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Pollen movement in a *Malus sylvestris* population and conclusions for conservation measures

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Abstract

Knowledge of pollen movement and frequency of interspecific hybridization in fragmented populations of rare species is a prerequisite for the implementation of conservation measures. In a large-scale study area (14,000 hectares) we analysed 297 *Malus sylvestris* trees with nine nuclear microsatellite markers. After open pollination of 564 offspring from 51 mother trees located in seven harvesting sites were investigated and genetic paternity analysis was performed. The paternal parent was identified for 213 offspring and the pollen dispersal distances between mother and pollen source were calculated. A large proportion of detected pollination events (42.4%) were observed within a radius of 50 m of the mother tree. The comparison of different tree densities indicated that with decreasing density the pollen dispersal distances increase. We observed pollination over long distances with a maximum of 10.7 km which is probably one of the reasons for a low spatial genetic structure within the *M. sylvestris* population and a stable genetic diversity in the offspring. Incorporating microsatellite data of 21 apple cultivars, a hybridization frequency of nearly 8% was determined. With decreasing tree density the number of hybridization events increased. Based on the results of our study an enhancement of the density of existing *M. sylvestris* populations is recommend to reduce the likelihood of hybridization. The production of young plants originated from seeds collected after open pollination is not advisable. Instead of that the seedlings for further reintroduction measures should be produced by controlled crossings in seed orchards to ensure 'true type' *M. sylvestris* individuals.

Keywords: crap apple; endangered; long-distance pollen dispersal; paternity analysis

Introduction

In recent years, numerous studies investigated the gene flow in insect-pollinated trees for the development of appropriate conservation strategies (Oddou-Muratorio *et al.*, 2005; Garcia *et al.*, 2007; Ahmed *et al.*, 2009; Kamm *et al.*, 2009; Sebbenn *et al.*, 2012). Thereby, several studies have observed that a reduction in population size resulted in increasing frequencies of long-distance pollen dispersal. The conclusion from these studies is that physically isolated trees are not necessarily reproductively isolated and thus the genetic diversity within the population can be maintained to a certain degree (reviewed in Ashley, 2010). On the other hand, this effect could also have negative consequences on the conservation of wild species, because it is expected that

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hybridization is not prevented due to spatial separation between related species since it may be compensated by longer pollen dispersal distances (Dick *et al*., 2008; Vranckx *et al*., 2012). Particularly, the extensive cultivation of domesticated crops greatly increases the likelihood of contact between domesticated and related wild species (Cornille *et al*., 2012).

Malus species are self-incompatible and need compatible pollination partners to maintain natural regeneration (Broothaerts, 2003). The pollination is provided by insects, mainly bees and bumblebees (Palmer-Jones and Clinch, 1968). No reproductive barriers inhibit hybridization of different species within the genus *Malus* (Larsen and Kjaer, 2009). It is assumed that hybrids have a higher degree of fitness and are more competitive than 'true type' wild species and over time the wild population is replaced by hybrids and at worst be finally extincted (Larsen *et al*., 2006).

Therefore, increasing efforts were performed to preserve wild relatives of cultivated crops as genetic resources for potential use in breeding programmes. *M. sylvestris* (L.) Mill. is the only indigenous wild relatives of the domesticated apple in Central Europe. This crab apple species became quite a rare species in recent years and is declared as an endangered species in Europe (Schnitzler *et al*., 2014; Schulze *et al*., 2013).

In order to maintain the crab apple as part of our ecosystem and genetic resource the implementation of conservation measures is recommended. For example, most crab apple trees seem to be resistant against powdery mildew (*Podosphaera leucotricha*) and could be a potential gene source for apple breeding (Büttner, 1999). Also its robustness against harsh weather conditions and its modest demands on soil and water conditions could be a potential genetic resource for apple breeding (Stephan *et al*., 2003).

The main objectives of our study were (1) the investigation of the genetic diversity of the pollen pool, parent genotypes and offspring within the study area using microsatellite markers (simple sequence repeats (SSR)), (2) estimation of the proportion of hybrids after open pollination and (3) the assessment of pollen dispersal distances within the *M. sylvestris* population in the East Ore Mountains. Based on these results conclusions were drawn to the implementation of conservation measures.

Material and methods

Study area and field sampling of Malus sylvestris *trees*

Germany has about 250 *M. sylvestris* populations with approximately 5500 trees (Schulze *et al*., 2013). The East Ore Mountains represents one of the rare populations in Germany with a relatively high number of *M. sylvestris* individuals (Schulze *et al*., 2013). Nevertheless, the existing crab apple trees are strongly fragmented within this area. The study area covered a region of about 14,000 hectares with a maximal east-west extension of 10 km and a maximal north-south extension of 20 km in the East Ore Mountains. The altitude in the study area ranges from 250 m up to 824 m with Mount Geising as the highest elevation.

Within the study area 625 potential *M. sylvestris* trees were mapped with GPS using the Gauss–Krueger coordinates. In total, 297 adult *M. sylvestris* trees that were distributed over the entire study area were chosen for the genetic analysis. The selection of each tree was based on several morphological characters, which indicated a 'true type' *M. sylvestris* tree (Reim *et al*., 2012). To collect seedlings after open pollination we chose seven sampling sites (Fig. 1). The selection of these sampling sites primarily focused on areas where the *M. sylvestris* trees were located in an appropriate distance (minimum 250 m) to domesticated apples, assuming no or low hybridization with *Malus* × *domestica*. Furthermore, it was intended to choose mother trees with at least four neighbouring trees in a radius of 500 m to provide an appropriate pollination. The selection of mother trees was also determined by the presence of fruit on the crab apple trees. Based on these criteria, in total 51 mother trees were chosen for seed collection. In the seven sampling sites 1–21 mother trees per site were sampled (Fig. 1). In autumn, 20–30 fruits from each mother tree were collected. Each fruit contained between two and ten seeds. The seeds were isolated and stratified at 4°C in autoclaved wet sand. After 90 days, 10–100 seeds per mother tree were sowed and cultivated in the greenhouse. After 4 to 6 weeks, the seeds germinated. Ten seedlings from each mother tree were usually sampled for paternity analysis. For single mother trees, the number of seedlings was lower than 10. To compensate this, the sample size of other mother trees was increased to 20 seedlings so that in total 564 seedlings for further genetic investigations were available.

DNA isolation and microsatellite analysis

Fresh leaf material of 297 adult *M. sylvestris* trees and 564 seedlings was collected in 2 ml reaction tubes and dried using silica gel according to a modified protocol by Chase and Hills (1991). In addition, seven genotypes ('Delicious', 'Fiesta', 'Prima', 'Worcester Pearmain', '*Malus floribunda* 821', '*Malus robusta* 5' and 'Malling 9') recommended by the European Cooperative Programme for Plant Genetic Resources (ECPGR) as standard apple genotypes (Govan *et al*., 2007) were included. Furthermore, 14 old apple cultivars often cultivated in the study area were analysed as out-group genotypes.

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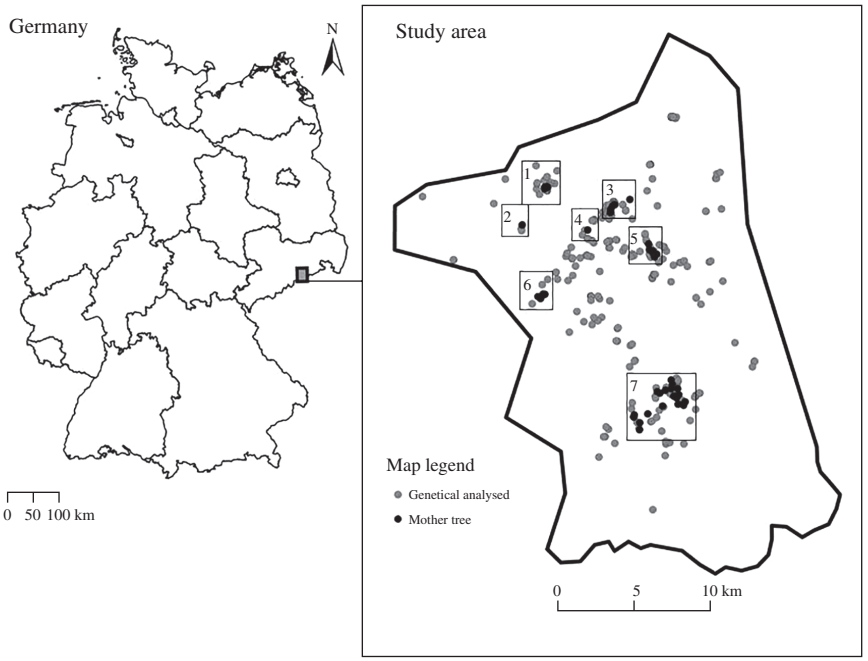

Sampling site	Mother trees (*n*)	Seedlings investigated (*n*)
1	5	50
2	1	10
3	6	60
4	1	10
5	12	158
6	6	68
7	20	208

Fig. 1. Map of the seven sampling sites within the study area including the position of all genetically analyzed *M. sylvestris* trees (grey points) and the mother trees (black points).

The leaf material was stored at room temperature until DNA isolation. DNA extraction and quantification was performed by the company LGC Genomics (Berlin, Germany). All samples were diluted to 10 ng/μl.

From a set of standard microsatellite primers, defined by ECPGR, nine primer pairs (CH01h01, CH04c07, CH01h01, Hi02c07, CH01f03b, GD147, CH01f02, CH02c09 and GD12) were selected for microsatellite analysis (Govan *et al.*, 2007). All these selected SSR markers have been developed for apple cultivars (Hokanson *et al.*, 1998; Liebhard *et al.*, 2002; Silfverberg-Dilworth *et al.*, 2006). The complete information about the molecular characteristics for each SSR primer can be found at http://www.hidras.unimi.it/HiDRAS-SSRdb/pages/index.php.

The PCRs were carried out following the manufactures guide of the 'type-it microsatellite kit'® (Qiagen, Germany). The electrophoresis was performed on a CEQ 8000 DNA Sequencer and the data were analysed using CEQ 8000 software (Beckman Coulter, Germany).

Paternity analysis

The quality of the chosen microsatellite primer set was estimated by calculating the frequency of null alleles on basis of the genotype data of the 297 candidate fathers using the software program CERVUS version 3.0 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007). Within our microsatellite set, the program CERVUS estimated null allele frequencies between a minimum value of −0.0380 (CH01H10) and a maximum value of 0.0383 (GD147). In the literature, the frequencies (*P*) of null

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alleles were almost always $P < 0.40$, and usually $P < 0.20$ (Dakin and Avise, 2004) indicating a good quality of the chosen primers in our study.

The calculation of the most probably fathers was based on multilocus genetic data of 564 offspring, a pool of 297 candidate fathers (including also the mother trees) and further 21 *Malus* reference genotypes using the software CERVUS version 3.0. For simulation of paternity analysis the following parameters were used: genotypes with less than four loci were excluded, 100,000 repetitions and a confidence level of 95 % (strict). The simulation parameter 'proportion of candidate parents sampled' was set to a value of 0.5. Also the offspring was checked statistically for self-fertilization using CERVUS version 3.0. For all further parameters standard settings were applied. The paternity exclusion probability (P_{ex}) of the second parent was calculated for the nine SSR loci and across all loci using also CERVUS version 3.0.

Based on the location of each identified father, the pollen dispersal distance to the respective mother tree was calculated. The *M.* × *domestica* descendants were excluded because the distance to the apple cultivars was unknown. Also the offspring resulted from self-pollination were excluded from further analysis. In order to compare the influence of tree density on the pollen dispersal distance, the mother trees were grouped into five density classes (21, 16–20; 11–15; 5–10; 0–4 nearby trees within a radius of 250 m for each mother tree). For each distance class we calculated the mean pollen dispersal distances and number of selfing and hybridization events. Finally, the relationship between the density and the mean pollen dispersal distance was calculated using the software SPSS version 19.

Genetic diversity parameters

The following genetic parameters were calculated using the software GENALEX version 6.5 (Peakall and Smouse, 2006, 2012): mean number of alleles by locus (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), the expected heterozygosity (H_e) and the fixation index (F). These genetic parameters were estimated for the 297 *M. sylvestris* individuals representing the crab apple pollen pool within the study area, the parent individuals (83 individuals) and for the offspring individuals with identified father (196 individuals). Hybrid genotypes were excluded from the further analysis.

Spatial genetic analysis

The spatial genetic analyses were implemented for the 297 genotyped trees. The correspondence between geographic and genetic distance was performed by Mantel test with statistical testing by 9999 permutations using the software GENALEX version 6.5 (Mantel, 1967). A correlation coefficient (r) equal to zero indicates that no genetic difference depending on the geographic distance is found. To estimate the relationship between genetic variables and geographic location on a smaller geographic scale we used the 'Multiple Distance Class' option in GENALEX version 6.5. For the calculation we set the base distance class size to 500 m for 20 runs (0–10,000 m).

Results

Paternity analysis

The paternity analysis was performed for 564 offspring genotypes with known mother involving the genetic data of 297 candidate fathers. All 564 offspring individuals and 297 candidate fathers showed a sufficient PCR amplification. Missing data occurred in single individuals but were highest at three loci, so that no genotype had to be excluded from the paternity analysis (see also Material and methods section).

Using a strict confidence level for parentage assignment (>95%) out of the 564 investigated seedlings the father could assign for 213 seedlings. Over the half (62%) of the seedling fathers remained undetected. In total, 69 different *M. sylvestris* individuals were responsible for the fertilization of the offspring. Nine seedlings originated from self-pollination, corresponding to a selfing rate of $s = 4.23\%$. Furthermore, 17 seedlings are the result of hybridization with *M.* × *domestica*, corresponding to a hybridization rate $h = 7.98\%$. These seedlings showed a perfect genotypic match with 'Baumanns Renette' (1x), 'Elstar' (1x), 'Goldparmäne Rogo' (2x), 'Großherzog Friedrich von Baden' (2x), 'Grahams Jubiläumsapfel' (1x), 'James Grieve' (6x), 'Ontario' (1x), 'Rote Sternrenette' (1x), 'Geheimrat Dr. Oldenburg' (1x) and 'Ruhm aus Kirchwerder' (1x), which were often cultivated in the study area. The probability of exclusion combined across all nine locus was $P_{ex} = 99.99\%$ indicating a high efficiency of the applied marker with an almost perfect exclusion of false fathers.

The calculated pollen dispersal distances range from minimum 6 m up to 10.7 km. The majority of the pollen dispersal occurred between the neighbouring trees. In total, 42.4% of the offspring were pollinated by trees standing in a distance of up to 50 m from the mother trees (Fig. 2). Within a radius of 100 m nearly 57% of all pollination events were observed. As might be expected, the proportion of pollination events decreased with increasing distance. Nevertheless, 3.2% of the seedlings

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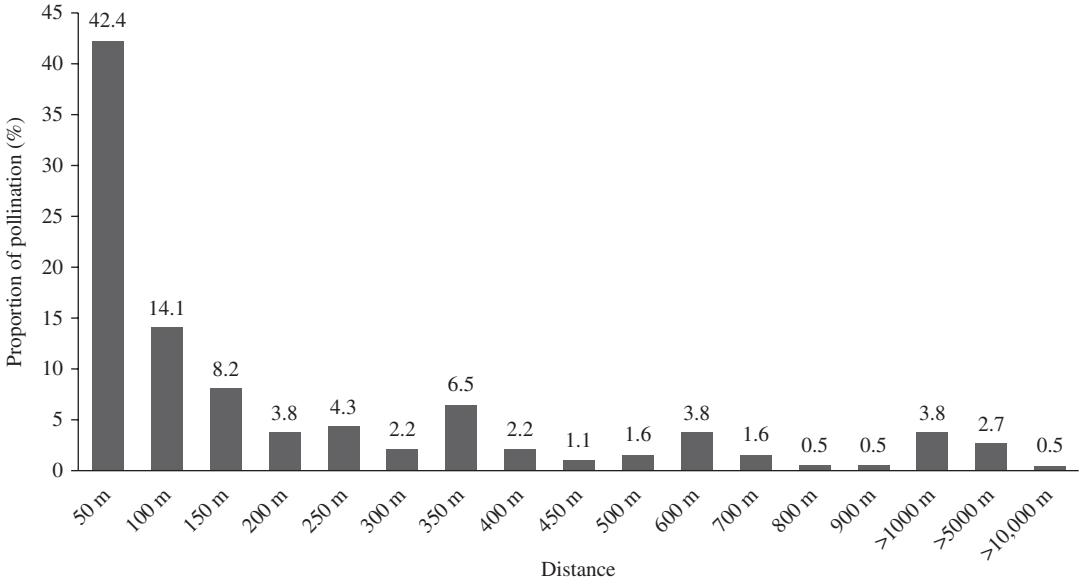

Fig. 2. Proportion of pollination in relation to the distance between mother and father trees.

were pollinated from fathers standing in a distance of over 5 km from the mother tree.

The calculation of Pearson's correlation between the density class and the mean pollen dispersal distance showed a significant strong negative correlation $r = -0.906$ ($P = 0.034$) between these two parameters. The highest density class (21 nearby trees in a radius of 250 m to the mother tree) showed the lowest mean pollen dispersal distance of 30 m (Fig. 3). In the other patches with a high density (16–20 nearby trees) and a moderate density (11–15 nearby trees), a pollen dispersal distance of up to 150 m was observed. In the lower density classes (5–10 nearby trees) the mean pollen dispersal distance increased up to 433 m. In the very low density plot with only 0 to 4 nearby trees the mean pollen dispersal distance was 957 m. No hybrids or selfing events were detected in the highest density class with 21 nearby trees. In the density classes with 16–20, 11–15 and 5–10

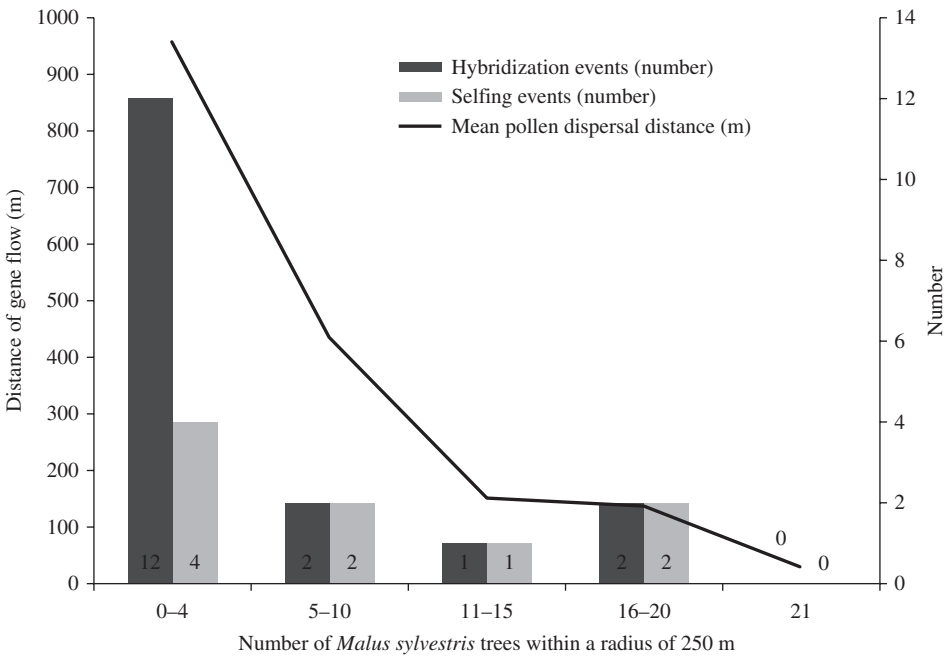

Fig. 3. Mean pollen dispersal distance, number of hybridization events and number of selfing events in each density class (number of nearby *M. sylvestris* trees within a radius 250 m to the mother tree).

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Table 1. Genetic diversity parameters estimated for nine SSR loci for the pollen pool and the offspring genotypes

		Pollen pool[1]						Parent genotypes[2]					Offspring[3]				
Locus	Null Allele[4]	N_a	N_e	H_o	H_e	F	P_{ex} (%)	N_a	N_e	H_o	H_e	F	N_a	N_e	H_o	H_e	F
CH01H10	−0.0012	23.0	7.1	0.86	0.86	0.00	75.4	15.0	6.2	0.89	0.84	−0.06	17.0	8.6	0.89	0.88	0.00
CH04C07	−0.0380	23.0	3.0	0.71	0.66	−0.07	45.4	11.0	2.7	0.70	0.63	−0.10	9.0	2.9	0.69	0.66	−0.06
CH01H01	0.0441	18.0	9.2	0.81	0.89	0.09	75.8	13.0	8.5	0.88	0.88	0.00	13.0	6.8	0.89	0.85	−0.04
Hi02C07	0.0037	25.0	5.9	0.82	0.83	0.01	68.1	16.0	5.3	0.80	0.81	0.02	15.0	6.4	0.83	0.84	0.01
CH01F03b	0.0377	16.0	4.5	0.73	0.78	0.06	59.8	10.0	4.2	0.65	0.76	0.15	8.0	3.9	0.69	0.74	0.07
GD147	0.0383	17.0	6.6	0.78	0.85	0.08	71.4	13.0	6.3	0.80	0.84	0.05	11.0	6.2	0.77	0.84	0.08
CH01F02	0.0138	20.0	8.6	0.86	0.88	0.03	77.3	18.0	7.5	0.86	0.87	0.01	18.0	8.0	0.85	0.88	0.03
CH02C09	0.0213	13.0	2.6	0.59	0.61	0.04	44.2	10.0	2.5	0.61	0.59	−0.04	10.0	2.6	0.58	0.61	0.05
GD12	0.0302	18.0	4.2	0.71	0.76	0.06	58.1	12.0	4.5	0.71	0.78	0.08	14.0	4.4	0.67	0.78	0.14
Mean		19.2	5.7	0.77	0.80	0.03		13.1	5.3	0.77	0.78	0.01	12.9	5.5	0.76	0.79	0.03

N_a, mean number of alleles by locus; N_e, effective number of alleles; H_o, observed heterozygosity (= number of heterozygotes/N); H_e, expected heterozygosity ($= 1 - \sum p_i^2$); F, fixation index $= (H_e - H_o)/H_e$. P_{ex}, probability of exclusion.
[1] 297 individuals genetically analysed. [2] 83 parent genotypes. [3] 196 individuals (descendants with identified father, the hybrids were excluded). [4] Null allele frequency calculated based on the 297 genotypes of the pollen pool.

nearby trees two or one hybridization and selfing events were estimated respectively (Fig. 3). In the lowest density plot (0 to 4 nearby trees) the number of hybridizations rose sharply to 12 events. The number of selfing events also increased to four in this density class.

Genetic diversity parameters

The genetic diversity parameters were calculated based on the allele frequencies of the candidate fathers (297 individuals), the 83 parent individuals and the offspring genotypes with assigned father (196 individuals). The 17 identified hybrids were not included in the analysis. The parameters of the genetic variability estimated for each SSR marker and each group are shown in Table 1.

The number of alleles (N_a) in the parent group ranged from 10 to 18 with an average number of alleles $N_a = 13$. In the offspring, the number of alleles (N_a) range from 8 to 18 per locus and the average number of alleles was with $N_a = 13$ the same as in the parent group. The average effective number of alleles was with $N_e = 5.3$ and 5.5 nearly equal for both groups.

The average expected heterozygosity (H_e) of the pollen pool, the parents and offspring genotypes was very similar with $H_e = 0.80$, 0.79 and 0.78, respectively.

The average fixation index (F) for each group was nearly zero indicating no heterozygote excess (outbreeding) or heterozygote deficiency (inbreeding).

Spatial genetic analysis

The Mantel test including the geographic and genetic distance matrix of 297 trees yielded in a significant ($P = 0.009$) correlation coefficient of $rxy = 0.057$ indicating a slight correlation of these two matrices. The patterns of spatial autocorrelation for each distance class were described by generating a correlogram (Fig. 4). The first distance class of 0–500 m showed the highest value with $r = 0.034$ indicating a low but significant spatial genetic structure. In the following distance classes r decreased but remained significant up to 9000 m. In the distance class beyond 9000 m, a genetic correlation was no longer detected suggesting a full spatial extent of autocorrelation.

Discussion

Pollen transport

The gene flow frequency was substantially in line with our expectations: a closer distance between the trees

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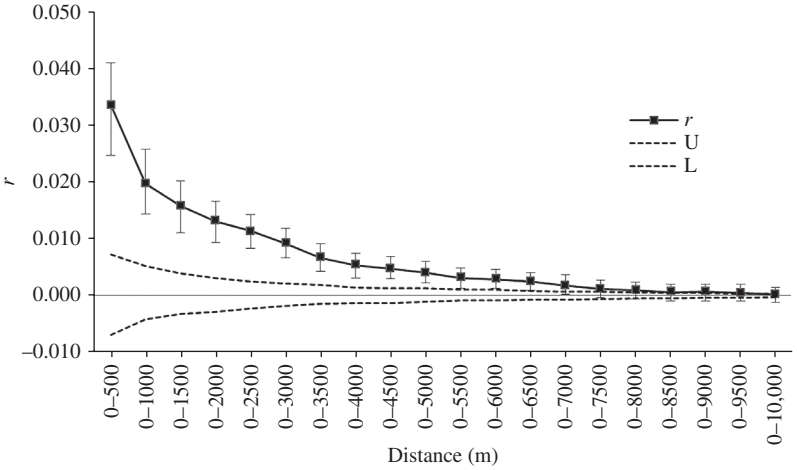

Fig. 4. Correlation (*r*) between multilocus data and spatial distribution of 297 *M. sylvestris* individuals based on an autocorrelation analysis for multiple distance class sizes. *U*: upper bounds of the 95 % confidence interval, *L*: lower bounds of the 95 % confidence interval.

increased the likelihood for their crossing. More than half of the pollen was derived from nearby trees standing in a distance of up to 100 m. With increasing distance between the trees the gene flow frequency decreased. Nevertheless, almost 20% of the descendants were pollinated from trees that were located in greater distances than 350 m. This is reflected in the low correlation between geographic and genetic distance in the *M. sylvestris* population. Kramer *et al.* (2008) assumed that fragmentation of populations or the reducing of population density lead to increasing pollen dispersal distances. This assumption was confirmed in our study. In the *M. sylvestris* population a lower density supported higher pollen dispersal distances. In such fragmented populations single trees act as stepping stones and bridge larger distances between groups of trees (Albaladejo *et al.*, 2012). This in turn is expected to reduce the spatial genetic structure of populations (Sebbenn *et al.*, 2012).

This observation might be explained by the main pollinators of the crab apple. Our European fruit species were mainly pollinated by honey bees and bumblebees which can travel up to 10 km (Beekman and Ratnieks, 2000). Honey bees are flower constant and collect their pollen mainly from one species while they visit up to 3000 flowers per day (Pickhardt and Fluri, 2000). Bumblebees even visit up to 5600 flowers per day (Pickhardt and Fluri, 2000). If there are not enough flowers nearby as result of a low tree density the bees are forced to extend their foraging flights to larger distances.

The pollen dispersal distance of almost 11 km measured in our *M. sylvestris* population confirmed that the pollinators are able to travel great distances during their foraging flight. Other studies have reported very large pollen transport distances, for example 3.2 km for *Dinizia excelsa* pollinated by African honeybees, 16 km for *Sorbus domestica* pollinated by bees and flies and even 160 km in an African *Ficus sycomorus* population that were pollinated by a fig wasp (Dick, 2001; Ahmed *et al.*, 2009; Kamm *et al.*, 2009). These results demonstrated that insect-pollinated trees, depending on the type of pollinator, are able to overcome great pollen dispersal distances, and thus to compensate habitat fragmentation to a certain degree.

The genetic diversity in the *M. sylvestris* offspring was not reduced compared with the parent generation. This indicated that the *M. sylvestris* population is not negatively affected by a reproductive decline. The strong fragmentation and the spatial isolation within the crab apple population were buffered by long-distance pollen dispersal. However, high pollen dispersal distances might have negative effects on the hybridization frequency. In our study, a hybridization rate with *M.* × *domestica* of almost 8% was detected, even though we selected mother trees that were located in a minimum distance of 250 m to possible apple cultivars. The highest number of hybridization events was observed in the low density class with <5 trees within a radius of 250 m. Here, it became apparent that a low density associated with an expansion of pollen dispersal distances had negative effects on the maintenance of 'true type' *M. sylvestris* individuals.

Self-fertilization occurred with a frequency of $s = 4.23\%$. Apples exhibit a gametophytic self-incompatibility controlled by a series of polymorphic S-alleles that prevent the process of pollen tube growth on the stigma of the same plant (Broothaerts, 2003). As a result, fertilization does not take place and there are no seeds formed. However, most self-incompatibility systems are incomplete and self-fertilization does occur at low frequencies (De Nettancourt, 2001). Therefore, it is

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not surprising that in other studies some levels of self-fertilization in cross-pollinated trees were observed, too (Carneiro *et al.*, 2009; Kamm *et al.*, 2009; Larsen and Kjaer, 2009). It is assumed that due to the absence of sufficient pollen sources in fragmented populations the selfing rate increases (Kamm *et al.*, 2009). Our results also indicated a slight increase of selfing events in plots with a low tree density.

Conclusions for conservation measures

For a long-term maintenance of pure *M. sylvestris* trees *in situ* it seems to be very important to improve the density of the existing *M. sylvestris* population. With increasing tree density the pollen dispersal distances could be reduced, pollination between nearby trees could be supported and hybridization with more distant *M.* × *domestica* trees could be prevented. In addition, the viability of existing *M. sylvestris* trees *in situ* can be improved by maintenance measures. Particularly crab apples in forest sites showed low or no fruit set and low vitality because of the disadvantageous light conditions. Pruning of the nearby competitor trees could improve the local light conditions of the crab apple trees resulting in an increasing vitality and flowering of *M. sylvestris* (Stephan *et al.*, 2003).

Another conservation measure can be reintroduction. Here, it is recommended to use identified 'true type' *M. sylvestris* trees. However, buying or producing them can be a problem because especially in endangered species such as *M. sylvestris*, there are often not enough seeds available. The results of our study show that it is inadvisable to collect seeds from *M. sylvestris* trees after open pollination for the production of seedlings. Even if *M. sylvestris* trees are located in greater distances to the next the *M.* × *domestica* an interspecific hybridization cannot be excluded, particular in plots with a low tree density. A controlled crossing *in situ* as an alternative is mostly not practicable, since the blossom (if any) is mainly located in the upper branches, the terrain is mostly inaccessible, the trees are far apart from each other, a permission of the tree owner is required, just to name a few reasons.

The better solution seems to be the planting of seed orchards for producing 'pure' *M. sylvestris* seeds. The establishment of seed orchards is associated with high efforts, costs and space requirements but ensures a long-term production of seedlings and extends the genetic basis of regeneration when planting the seedlings in the natural habitat (Stephan *et al.*, 2003). But here also a contamination with pollen of apple cultivars cannot be excluded completely. To avoid hybridization in *M. sylvestris* seed orchards alternative strategies have to be established. One approach to produce 'pure' seeds is to cover the trees with plastic tunnels during flowering time to protect them from pollen contamination. The pollination is then performed by purchased bumblebees (Kleinschmit, pers. communication) or by hand. However, whether the proportion of hybridization in seed orchards really exceeds a tolerable amount (< 1%) must be investigated in further studies.

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