML paternity analysis *ante* seed dispersal					**established seedlings: ML parent-pair analysis[*] *post* establishment**						
	origin of pollen (N_{seed})			**gene flow estimates**		**origin of pollen and seed (N_{seed})**				**gene flow estimates**		
	sample size[#]	**local seed**		**pollen flow**	**total gene flow via pollen**	**sample size**	**local seed**		**foreign seed**	**potential pollen flow**	**realised pollen flow**	**total gene flow**
		local pollen	**foreign pollen**				**local pollen**	**foreign pollen**				
remnant	C[§]		A[§]		T_p[§]	C' [†]		A' [†]	B' [†]	*local seed*		T'_s [†]
CDa	11/282	81	201	**71%**	**35%**	20	3	7	10	**70%**	**17.5%**	**67.5%**
CMa	2/32	1	31	**97%**	**48%**	20	0	5	15	**100%**	**12.5%**	**87.5%**
SCa	6/108	37	71	**66%**	**33%**	20	2	7	11	**78%**	**17.5%**	**72.5%**

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Supplementary Table S1. Individual contribution of *Fraxinus excelsior* trees to effective pollination of non-dispersed seeds sampled in three forest remnants of the Moffat Dale catchment. Results are based on the 422 seeds sampled in remnants CDa, CMa and SCa which displayed a genotype compatible with that of their mother, transformed to minimise genotyping error, considering all 48 sires growing in the three remnants. The most-likely number of seeds sired by individual trees was determined by means of maximum-likelihood paternity analysis in FAMOZ (Gerber et al. 2003) considering 48 candidates genotyped and assigning paternity at a threshold TF of 2.90 (Type I error <0.05, Type II error <0.22). When more than one likely father was identified (unresolved assignment), a fraction of the seed was assigned to all likely fathers evenly and proportionally to the number of likely fathers.

		MOTHER TREE																						
		CDa												CMa				SCa						
	SIRE	102	105	108	111	112	118	123	125	126	129	130	**CDa all**	24	26	**CMa all**	33	34	35	36	38	41	**SCa all**	**All**
	n	*22*	*29*	*23*	*29*	*24*	*21*	*27*	*26*	*23*	*30*	*29*	***283***	*20*	*11*	***31***	*22*	*2*	*28*	*24*	*3*	*29*	***108***	***422***
CDa	19					0.33	1.00		9.33	8.00	1.00		19.67											**19.67**
	130	1.00	1.00					4.00			1.00		7.00											**7.00**
	107			1.00	2.00						3.00		6.00											**6.00**
	109		0.33	1.33		2.00	1.33	1.00					6.00											**6.00**
	114		1.00			1.00		1.00			2.33		5.33							0.50			0.50	**5.83**
	110		3.00		0.75	1.00				1.00			5.75											**5.75**
	113								1.00		0.50		1.50	0.50		0.50	1.00		1.00	0.67			2.67	**4.67**
	116		1.00			1.00						1.00	3.00	1.50		1.50								**4.50**
	119	0.33	1.00					1.00			2.00		4.33											**4.33**
	115	0.33				0.33	1.00		0.83		0.50		3.00	1.00		1.00								**4.00**
	125		0.33	0.33	1.00		1.33	1.00					4.00											**4.00**
	120													1.00	2.00	3.00								**3.00**
	101				0.25								0.25		0.50	0.50			1.00	1.00			2.00	**2.75**
	111				1.50							1.00	2.50											**2.50**
	129		0.39			1.00	1.00						2.39											**2.39**
	104		0.25								0.83		1.08	0.50		0.50				0.50			0.50	**2.08**
	118		0.14										0.14	0.50		0.50				0.17		1.00	1.17	**1.81**

	127						0.50		0.50		0.50		1.50									**1.50**
	128				0.50	1.00							1.50									**1.50**
	102		0.14		1.25								1.39									**1.39**
	112	0.33	1.00										1.33									**1.33**
	105		0.48	0.33			0.33						1.14					0.17		0.17		**1.31**
	126		1.00		0.25								1.25									**1.25**
	124		1.00										1.00									**1.00**
	121		0.14										0.14	0.50		0.50		0.17		0.17		**0.81**
	122		0.14										0.14					0.17		0.17		**0.31**
	103																					
	108																					
	117																					
	123																					
	CDa all	**2.00**	**12.36**	**3.00**	**7.50**	**7.67**	**6.50**	**8.00**	**11.67**	**9.00**	**11.67**	**2.00**	**81.36**	**5.50**	**2.50**	**8.00**	**1.00**		**2.00**	**3.33**	**1.00**	**7.33**	**96.69**
CMa	26				0.50	1.33			1.33				3.17									**3.17**
	24													0.50	0.50	1.00						**1.00**
	27																					
	28																					
	CMa all				0.50	1.33			1.33				3.17	0.50	0.50	1.00						**4.17**
SCa	32																3.50	1.00	4.00	5.00		13.50	**13.50**
	37																0.50		7.00	4.00	1.00	12.50	**12.50**
	42																3.00			1.00		4.00	**4.00**
	33																3.00			0.50		3.50	**3.50**
	38		0.14									1.00	1.14					1.00		0.17	1.00	2.17	**3.31**
	43											0.50	0.50										**0.50**
	41																			0.50		0.50	**0.50**
	40						0.50						0.50										**0.50**

36																		0.50			0.50	**0.50**
31											0.50	0.50										**0.50**
34		0.25										0.25										**0.25**
35																						
SCa all		0.39				0.50					2.00	2.89				10.00	2.00	11.00	11.67	0.00	2.00	36.67	**39.56**
B		0.25								0.33		0.58											**0.58**
A																							
All sires	**2**	**13**	**3**	**8**	**9**	**7**	**8**	**13**	**9**	**12**	**4**	**88**	**6**	**3**	**9**	**11**	**2**	**13**	**15**	**0**	**3**	**44**	**141**

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Supplementary Table S2. Individual contribution of *Fraxinus excelsior* trees to effective pollination of non-dispersed seeds sampled in three forest remnants of the Moffat Dale catchment. Results are based on the 422 seeds sampled in remnants CDa, CMa and SCa which displayed a genotype compatible with that of their mother, transformed to minimise genotyping error, considering 88 sires. 20 trees were genotyped in each of remnant SBa and Wa as an indication of potential local sources of immigrant pollen. The most-likely number of seeds sired by individual trees was determined by means of maximum-likelihood paternity analysis in FAMOZ (Gerber et al. 2003) considering all 88 trees genotyped and assigning paternity at a threshold TF of 2.92 (Type I error <0.05, Type II error <0..28). When more than one likely father was identified (unresolved assignment), a fraction of the seed was assigned to all likely fathers evenly and proportionally to the number of likely fathers.

		MOTHER TREE																						
		CDa												CMa				SCa						**All**
	SIRE	102	105	108	111	112	118	123	125	126	129	130	**CDa all**	24	26	**CMa all**	33	34	35	36	38	41	**SCa all**	
	n	*22*	*29*	*23*	*29*	*24*	*21*	*27*	*26*	*23*	*30*	*29*	***283***	*20*	*11*	***31***	*22*	*2*	*28*	*24*	*3*	*29*	***108***	***422***
CDa	19					0.25	1.00		9.33	8.00	1.00		19.58											**19.58**
	130	1.00	1.00					4.00			1.00		7.00											**7.00**
	109		0.33	1.33		2.00	1.33	1.00					6.00											**6.00**
	110		3.00		0.70	1.00				1.00			5.70											**5.70**
	107			1.00	1.50						2.50		5.00											**5.00**
	114		0.50			0.50		1.00			2.25		4.25							0.50			0.50	**4.75**
	113								1.00		0.50		1.50	0.50		0.50	1.00		1.00	0.50			2.50	**4.50**
	119	0.33	1.00					1.00			2.00		4.33											**4.33**
	125		0.33	0.33	1.00		1.33	1.00					4.00											**4.00**
	115	0.33				0.25	1.00		0.83		0.50		2.92	1.00		1.00								**3.92**
	120													1.00	2.00	3.00								**3.00**
	116		0.67			0.50						1.00	2.17	0.75		0.75								**2.92**
	101				0.20								0.20		0.50	0.50			1.00	1.00			2.00	**2.70**
	111				1.50							1.00	2.50											**2.50**
	118		0.14										0.14	0.50		0.50				0.17		1.00	1.17	**1.81**
	129		0.24			1.00	0.50						1.74											**1.74**

	104		0.10								0.75		0.85	0.50		0.50			0.33		0.33	**1.68**
	128				0.50	1.00							1.50									**1.50**
	102		0.15		1.20								1.35									**1.35**
	112	0.33	1.00										1.33									**1.33**
	127						0.33		0.50		0.50		1.33									**1.33**
	105		0.48	0.33			0.33						1.14						0.17		0.17	**1.31**
	126		1.00		0.20								1.20									**1.20**
	121		0.14										0.14	0.50		0.50			0.17		0.17	**0.81**
	124		0.67										0.67									**0.67**
	122		0.14										0.14						0.17		0.17	**0.31**
	103																					
	108																					
	117																					
	123																					
	CDa all	2.00	10.90	3.00	6.80	6.50	5.83	8.00	11.67	9.00	11.00	2.00	76.70	4.75	2.50	7.25	1.00		2.00	3.00	1.00	7.00	90.95
CMa	26				0.50	1.25			1.33				3.08									**3.08**
CMa	24													0.25	0.50	0.75						**0.75**
CMa	27																					
CMa	28																					
	CMa all				0.50	1.25			1.33				3.08	0.25	0.50	0.75						**3.83**
SCa	32																3.50	1.00	4.00	5.00	13.50	**13.50**
SCa	37																0.50		7.00	4.00	1.00	12.50	**12.50**
SCa	42																1.75			1.00		2.75	**2.75**
SCa	33																1.75			0.50		2.25	**2.25**
SCa	38		0.14									0.50	0.64					0.50		0.17	0.50	1.17	**1.81**
SCa	41																			0.50		0.50	**0.50**
SCa	36																			0.50		0.50	**0.50**

	43										0.33	0.33										**0.33**
	40					0.33						0.33										**0.33**
	31										0.33	0.33										**0.33**
	34		0.10									0.10										**0.10**
	35																					
	SCa all		0.24			0.33					1.16	1.73				7.50	1.50	11.00	11.67		1.50	33.17	**34.90**
	B		0.10							0.25		0.35											**0.35**
	A																						
CDa+CMa+SCa		2	11	3	7	8	6	8	13	9	11	3	82	5	3	8	9	2	13	15	0	3	40	**130**
SBa	20		2.67		0.50	0.50							3.67	0.50		0.50								**4.17**
	1			3.00				1.00					4.00											**4.00**
	6	0.33	0.50		0.70	0.50					0.50		2.53											**2.53**
	4		0.10								0.25		0.35		1.00	1.00								**1.35**
	7	0.33			0.50		0.50						1.33											**1.33**
	2									1.00			1.00	0.25		0.25								**1.25**
	18	1.00											1.00											**1.00**
	9						0.50						0.50											**0.50**
	10				0.50								0.50											**0.50**
	12						0.50						0.50											**0.50**
	16																0.50						0.50	**0.50**
	11													0.25		0.25								**0.25**
	15					0.25							0.25											**0.25**
	5		0.10										0.10											**0.10**
	17		0.10										0.10											**0.10**
	3																							

	8																				
	13																				
	14																				
	19																				
	SBa all	1.67	3.47	3.00	2.20	1.25	1.50	1.00		1.00	0.75		15.83	1.00	1.00	2.00	0.50				0.50	**18.33**
Wa	11																	0.50	1.00	0.50	2.00	**2.00**
	15		1.00										1.00						1.00		1.00	**2.00**
	16	0.33			0.50		0.50						1.33				0.50				0.50	**1.83**
	13											0.33	0.33							1.00	1.00	**1.33**
	12																1.25				1.25	**1.25**
	19																1.25				1.25	**1.25**
	4																		1.00		1.00	**1.00**
	6											1.00	1.00									**1.00**
	14														1.00	1.00						**1.00**
	18													1.00		1.00						**1.00**
	1											0.50	0.50									**0.50**
	7																		0.33		0.33	**0.33**
	3		0.10										0.10									**0.10**
	8		0.10										0.10									**0.10**
	17		0.10										0.10									**0.10**
	2																					
	5																					
	9																					
	10																					
	20																					

Wa all	0.33	1.30		0.50		0.50					1.83	4.47	1.00	1.00	2.00	3.00	0.50		3.33		1.50	8.33	**14.80**
SBa+Wa	2	5	3	3	1	2	1	0	1	1	2	20	2	2	4	4	1	0	3	0	2	9	**33**
All sires	**4**	**16**	**6**	**10**	**9**	**8**	**9**	**13**	**10**	**12**	**5**	**102**	**7**	**5**	**12**	**12**	**2**	**13**	**18**	**0**	**4**	**49**	**163**