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ORIGINAL ARTICLE

Frequent long-distance gene flow in a rare temperate forest tree (*Sorbus domestica*) at the landscape scale

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Precise empirical data on current gene flow by pollen, both with respect to distance and abundance, is crucial to understand whether habitat fragments are functionally connected. Based on a large-scale inventory (≈100 km²) in which all individuals of a naturally scattered forest tree (*Sorbus domestica*) were mapped, we inferred current gene flow by pollen using genetic paternity analysis. We detected an extensive network of effective pollen transfer. Although short pollen flow distances were most abundant, 10% of the assigned pollen donors were more than 2 km away from their female mating partners, and 1.8% were even at a distance of 12–16 km. This latter pollen flow shows that current long-distance gene flow over a fragmented landscape clearly occurs. Pollen dispersal was well described by a fat-tailed inverse curve. Using parentage analysis of established trees, maternally inherited chloroplast markers and diameter at breast height measurements as an indicator of individual tree age, we were able to infer regular seed dispersal distances over several hundred metres up to more than 10 km. We conclude that in temperate, insect-pollinated and animal-dispersed tree species such as *S. domestica*, fragmented subpopulations are functionally connected by gene flow through both pollen and seed.
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Introduction

Obtaining reliable measures of long-distance gene flow (LDGF) is relevant in various ecological contexts, including studies of habitat fragmentation (Sork and Smouse, 2006), the spread of invasive species (Dunphy and Hamrick, 2005), range shifts due to climatic change (Reusch and Wood, 2007) and the introduction of genetically modified crops (Williams and Davis, 2005). Thanks to improved molecular, genetic and statistical methods such as parentage analysis (Sork *et al*., 1999), it has lately become easier to directly investigate patterns of current gene flow. In accordance, gene flow distances far exceeding those reported in traditional ecological experiments (Ellstrand, 1992) have recently been demonstrated for several woody species, among them many tropical, insect-pollinated and animal-dispersed trees (White *et al*., 2002; Lowe *et al*., 2005; Ward *et al*., 2005; Hardesty *et al*., 2006).

Despite the potential of genetic approaches to investigate LDGF, most empirical studies of tree populations have been limited in scope, either because they were restricted to relatively small areas within continuous populations (Ward *et al*., 2005) or because they evaluated current patterns of gene flow among small forest fragments (Lowe *et al*., 2005; Bacles and Ennos, 2008). The first type of study provides detailed information on current gene flow patterns at comparatively small spatial scales. As these direct measures of gene flow are restricted by the spatial extent of the empirical data (Nathan, 2006), the probability of dispersal of pollen or seed to larger distances is usually extrapolated using appropriate mathematical models (that is, dispersal kernels; Oddou-Muratorio *et al*., 2006). However, such models are notoriously unreliable in characterizing the tail of the dispersal curve, that is, the part of the curve representing LDGF. In the second approach, individual cases of long-distance dispersal are detected, but it is impossible to quantify the distances over which such events occur, unless all fragments in a given landscape are studied (Bacles and Ennos, 2008). Corresponding studies also often find a weak relationship between siring success and the distance between mating partners and thus question that most pollinations occur among nearest neighbors (Kramer *et al*., 2008).

Considering the above limitations, spatially explicit data on effective long-distance pollen movement and seed dispersal are needed on the landscape scale to reliably quantify the distance and frequency of LDGF. Such spatially explicit data on effective long-distance pollen and seed dispersal are needed to understand functional connectivity among forest fragments. A better empirical basis on functional connectivity by gene flow

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within landscapes would substantially contribute to the open discussion on the paradox of forest fragmentation genetics (Kramer *et al*., 2008).

The aim of this study was to directly investigate current gene flow by pollen as well as recent gene flow by seed (that is, one generation earlier) at the landscape scale using parentage and paternity analysis. Parentage analysis, the currently most effective approach for the precise description of current pollen flow and seed dispersal patterns, requires that all potential parents of open-pollinated progenies within a study area are mapped, sampled and genotyped (Chase *et al*., 1996). When aiming at data on gene flow distances at the landscape level, study areas are comparably large, which leads to considerable effort for identifying all parents in the field and genotyping them in the laboratory (Manel *et al*., 2003). Species that naturally occur in low densities are particularly suitable for this purpose, as it is realistic to map and genotype all potential parents in a landscape.

By relying on a well-known population of the naturally scattered tree *Sorbus domestica* (L.) monitored for more than 20 years, we could be reasonably certain of having located all mature trees of this species in a study area of over 100 km² (Figure 1). Maximum distances among potential mates were up to 25 km. Thus, the data set offered an extraordinary framework to investigate the functional connectivity of a forest tree, both through gene flow by pollen and seed. We asked the following questions: (1) To what extent are the individuals of the insect-pollinated *S. domestica* genetically connected by current pollen transfer? (2) Which mathematical function adequately describes the pollen dispersal curve detected? (3) Is there evidence for recent long-distance seed dispersal?

Materials and methods

Study species

The service tree, *Sorbus domestica* L. (Rosaceae), naturally occurs in low densities in Central Europe (Barengo *et al*., 2001). It is an insect-pollinated, fleshy fruited, temperate forest tree. Like other woody species of the Rosaceae, its white hermaphroditic flowers attract a large variety of generalist pollinators such as Diptera, bees and bumblebees (Raspé *et al*., 2000; Oddou-Muratorio *et al*., 2006). The stamens mature slightly before the styles, but flowering is not synchronous within corymbs and individual trees, allowing for sequential visits by pollinators. Observations of flowering phenology of the closely related *S. torminalis* showed that individual trees differ in the flowering period, but that most if not all trees within populations overlap in flowering to a certain degree (Hoebee *et al*., 2007). We assumed that individuals of *S. domestica* could thus potentially mate with each

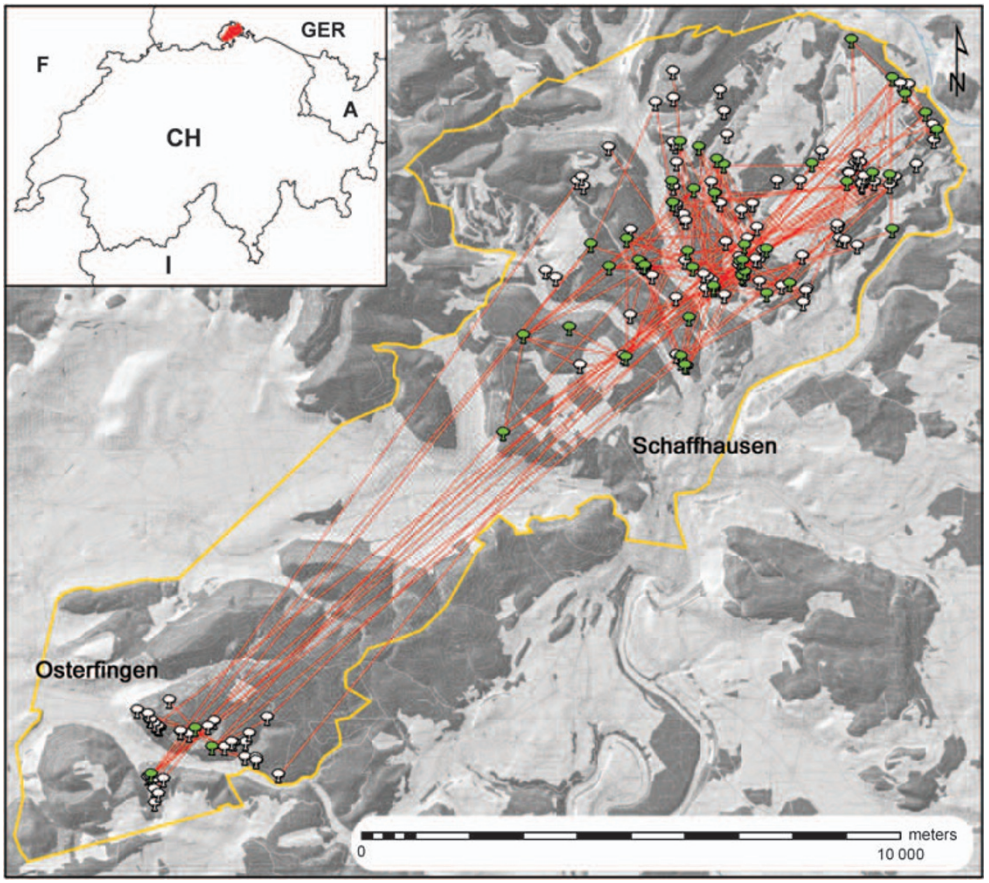

Figure 1 Current gene flow patterns by pollen in *Sorbus domestica* at the landscape scale in Schaffhausen, Switzerland. The yellow line delimits the study area (>100 km²) within which all *S. domestica* individuals were genotyped (tree symbols). Forest areas are displayed in dark grey, and green tree symbols indicate mother trees from which offspring was genetically analysed. Red lines connect mating partners identified by paternity analysis and thus represent current gene flow events by pollen. The inset gives the position of the study area (red) within Switzerland (CH; neighbouring countries: GER: Germany; A: Austria; I: Italy; F: France).

other. A characteristic that *S. domestica* probably shares with other woody Rosaceae is a gametophytic self-incompatibility system (Raspé and Kohn, 2002; Holderegger *et al*., 2008). However, self-incompatibility systems may break down when pollination is delayed or during periods of adverse weather conditions (De Nettancourt, 2001; Garcia *et al*., 2005), and individual trees may therefore exhibit substantial selfing rates (Oddou-Muratorio *et al*., 2006; Hoebee *et al*., 2007). *Sorbus domestica* is representative of a group of insect-pollinated, fleshy fruited and naturally scattered rare forest tree species of Central Europe such as *Pyrus pyraster*, *Malus silvestris*, *Prunus mahaleb* or *S. torminalis* (Barengo *et al*., 2001).

Study area and sampling

Within the study area in the Canton of Schaffhausen in northern Switzerland, *Sorbus domestica* occurs as scattered individuals within extensive areas of forest dominated by *Fagus sylvatica*. We based our study on a comprehensive inventory compiled over the past 20 years, in which all known trees of *S. domestica* had been recorded for the purpose of conservation and seed collection. We supplemented this inventory with additional information on tree positions from regional forest inventories and our own systematic survey of the area. We were thus confident that the vast majority if not all reproductive individuals of *S. domestica* in the study area had been located. We verified all reported locations and mapped them by using a hand-held GPS receiver. The trees varied in their degree of spatial isolation from other trees of *S. domestica*. At a larger scale, they formed two fairly distinct subpopulations (Schaffhausen and Osterfingen; Figure 1). Densities were 0.042 individuals per hectare for Schaffhausen and 0.138 individuals per hectare for Osterfingen. Our study area adjoined the Swiss–German border (Figure 1). In adjacent Germany, however, *S. domestica* is supposed to be absent or at least very rare, probably because of historically different forest management strategies in the two countries.

We collected fresh leaves or buds from all known 189 *S. domestica* trees and determined their diameters at breast height. We visited fruiting individuals up to three times during September–October to collect fruits. Leaves were dried in silica gel, whereas fruits and buds were stored at $-20\,^{\circ}C$. Over all, the fruit set of most trees was low. We thus only used maternal trees from which we were able to collect at least 25 fruits ($N = 49$).

DNA extraction and microsatellite genotyping

We extracted total genomic DNA following a slightly modified DNeasy plant kit protocol (Qiagen, Hilden, Germany). For adult trees, DNA was isolated from 15 mg of dried leaf material or 50 mg of frozen bud tissue. We also extracted DNA form lyophilized embryos and cotyledons that had been carefully excised from seeds ($N = 1183$ seeds; one seed per fruit).

Nuclear microsatellite markers (nSSRs) were transferred from closely related woody Rosaceae species. We tested 28 nSSR primer pairs designed for *Malus* (Gianfranceschi *et al*., 1998; Liebhard *et al*., 2002), *Pyrus* (Yamamoto *et al*., 2002a, b) and *Sorbus* (Oddou-Muratorio *et al*., 2001; Robertson *et al*., 2004). PCR products were screened for polymorphism on high-resolution Spreadex gels (Elchrom, Cham, Switzerland). Finally, we chose nine variable nSSR markers exhibiting highly reproducible amplification patterns (Table 1). Primers were combined in two multiplex-PCR amplifications comprising five and four primer pairs, respectively. Each reaction volume of 10 µl contained 0.5 µl template DNA ($\approx 4\,ng\,\mu l^{-1}$), 5 µl Multiplex-Master mix (Qiagen) and 1 µl of each primer (2 µM). PCRs were performed on PTC-100 thermocyclers (MJ Research, Waltham, MA, USA) starting with initial denaturation at 95 °C for 15 min, running 30 cycles with 94 °C for 30 s, 50 or 60 °C for 90 s (depending on the multiplex reaction; Table 1) and ending with a final extension at 72 °C for 30 min. Amplified fragments were analysed on an ABI 3100-Avant capillary sequencer (Applied Biosystems, Foster City, MA, USA). Fragment sizes were assessed using GENOTYPER 3.5 (Applied Biosystems) relative to a ROX 400 HD size standard (Applied Biosystems). Goodness-of-fit Hardy–Weinberg equilibrium tests and estimates of the frequency of null alleles were carried out using CERVUS 2.0 (Marshall *et al*., 1998).

Table 1 Characteristics of nine nuclear microsatellite loci in *Sorbus domestica* with primer sequence, number of alleles observed and their size range, annealing temperature (T_a) and corresponding reference

Locus	*Primer sequence [5′-3′]*	*Number of alleles*	*Size range [bp]*	T_a *[°C]*	*Reference*
MSS5	F:CCCCAACAACATTTTTCTCC R:CCTCTCGCTCTTTGCCTCT	5	117–127	60	Oddou-Muratorio *et al*. (2001)
MSS16	F:CTCCCCTTGTGTGATGCC R:TTGCCCTCAAAGAATGCC	4	143–161	60	Oddou-Muratorio *et al*. (2001)
CH01h10	F:TGCAAAGAAGGTAGATATATGCCA R:AGGAGGGATTGTTTGTGCAC	11	105–136	60	Gianfranceschi *et al*. (1998)
CH01h01	F:GAAAGACTTGCAGTGGGAGC R:GGAGTGGGTTTGAGAAGGTT	5	87–137	60	Gianfranceschi *et al*. (1998)
CH02c09	F:TTATGTACCAACTTTGCTAACCTC R:AGAAGCAGCAGAGGAGGATG	6	223–243	60	Liebhard *et al*. (2002)
BGT23b	F:CACATTCAAAGATTAAGAT R:ACTCAGCCTTTTTTTCCCAC	4	190–196	50	Yamamoto *et al*. (2002b)
CH02B03b	F:ATAAGGATACAAAAACCCTACACAG R:GACATGTTTGGTTGAAAACTGG	7	87–103	50	Gianfranceschi *et al*. (1998)
MS14H03	F:CGCTCACCTCGTAGACGT R:ATGCAATGGCTAAGCATA	9	162–181	50	Liebhard *et al*. (2002)
CH02d08	F:TCCAAAATGGCGTACCTCTC R:GCAGACACTCACTCACTATCTCTC	11	237–265	50	Liebhard *et al*. (2002)

Abbreviations: F: forward primer; R: reverse primer.

Current pollen flow

Mating patterns of individual mother trees were determined in a paternity analysis of their open-pollinated offspring. Paternity was assigned using the maximum likelihood-based method (Meagher, 1986) implemented in CERVUS 2.0 (Marshall *et al*., 1998). The program uses simulation-based data to assign paternity at a specified significance threshold. To run simulations, we used the following parameters: 10 000 simulated mating events; minimum number of matching loci equals eight; error rate of zero (Slate *et al*., 2000, Slavov *et al*., 2005; note that using an error rate of 0.01 had no relevant effects on paternity assessment); all adult trees as candidate parents ($N = 167$; each clonal cohorts were counted as single genetic individuals), complete sampling of candidate parents; all loci typed for all samples. We applied a 95% significance threshold to ensure valid assignment, thus accepting missing cases of true paternity. Special attention was given to long-distance pollen flow events between the two subpopulations. Here, we conservatively used a 100% significance threshold.

We used different curve estimation regression models (SPSS 14.0, SPSS, Chicago, and TABLECURVE 2D, SYSTS, Richmond) to determine the function that best fitted the empirical data on pollen flow with increasing geographical distance.

Recent seed dispersal

To obtain information about the distances between maternal trees and their established progeny (that is, seed dispersal across one generation), we determined the maternally inherited (Raspé, 2001) cpDNA haplotypes of all adult *S. domestica* trees in our study area. Screening for variation was performed using 18 universal cpDNA primer pairs described in Demesure *et al*. (1995), Dumolin-Lapègue *et al*. (1997), Grivet *et al*. (2001) and Sang (1997), which were well distributed over the large single copy (LSC) region (Grivet *et al*., 2001), on 18 *S. domestica* individuals distributed over the whole study area. Amplification was done in 20 µl volumes containing 1.0 µl template DNA (≈ 4 ng µl^{-1}), 10 µl Multiplex-Master Mix (Qiagen) and 0.1 µl of each primer (2 µM). PCR amplifications were performed on PTC-100 thermocyclers (MJ Research), using initial denaturation at 95 °C for 15 min, 34 cycles with 94 °C for 30 s, 48–60 °C (depending on the given primer) for 90 s and 72 °C for 3 min, followed by a final extension at 72 °C for 10 min. Fragments were amplified from 13 primer pairs, and the PCR products were then screened for restriction fragment length polymorphisms using 14 restriction endonucleases. PCR products (1 µl) were digested in 10 µl reaction volumes for 3 h with 1 U of restriction enzyme at 37 °C in an oven. Restriction fragments were separated by electrophoresis on 2% agarose gels in 1 × TBE buffer at 110 V for 2 h. For visualization, gels were stained with ethidium bromide and photographed under UV (UVP, Upland, CA, USA). We only found two different cpDNA haplotypes, with frequencies of 0.114 and 0.886, respectively (*trn*K [tRNA-Lys (UUU) exon 1] and *trn*K [tRNA-Lys (UUU) exon 2], RÉ *Alu*I; for primers described in Demesure *et al*., 1995). In addition, we tested the universal cpDNA microsatellite primers described by Deguilloux *et al*. (2003), but could not find any variation.

For trees from subpopulation Schaffhausen (Figure 1) with the rare cpDNA haplotype, we performed a parentage analysis using the nuclear microsatellite data to assign the most likely parents (CERVUS; significance level 80%). If the most likely parent had the same rare cpDNA haplotype as the individual tested and also exhibited a larger diameter at breast height (as rough indicator of tree age), it was considered to be the mother tree and, hence, the source plant of seed dispersal.

Results

We detected 62 alleles in the nine nuclear microsatellite markers, with four to eleven alleles per locus (Table 1). Two private alleles, each with a single occurrence, were detected in the offspring. All loci showed diploid banding patterns, and Mendelian inheritance was observed when genotypes of offspring and corresponding mothers were compared. One locus (MSS 16) showed evidence for the occurrence of a null allele (null allele frequency estimated by CERVUS: 0.0104, $P < 0.01$). However, mother–offspring comparisons resulted in no obvious signs of null alleles at this locus, and it was therefore retained in the analysis.

CERVUS analysis resulted in a high exclusion probability of 0.993. In accordance, 62.9% ($N = 744$) of the offspring could be assigned with a high confidence of $\geqslant 95\%$ to a single pollen donor. For 27.4% ($N = 324$) of the offspring, several potential fathers were found (that is, unassigned gene flow within the population), whereas for 9.7% ($N = 115$), no matching father could be found. In view of the exhaustive sampling of the adult trees and the above mentioned high exclusion probability, these non-matching offspring genotypes were attributed to pollen immigration (Garcia *et al*., 2005). Using these data, we assessed the selfing rate per mother tree and the straight-line distances between mothers and unambiguously identified father trees. Overall selfing rate was 33.5% ($N = 249$), but variation in selfing rates was considerable, with individuals having no selfed offspring (34.7% of mothers) to individuals with over 80.0% of selfed seeds (8.0% of mothers).

The mean pollen flow distance was 1.2 km ($N = 495$), but the shape of the frequency distribution of pollen dispersal was leptokurtic, with a rapid decrease within the first 200 m, indicating a predominance of near-neighbour gene exchange (Figure 2). However, a surprisingly high proportion ($> 33\%$) of pollen donors were located at a distance of more than 1 km from the mother trees (Figure 2). We also detected 13 long-distance pollen flow events between the two subpopulations at distances of 12–16 km (Figure 1), six of them being supported at a 100% level of statistical confidence.

Pollen dispersal was well described by a simple inverse curve ($y = -0.067 + 2551.54/x$; $R^2 = 0.985$), with a long tail (Figure 2). Alternative curve estimation models such as power ($R^2 = 0.843$) or exponential models ($R^2 = 0.485$) less fitted the pollen dispersal data.

We could only infer 10 unambiguous events of recent seed dispersal (Figure 3). These seed dispersal events covered distances between 12 m and 12 357 m, with most dispersal events at distances between 1–2 km.

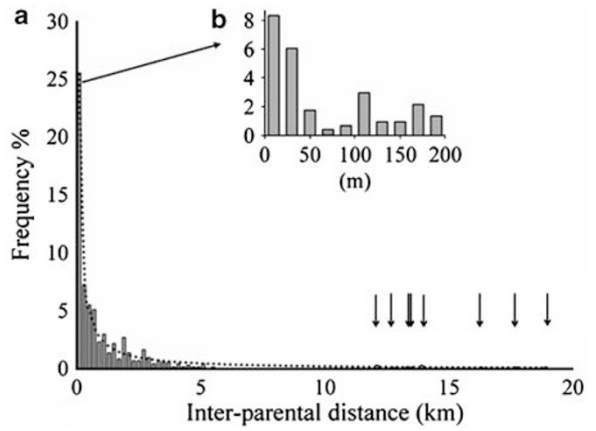

Figure 2 Frequency distribution of current pollen flow in *Sorbus domestica* (grey bars). (**a**) Frequency distribution in 200-m distance classes. The dotted line represents the best fitting curve ($y = -0.0674 + 2551.54/x$), and the arrows indicate long-distance events of pollen flow. (**b**) Inset for distances of 0–200 m in 20-m distance classes.

Discussion

Our results show that, despite the heterogeneous landscape and the low density of *S. domestica* in the study area, most adult trees formed part of an extensive network of pollen flow spanning deep valleys and large unforested areas comprising intensively used agricultural land and built areas (Figure 1). Interestingly, most of the offspring for which no matching father could be assigned were from mothers growing close to the northern border of the study area (data not shown). In contrast to our survey, this result indicated that at least some unknown *S. domestica* adult individuals grow outside our study area in adjacent Germany.

The average pollination distance of about 1.2 km for 495 outcrossed offspring assigned to single pollen donors was larger than those that have been reported for closely related temperate (Oddou-Muratorio *et al*., 2005; Hoebee *et al*., 2007) or tropical tree species (Ward *et al*., 2005). However, the maximum distance of pollen flow of 16 km is, to our knowledge, the largest distance that has been directly measured so far. These large pollen flow distances reflected the large size of our study area, illustrating the limited value of estimates of average pollen flow distances derived from spatially restricted studies. Based on considerations of tree density, it has been deduced that gene flow by pollen in tropical fig species should occur over similar distances as reported here (Nason *et al*., 1998). However, unlike *S. domestica*, tropical figs have highly specialized insect pollinators that have evolved the ability to locate their widely spaced host plants in tropical rainforest (Ollerton *et al*., 2006). In contrast, *S. domestica* relies on a wide spectrum of generalist pollinators (Raspé *et al*., 2000). We conclude that at least some pollinators that visit *S. domestica* locate trees of the same species across large areas of open habitat. Several insect pollinators are indeed known to cross large patches of unsuitable habitat (Steffan-Dewenter and Kuhn, 2003), suggesting that foraging distances of insects may have generally been underestimated. For *S. domestica*, the most likely long-distance pollen vectors

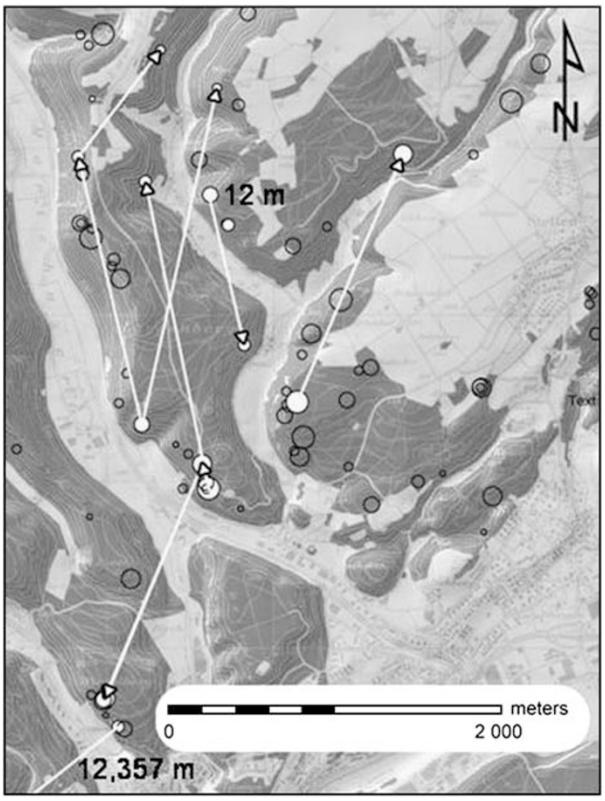

Figure 3 Recent gene flow by seed in *Sorbus domestica* at the landscape scale in Schaffhausen, Switzerland. White circles indicate trees with the rare cpDNA haplotype (all trees with the rare haplotype from subpopulation Schaffhausen are shown), whereas open circles show trees having the frequent cpDNA haplotype. Circle sizes are proportional to tree diameters at breast height. Arrows represent inferred recent events of gene flow by seed. Numbers indicate the shortest and the longest dispersal event. Note that this is a zoom-in from Figure 1.

are bumblebees and honey bees, as these insects are known to have large foraging ranges (Kreyer *et al*., 2004).

The shape of the curve describing the frequency distribution of pollen flow events with increasing geographical distance was consistent with other studies on pollen flow in trees also reporting leptokurtic pollen dispersal curves with fat tails (Oddou-Muratorio *et al*., 2005). Accordingly, modelling approaches for several forest tree species strongly indicated that fat-tailed pollen dispersal curves are much more likely than thin-tailed pollen dispersal curves (Austerlitz *et al*., 2004). However, although most published pollen dispersal curves relied on extrapolation from observations of pollen flow over rather short distances, our dispersal curve was empirically also supported for larger distances. The existence of a fat tail is significant because it implies that even isolated trees receive pollen from a genetically diverse donor pool (Klein *et al*., 2006). Nonetheless, the total number of foreign pollen received by isolated trees is likely to be small, which might explain the high selfing rates observed in some trees (U Kamm unpublished data). Several woody Rosaceae are known to exhibit weak self-incompatibility which breaks down if cross-pollination is delayed (Garcia *et al*., 2005).

Seed dispersal is another process enabling plants to move genes in space. As in the case of pollen, recent

molecular genetic studies have demonstrated the importance of long-distance dispersal of seeds (Bacles *et al*., 2006; Hardesty *et al*., 2006) for colonization as well as for maintaining functional connectivity of spatially isolated populations. In the case of the wind-pollinated and wind-dispersed tree *Fraxinus excelsior*, seed dispersal was shown to be more effective than pollen dispersal in maintaining functional connectivity (Bacles *et al*., 2006).

Long-distance dispersal of seed is supported by studies using both direct (Godoy and Jordano, 2001) and indirect (Mohanty *et al*., 2002) genetic methods in temperate forest trees with fleshy fruits. As shown here, it seems that *S. domestica* seeds are regularly dispersed to distances of several hundred meters. Most dispersal events observed were along forested hill ranges, but also traversed valleys between inferred mother–offspring pairs (Figure 3). In view of the high mobility of seed vectors such as birds and large mammals (for example, roe deer, wild boar; Myers *et al*., 2004), we assume that the seed dispersal distances of up to 12 km detected in our study probably do not represent the maximum distances that may occur.

Although the extensive gene flow network by pollen detected in *S. domestica* enables the formation of a genetically diverse seed pool, natural regeneration in the study area was low or even absent (U Kamm, personal observation). This was probably due to low fruit production combined with a low availability of suitable regeneration niches. In central Europe, the habitat suitable for the recruitment of light-demanding forest species such as *S. domestica* has declined as forests have become generally older and darker over the past 100 years (Wohlgemuth *et al*., 2002). In addition, the low density of pollen donors, reflected by generally higher selfing rates of isolated trees (Larson and Barrett, 2000), was probably responsible for low seed production in our study species. Indeed, Aguilar *et al*. (2006) have argued that the most common proximate cause of reproductive impairment in fragmented habitats may be pollination limitation. In our study area, *S. domestica* produced genetically diverse offspring, but because suitable habitat patches for regeneration were widely scattered, the few seeds produced may be insufficient to maintain the population.

The extensive network of pollen flow revealed in this study has implications for conservation. First, *S. domestica* is representative for several rare forest trees in Central Europe that are insect-pollinated, fleshy fruited and persist as scattered individuals in managed high forests. As well as securing natural regeneration, management strategies for such species should aim to maintain the number of local populations, even if they appear to be small and geographically isolated, as they may form part of an interbreeding network of stands. Second, in assessing the effectiveness of current programmes to increase habitat connectivity, we need to shift from descriptive studies of structural habitat networks to a more quantitative analysis of their functional connectivity (Nathan, 2005; Holderegger and Wagner, 2008).

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