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Assessing gene flow in apple using a descendant of *Malus sieversii* var. *sieversii* f. *niedzwetzkyana* as an identifier for pollen dispersal

Stefanie REIM[1], Henryk FLACHOWSKY[1**], Maria MICHAEL[2] and Magda-Viola HANKE[1*]**

[1] Federal Centre for Breeding Research on Cultivated Plants, Institute for Fruit Breeding, Pillnitzer Platz 3a, 01326 Dresden, Germany
[2] University of Applied Science Zittau/Görlitz, Theodor-Körner-Allee 16, 02763 Zittau, Germany

The release of genetically engineered apple trees raises the question of their potential environmental impact, and the transfer via pollen of transgenes to cross-compatible cultivars of *Malus domestica* and *Malus* species is deemed to be the greatest source for environmental exposure. The hybrid TNR 31-35, a descendant of *Malus sieversii* var. *sieversii* f. *niedzwetzkyana*, carrying a homozygous, dominant gene responsible for red pigmentation in all plant parts, was used to assess gene flow in an apple scion repository of genetic resources. The red pigmentation provides a morphological marker that enables large-scale evaluation of cross-fertilization under natural conditions. In two consecutive years, 60 and then 56 apple trees of 38 different *Malus domestica* cultivars were selected to serve as pollen-receptor trees. In these two years, 6876 and then 5513 seeds, respectively, were gathered from pollen-receptor trees located at different distances, 2–100 m from 15 pollen-dispenser trees. In total, 11 797 seedlings were examined. An average of 1.8% and 1.4%, respectively, of all seedlings obtained showed red-colored leaves. Considering both years of sampling, 69% of the seeds fertilized by TNR 31-35 were found at less than 10 m from the nearest pollen-dispenser tree. Almost 91% of all seeds fertilized by TNR 31-35 were found at less than 60 m from the nearest pollen-dispenser tree, which is equal to 30 adjacent trees along the row. In this study, pollen was dispersed at least 104 m. After phenotypical evaluation, seedlings selected as red-colored were investigated by simple sequence repeat (SSR) analysis. Each seedling was tested with at least one heteromorphic SSR-marker, which allows the verification of TNR 31-35 as the male parent. All but four seedlings showed one allele specific for the appropriate fruiting tree and the second allele specific for the pollen-dispenser TNR 31-35.

Keywords: apple / cross-pollination / gene flow / *Malus* / red-marker gene / SSR-marker / vertical gene transfer

INTRODUCTION

Apple is one of the most important tree fruit crops in Europe and worldwide. According to the European orchard surveys, which covered 15 European member states in 2002, the area of table apple trees was around 225 433 ha, with France, Italy, Spain and Germany as the major apple producers (URL: http://epp.eurostat.ec.europa.eu). Apple production is dominated by ten top world cultivars (O'Rourke et al., 2003). Because the leading cultivars are susceptible to diseases which require the application of pesticides, breeding resistant cultivars in apple is of high relevance, both to protect the environment and consumers' health. In this context, the main focus of most of the world's apple breeding programs is disease resistance, in addition to improvement of fruit quality. Conventional breeding in apple is time- and labor-consuming, due to a long reproduction cycle with long juvenile phases, which can take up to ten years in the case of hybridization between the domesticated apple and wild species. Using genetic engineering to transfer apple-specific genes that are involved in resistance gives a promising opportunity to shorten the breeding cycle. Although the commercial production of transgenic annual plants is a reality, commercialization of genetically-modified (GM) fruit

* Corresponding author: v.hanke@bafz.de
** These authors contributed equally to this work.

trees is still uncommon (Petri and Burgos, 2005). Nevertheless, since the early 1990s, many GM apple plants have been tested in field trials. According to the Environmental Releases Database for the U.S. (http://www.isb.vt.edu/cfdocs/fieldtests1.cfm), 47 field test records were found for apple. The main interest in these trials is in improvement of fruit quality (fruit ripening and storability) by alteration of ethylene and carbohydrate metabolism and in insect, fungal and bacterial resistances. In the European Union, releases of GM plants have to be notified according to Directive 2001/18/EC. A total of eight summary notifications concern environmental releases of apple: two from Belgium, one from Germany, three from the Netherlands, and two from Sweden (http://gmoinfo.jrc.it; http://biotech.jrc.it/deliberate/dbplants.asp).

Despite the potential benefits of genetically engineered trees, there is also concern about their potential agronomical and environmental impact if transgenes should escape. The prerequisite for hybridization to take place is the presence of other cultivars of the domesticated species or wild relatives and their compatibility under natural conditions. The potential for pollen-based gene flow depends on the geographic distribution of the different compatible species. From the agronomical point of view, the transfer of novel genes from GM apple plants to other non-transgenic cultivars of the domesticated species may have implications on the marketability of fruit due to transgenic seeds, especially if organic fruit-growing orchards are exposed. However, there can be no concern over the propagation of apple cultivars, since apple trees are propagated by grafting vegetative buds onto rootstocks. The sexual transfer of transgenes to wild species is deemed to be the greatest source of environmental exposure. Eastham and Sweet (2002) consider that it is difficult to predict how widespread gene flow and introgression of GM apple crops into wild species may be, and Raybould and Gray (1993) included apple into the group of plants with a high probability of gene flow.

The dissemination of novel genes into the environment occurs mainly by pollen or seeds (Schütte et al., 2001). Seeds are transported over greater distances than pollen, but the dissemination of seeds is easier to control, which is why pollen flow is under more investigation for risk assessment. Apple pollen is distributed mainly by vectors but also by wind. There are different factors affecting pollen dispersal and cross-pollination, such as size of pollen source and sink, pollination vectors, environmental factors (weather, local environment, physical barriers), pollen viability and competitive ability, level of outbreeding in the crop and degree of synchrony in flowering times (Eastham and Sweet, 2002). Apple is a heterozygous, self-incompatible crop that relies on fertilization by pollen of other cultivars or, as common in commercial apple production, of wild species. There are several studies on pollen dispersal in apple orchards aimed at maximizing cross-fertilization in apple and achieving optimal orchard design, especially with regard to the place and distribution of pollen-dispenser trees in commercial high-density orchards (Free, 1962; Free and Spencer-Booth, 1964; Kron et al., 2001a; Kron et al., 2001b; Wertheim, 1991). Pollination in apple is mainly due to insects and in commercial orchards honey bees and bumblebees are used as pollinators. The distance that pollen actually travels depends largely upon the foraging behavior of these vectors, which may be influenced by flower morphology, flowering time and spatial position of the apple cultivars (Free, 1966; Mayer et al., 1989).

Most investigations on vertical gene transfer between crops and wild relatives have been carried out on annual crops (Gerdemann-Knörk and Tegender, 1997; Neuroth, 1997). Increasing numbers of transgenic perennials are being developed, but the potential for gene escape from these crops has seldom been examined. In particular, the long life cycle of fruit tree species may have significant effects on the extent of crop gene dispersal. Gene flow can be measured in various ways. A simple method is to introduce a plant with an easy-to-follow marker into a population and follow the appearance of the marker carried by pollen into the next generation of progenies (Eastham and Sweet, 2002). Morphological markers, such as leaf color (Hanna and Burton, 1992; Lespinasse et al., 1983; UrRahman et al., 1997) or growth type (Lapins, 1976) have the advantage of being inexpensive tools for analyzing a large number of samples if the inheritance of the gene of the morphological feature is homozygous and dominant with a stable expression. In contrast, molecular markers provide a wide choice of environmentally independent analyses, and have been used for examination of vertical gene transfer several times. Another possibility in the study of vertical gene transfer is to release transgenic plants carrying specific marker genes, such as *gus* or *gfp* (Ottenschlager et al., 1999).

Using the hybrid TNR 31-35, a descendant of *Malus sieversii* var. *sieversii* f. *niedzwetzkyana* (Dieck) (Hanelt, 2006) as a pollen-dispenser facilitates the assessment of the gene flow rate between apple cultivars under natural conditions, using both a morphological marker and a molecular marker. This hybrid carries a homozygous, dominant gene which causes red pigmentation of leaves (Lespinasse and Godicheau, 1980). Red color of apple plants has been used as a morphological marker several

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times (Lespinasse et al., 1983; Lespinasse and Godicheau, 1980; UrRahman et al., 1997; Wertheim, 1991; Williams et al., 1979).

This paper reports on a study pertinent to issues that should be clarified in considering bringing GM fruit trees into the environment. The primary goal was to use morphological and molecular markers to estimate gene dispersal in an apple orchard representing a collection of genetic resources of apple, such as various cultivars, wild species and breeding material. This study on gene flow provides a preliminary baseline for assessing the probability of vertical gene transfer from GM trees developed in our laboratory (Hanke et al., 2003). An approach to assess gene flow in an apple orchard is described using the hybrid TNR 31-35 of *M. sieversii* var. *sieversii* f. *niedzwetzkyana* as a pollen-dispenser, whose paternity could be tracked by the red color of tissue and by specific molecular markers. Simple sequence repeat (SSR) markers were used as they are PCR-based, highly reproducible, polymorphic, generally codominant and abundant in the plant genome (Powell et al., 1996). The SSR marker system has also been developed in apple and it was shown to be a powerful tool (Hokanson et al., 1998). By screening seedlings obtained from a large number of cultivars within a sector of the orchard, gene flow was estimated. The following specific questions were of interest: (1) what proportion of seeds obtained is red-colored as a result of pollination by TNR 31-35; (2) what is the maximum distance of pollen dispersal occurring in a multi-genotype orchard; and (3) what is the distance of wind-transported pollen dispersal?

RESULTS

Flowering phenology

Through preliminary selection, appropriate genotypes were identified among 50 different apple scion cultivars growing at the experimental site which overlap in flowering period with the pollen-dispenser TNR 31-35 used in this study. Such an overlap is crucial for cross-pollination. The blooming time of the 50 cultivars was estimated as early, medium and late according to the classification used for apple cultivars in the databank http://www.genres.de/eva/apfel.htm. Out of 50 cultivars, 38 recorded as early to medium blooming were included in the experiment (Tab. 1).

In 2003, the pollen-dispenser TNR 31-35 was in full bloom on April 28th. Among the pollen-receptor cultivars, 'Discovery' flowered first, two days after the pollen-dispenser genotype. The remaining cultivars followed two and three days later. The latest flowering cultivars were 'Cox Orangen Renette', 'Oldenburg', 'Reka' and 'Rewena'. These cultivars were in full blossom on May 3rd. In 2004, apple bloom started with the pollen-dispenser genotype on April 25th. The cultivars 'Liberty' and 'McIntosh' followed three days later. The main part of the pollen-receptor cultivars started to flower on April 29th and 30th. Only 'Reanda' and 'Rewena' started to flower on May 1st. Summarizing both years, the pollen-dispenser genotype started flowering two or three days earlier than the pollen-receptor cultivars. The blossom of TNR 31-35 overlapped the blossom of all pollen-receptor cultivars by at least one week. It is assumed that spontaneous hybridization between the domesticated apple cultivars and the wild relative depends upon the duration of the overlap in flowering periods (Tab. 1).

Pollen vitality and germination

The pollen of TNR 31-35 was highly vital. About 96% of 1000 investigated pollen grains were orange-red colored after staining in carmine-acetic-acid. The capacity of pollen to germinate was also very high, since 91% of 2000 pollen grains developed pollen tubes.

Cross-compatibility

Cross-compatibility was studied between 27 and 28 cultivars of the domesticated apple and the pollen-dispenser TNR 31-35 in 2003 and 2004, respectively (Tab. 2). In 2003, the average fruit set obtained after June drop was 20.1%. Out of these 27 cultivars, 18 were classified as highly cross-compatible in combination with TNR 31-35, with a fruit set higher than 15%. Eight cultivars were classified as moderately compatible, showing a fruit set of less than 15%. The cultivar 'Idared' was low in compatibility, with a fruit set of 5%. In 2004, the average fruit set of 36.6% was higher than in the previous year. Twenty-seven cultivars showed a fruit set higher than 15%. A high compatibility for these cultivars was assumed. Only one cultivar ('Greensleeves') was classified as moderately compatible. From these experiments, it can be stated that the cross-compatibility of TNR 31-35 is high, in some cases moderate, with all the apple cultivars used in this study.

Analysis of progenies obtained from hand-pollination

The phenotype of 4008 seedlings resulting from artificial hand-pollination between TNR 31-35, used as the male

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Table I. Characterization of trees used for pollen-mediated gene flow sampling.

Identifier	Row number	Tree number	Distance from the pollen-dispenser (m)	Genotype	Flowering category[1]	Date of full bloom	
1	1	3	104	G.Delicious[2]	4	02.05.2003	29.04.2003
2	1	17	77	Prima	3	01.05.2003	30.04.2004
3	1	38	35	McIntosh	3	01.05.2003	28.04.2004
4	1	56	9	Remo	3	n.a.	30.04.2004
5	1	61	9	Remo	3	n.a.	30.04.2004
6	1	64	9	Piglos	n.a.	n.a.	n.a.
7	1	65	9	Piglos	n.a.	n.a.	n.a.
8	1	66	9	Piglos	n.a.	n.a.	n.a.
9	1	67	9	Greensleves	3	n.a.	30.04.2004
10	1	80	24	Pinova	4	02.05.2003	29.04.2004
11	1	100	62	Oldenburg	3	03.05.2003	29.04.2004
12	2	3	104	Discovery	3	30.04.2003	n.a.
13	2	11	88	Reka	4	03.05.2003	30.04.2004
14	2	15	80	Reglindis	3	02.05.2003	29.04.2004
15	2	25	60	Rome[3]	5	02.05.2003	30.04.2004
16	2	30	50	Freedom	3	02.05.2003	30.04.2004
17	2	44	22	AS 21,73	n.a.	01.05.2003	29.04.2004
18	2	50	10	AS 21,73	n.a.	01.05.2003	29.04.2004
19	2	60	8	Idared	3	01.05.2003	n.a.
20	2	61	4.5	Jonathan	4	n.a.	29.04.2004
21	2	62	4.5	Jonathan	4	n.a.	29.04.2003
22	2	63	5	G.Delicious[2]	4	02.05.2003	29.04.2003
23	2	64	4.5	G.Delicious[2]	4	02.05.2003	29.04.2003
24	2	66	4.5	Pinova	4	02.05.2003	29.04.2004
25	2	67	4.5	J.Grieve[4]	3	n.a.	29.04.2004
26	2	68	4.5	J.Grieve[4]	3	n.a.	29.04.2003
27	2	69	4.5	Starkrimson	n.a.	n.a.	n.a.
28	2	70	4.5	Starkrimson	n.a.	n.a.	n.a.
29	2	71	6	Remo	3	n.a.	30.04.2004
30	2	72	8	Remo	3	n.a.	30.04.2004
31	2	74	11	Remo	3	n.a.	30.04.2004
32	2	81	24	Liberty	3	01.05.2003	28.04.2004
33	2	87	36	Prima	3	01.05.2003	30.04.2004
34	2	96	54	Rewena	5	03.05.2003	01.05.2004
35	3	3	104	Realka	4	n.a.	30.04.2004
36	3	10	92	Spartan	4	01.05.2003	29.04.2004
37	3	12	86	Gloster	5	n.a.	29.04.2004
38	3	16	78	Rome[3]	5	02.05.2003	30.04.2004

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Table I. Continued.

Identifier	Row number	Tree number	Distance from the pollen-dispenser (m)	Genotype	Flowering category[1]	Date of full bloom	
39	3	20	70	Priam	3	n.a.	28.04.2004
40	3	23	64	Clivia	3	01.05.2003	29.04.2004
41	3	27	56	Piglos	3	n.a.	n.a.
42	3	36	38	Freedom	3	02.05.2003	30.04.2004
43	3	40	30	Oldenburg	3	03.05.2003	29.04.2004
44	3	42	26	Oldenburg	3	03.05.2003	29.04.2004
45	3	44	22	Reanda	3	02.05.2003	01.05.2004
46	3	45	20	Releta	3	02.05.2003	01.05.2004
47	3	48	14	Releika	3	n.a.	29.04.2004
48	3	50	10	Resi	5	02.05.2003	30.04.2004
49	3	51	8	Retina	3	n.a.	30.04.2004
50	3	52	4.5	Retina	3	n.a.	30.04.2004
51	3	73	8	McIntosh	3	01.05.2003	28.04.2004
52	3	74	10	McIntosh	3	01.05.2003	28.04.2004
53	3	76	14	Carola	3	02.05.2003	n.a.
54	3	78	18	Undine	3	02.05.2003	n.a.
55	3	80	22	Pikant	4	02.05.2003	30.04.2004
56	3	82	26	Jonadel	4	02.05.2003	n.a.
57	3	84	30	Cox[5]	4	03.05.2003	n.a.
58	3	88	38	Piros	4	02.05.2003	n.a.
59	3	93	48	Auralia	4	02.05.2003	30.04.2004
60	3	96	54	Jonagold	3	02.05.2003	n.a.
61	3	55-69	0	TNR 31-35	3	28.04.2003	25.04.2004

[1] Scale for blooming time: 3: early; 4: medium; 5: late.
[2] Golden Delicious; [3] Rome Beauty; [4] James Grieve; [5] Cox Orangen Renette; n.a.: not assessed.

parent, and 36 pollen-receptor cultivars, used as the female parents, was determined from the seedlings' leaf color. From 2504 seeds that were sown in a glasshouse, 2359 seedlings were obtained in 2003 (germination rate: 92.1%). In 2004, 1649 seeds germinated out of 2392 (germination rate: 66.9%).

The majority of germinated seedlings, in total 3921, had red leaves. Eighty-seven seedlings (2.2%) showed green-colored leaves. These seedlings descended from different female parents (Tab. 2). All seedlings with green-colored leaves, except for one seedling of 'Golden Delicious', were analyzed by SSR markers. The DNAs of the appropriate mother cultivars, of the pollinator TNR 31-35 and five seedlings with red leaves of each cross combination were also included in this analysis. Each seedling was characterized by two to five SSR primer combinations, depending on the unambiguous classification of genotype-specific markers that have to be used for genotype identification as previously determined (Tab. 3). No marker of the pollinator was detectable for eight green-colored seedlings (0.2%). All SSR alleles determined were specific for the female parent. For these plants, self-pollination was suggested. Seventy-eight seedlings (2%) originated from cross-pollination, but the male parent was not the pollen-dispenser TNR 31-35. Only one offspring of 'Idared' showed fragments that were equal to the alleles of both parents. In contrast to the green-colored seedlings, all investigated plants with red leaves showed one allele of the female parent and one allele of the pollen dispenser for each SSR marker.

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Table 2. Phenotypical evaluation of seedlings obtained from hand-pollination using different cultivars of *Malus domestica* as female parents and TNR 31-35 as the male parent.

Cultivar	Cross-compatibility[1]		Number of seedlings[2]			Origin of the green colored seedlings[3]
	2003	2004	Total	Red	Green	
Auralia	++	+++	48	48	0	
Carola	+++	n.a.	146	144	2	2 cross
Clivia	+++	+++	236	234	2	2 cross
Cox	++	n.a.	11	11	0	
Discovery	++	n.a.	7	7	0	
Freedom	+++	+++	134	120	14	14 cross
Gloster	n.a.	+++	35	35	0	
G. Delicious	+++	+++	394	390	4[4]	1 self + 2 cross
Greensleeves	n.a.	++	11	11	0	
Idared	+	n.a.	26	22	4	4 cross*
J.Grieve	n.a.	+++	16	16	0	
Jonadel	+++	n.a.	52	49	3	3 cross
Jonagold	+++	n.a.	37	37	0	
Jonathan	n.a.	+++	115	115	0	
Liberty	++	+++	70	70	0	
McIntosh	+++	+++	131	131	0	
Oldenburg	+++	+++	159	159	0	
AS 21,73	++	+++	185	184	1	1 cross
Pikant	++	+++	67	67	0	
Pinova	+++	+++	251	241	10	2 self + 8 cross
Piros	++	n.a.	27	27	0	
Priam	n.a.	+++	132	131	1	1 cross
Prima	+++	+++	208	203	5	5 cross
Realka	n.a.	+++	31	31	0	
Reanda	+++	+++	121	118	3	3 cross
Reglindis	+++	+++	201	175	26	2 self + 24 cross
Reka	+++	+++	132	131	1	1 self
Releika	n.a.	+++	46	46	0	
Releta	+++	+++	169	168	1	1 cross
Remo	n.a.	+++	59	59	0	
Resi	+++	+++	179	174	5	2 self + 3 cross
Retina	n.a.	+++	36	35	1	1 cross
Rewena	++	+++	115	115	0	
Rome	+++	+++	220	216	4	4 cross
Spartan	+++	+++	134	134	0	
Undine	+++	n.a.	67	67	0	

[1] (+) weakly compatible, (++) moderately compatible, (+++) highly compatible; n.a.: not assessed.
[2] Results of 2003 and 2004 combined; [3] number of seedlings from self pollination (self) or cross pollination (cross) when TNR 31-35 was excluded as the male parent, based on molecular evaluation, except for one seedling* of 'Idared'; [4] one seedling was not analyzed.

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Table 3. Specific SSR markers used for the discrimination between different apple cultivars and TNR 31-35. Different alleles are recorded in bp.

	SSR-Marker[6]									
Cultivar	CH05A05	CH04E03	CH05C07	CH03D11	CH04A12	CH04E02	CH04E05	CH02C02b	CH03B10	CH02F06
Alkmene	0/0		124/142	120						
Auralia	221/233		122							
Carola	199/223		124/134	116/120						
Clivia		0/0	112/124		159/165					
Cox[1]	0/0		122/124					105/117		
Discovery	217/233	191/199		120	159			115/123		
Elstar	219	191/199		120	0/0					
Empire	219/227					143/153			98/108	
Freedom				120						152/154
Gibbs[2]	219			120/128						
Gloster	219/221	198				143/153				
G.Delicious[3]	217/221	198		120						
Greensleves	217	191/198		120						
Idared	197/229									
J.Grieve[4]	215/219	191		120				75/115		
Jonadel	197/213	198								
Jonafree	221		134							
Jonagold	219/221		142/152				194/206			0/0
Jonathan	197/221									
Juno	217/221	198		120	161/181		198/210			
Liberty	219/221	179/198		120	169/173					0/0
McIntosh	219/225					143/165				0/0
Oldenburg		200/213								
AS 21,73	219/229	192/198	110/122		161/181		194/196			
Piglos	219/221	198				143/153				0/0
Pikant	0/0		122/124		167					0/0
Pilot		198								
Pinova		198			167/181		0/0			0/0
Piros		0/0	112/128	120						
Priam	199/221	0/0								0/0
Prima	199/215		140/142		169/173					
Priscilla	217	0/0			173				98/108	

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Table 3. Continued.

	SSR-Marker[6]									
Cultivar	CH05A05	CH04E03	CH05C07	CH03D11	CH04A12	CH04E02	CH04E05	CH02C02b	CH03B10	CH02F06
Realka					173/181					
Reanda			112/114							
Redfree	0/0		114	120	0/0		0/0			
Reglindis	219/231	198	112	120						0/0
Reka	219	189/198	124/126	116/119						
Releika	221/231		112/126	120	161/181					
Releta			114/142							
Remo		179/191	112/124	120						
Rene		205		114/116						
Resi		200								
Retina	0/0		112/114							0/0
Rewena	0/0	179/207	112/114	119						
Rome[5]				0/0						
Sire Prize	217/221	198			169/181					
Spartan	221/225	198				143/153				
Starkrimson	219/221					153/155				
Undine	199/221	198	114					117		
TNR 31-35	203/205	185/195	138/146	124	177/191	149/159	174/202	109/111	104/118	148/158

[1] Cox Orangen Renette; [2] Gibbs Golden Gage; [3] Golden Delicious; [4] James Grieve; [5] Rome Beauty.

[6] The sequence of SSR primers according to Liebhard et al., 2002. Genotype-specific markers can be used for pollen parent identification in seedlings obtained from open pollination. Using different primer combinations, TNR 31-35 can be excluded as pollen parent among the other cultivars. Genotypes presenting only one allele are of genotype AA or A0.

Pollen-mediated gene flow

The evaluation of seedlings obtained from open pollination under natural conditions was performed using 60 (2003) and 54 (2004) pollen-receptor plants, respectively. Six pollen-receptor plants (Identifier numbers 23; 51; 52; 53; 56; 58; Tab. 1) were not analyzed in 2004 due to their biannual fruit bearing. Altogether, a total of 11 797 seedlings were examined. In 2003, from 6876 seeds sown, 6284 seedlings were obtained (average germination rate: 91.7%). One hundred and fifteen seedlings were sired by TNR 31-35 and showed red-colored leaves, an average of 1.8% of all seedlings obtained from apple sampled at different distances from the pollen-dispenser. The percentage of red-colored seedlings per individual tree used for sampling ranged from 0 to 16.7%. In 2004, 5513 seeds were collected and sown. The average germination rate was 88.1%, nearly 10% lower than the previous year, so that 4774 seedlings were obtained. Sixty-nine seedlings showed red-colored leaves, a mean value of 1.4% of all seedlings obtained.

Pollen-mediated gene flow from the pollen-dispenser was measured as the percentage of seedlings bearing the red marker gene of TNR 31-35 at increasing distances from the pollen-dispenser (Tab. 4). Considering both years of sampling, 69% of all observed seeds fertilized by TNR 31-35 were found at a distance of less than 10 m from the nearest pollen-dispenser tree. This means, on average, not further away than five adjacent trees along the row or two adjacent trees across the row. Almost 91% of all seeds fertilized by TNR 31-35 were found at a distance less than 60 m from the nearest pollen-dispenser tree, which is equal

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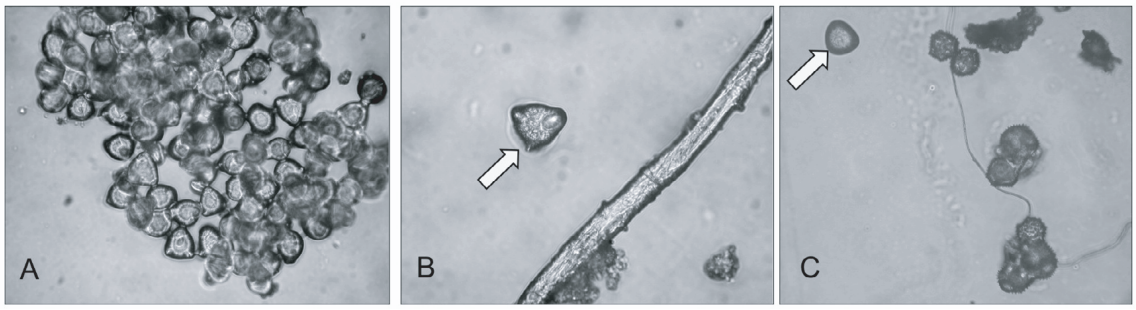

Figure 1. Wind-transported apple pollen grains observed on pollen traps at different distances and orientations from the pollen-dispenser tree. A. Apple pollen grains 4 m north of the pollen dispenser tree. B. Apple pollen grains (white arrow) 14 m west of the pollen dispenser tree. C. Apple pollen grains (white arrow) 20 m north of the pollen-dispenser tree. The remaining pollen grains originated from *Taraxacum officinalis.*

Table 4. Pollen-mediated gene flow using TNR 31-35 as a pollen-dispenser, measured as percentage of red-colored seedlings sired by the pollen-dispenser genotype at increasing distances from the pollen-dispenser trees.

Distances from the pollen-dispenser (m)	Number of seedlings obtained from pollen-receptor trees		Number of red-colored seedlings		Percentage of seedlings sired by the pollen-dispenser genotype	
	2003	2004	2003	2004	2003	2004
0–10	1757	1440	88	39	5.0	2.7
11–30	1786	1287	15	26	0.8	2.0
31–60	1272	677	5	0	0.4	0.0
61–105	1469	1370	7	4	0.5	0.3
Total	6284	4774	115	69		

to 30 adjacent trees along the row. In this study, pollen dispersed was observed at 104 m.

After phenotypical evaluation, seedlings selected as red-colored were investigated by SSR-analysis. Each seedling was tested with at least one heteromorphic SSR-marker that allows the verification of TNR 31-35 as the male parent. All tested seedlings, except four, showed two specific alleles, one allele specific for the appropriate female parent and the second allele specific for the pollen-dispenser TNR 31-35. Two seedlings of 'Greensleeves' (Identifier number 9) and 'Idared' (Identifier number 19, Tab. 1) in 2003 and two seedlings of 'Prima' (Identifier number 2) and 'Discovery' (Identifier number 12) in 2004 did not show any allele specific for TNR 31-35.

Estimation of pollen dispersal by wind

In both experiments, performed in 2005 and 2006, a sufficient amount of pollen was caught in pollen traps, but the number of grains in 2006 was higher than in 2005. The results of both experiments were comparable. At least one apple pollen grain was found in traps in each direction within a radius of 6 m from the pollen-dispenser tree. In general, apple pollen grains were detected at a distance up to 20 m depending on the direction (Fig. 1). In both years, the greatest amount of apple pollen was found in pollen traps in the north and west directions, in particular the northern direction exhibited a high number of apple pollen grains. In both directions, west and north, apple pollen was found at a distance of 20 m (Fig. 2). In eastern and southern directions, the number of apple pollen grains was lower.

DISCUSSION

This study describes the pattern of pollen gene flow from a pollen-dispenser, TNR 31-35, throughout a three-row area consisting of 50 different apple scion cultivars used as a repository of apple genetic resources. The restricted value of results obtained in this study, due to an experimental layout that was not designed especially for it, has to be considered. The pattern of gene movement observed in this study may be interpreted as 'realized' pollen dispersal, according to Kron et al. (2001a). This reflects not only where pollen is dispersed, but also how likely it was that the pollen flow resulted in fertilization

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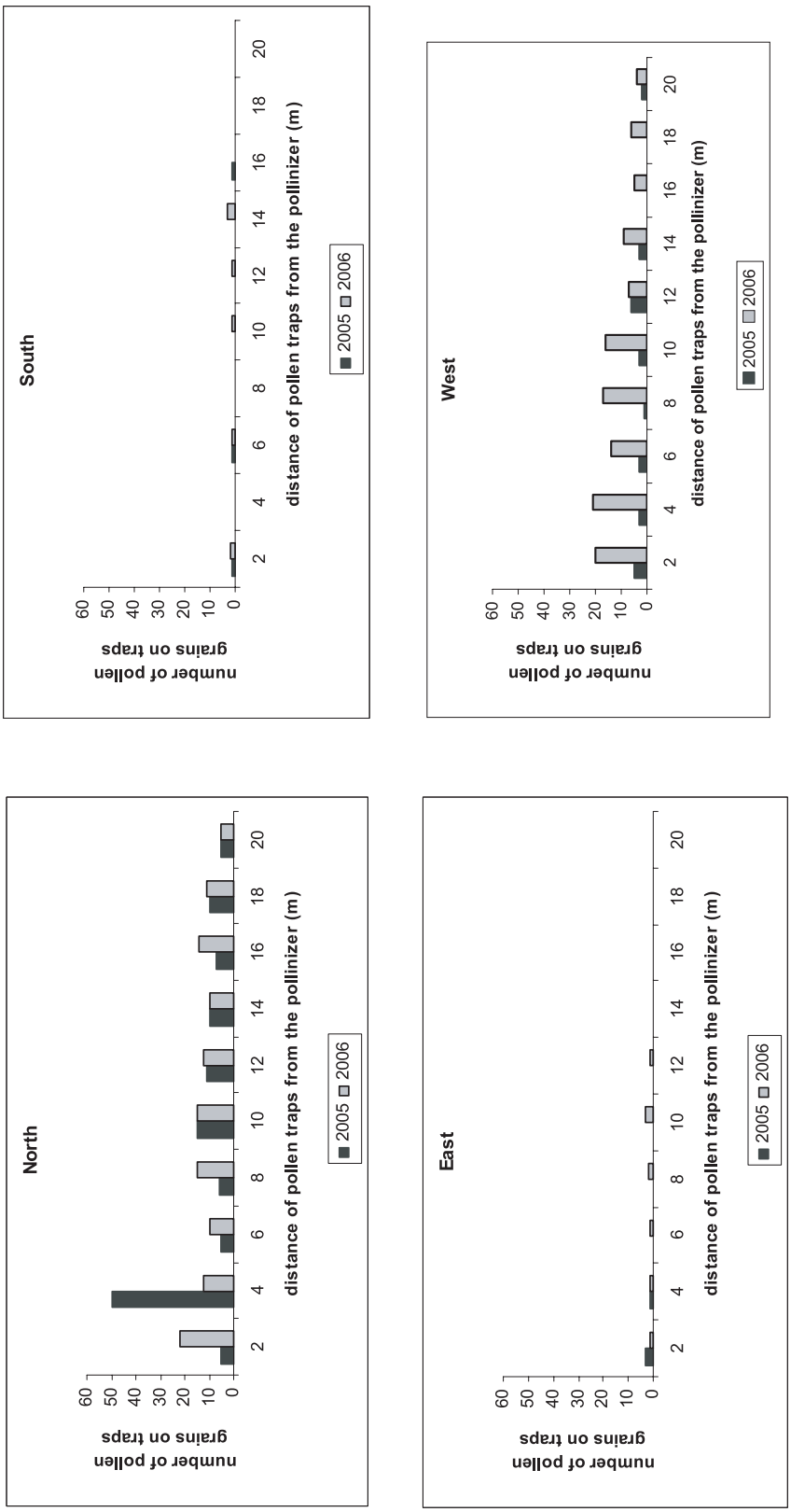

Figure 2. Number of wind-transported apple pollen grains detected in pollen traps depending on the distance and direction to the pollen-dispenser tree.

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of the ovule. As apple flowers received pollen from a range of other cultivars, the measured pollen dispersal represents the minimum values of the amount of pollen from the pollinator actually deposited on the stigmas (Kron et al., 2001a).

Gene flow was monitored using both a morphological and some molecular markers. Using an apple hybrid, originating from a wild species, which carries a homozygous dominant red marker gene as a pollen-dispenser is an easy and inexpensive method for monitoring pollen dispersal in apple orchards. In this study, in two consecutive years, 11 797 seedlings obtained from open pollination were examined. In the two years, the average of red-leaf colored seedlings was 1.8% and 1.4%, respectively, of seedlings tested. The pollen-dispenser used for this study is homozygous for the screened marker trait, whereas there are usually two or more different alleles of a given trait in the highly heterozygous cultivars of the domesticated apple. Also, genetically engineered apple pollen donors will be hemizygous for the transgenic trait. By using TNR 31-35 as a pollen donor for gene flow studies, the measured cross-fertilization rate was twice that of a pollen donor that is hemizygous for the trait. It was found that pollen was dispersed at least 100 m away from the pollen dispenser. Of all observed seeds fertilized by TNR 31-35, 69% were found at a distance less than 10 m from the nearest pollen-dispenser tree and almost 91% at a distance less than 60 m. Williams et al. (1979) was the first author who used descendants of *M. sieversii* var. *sieversii* f. *niedzwetzkynana* cv. Baskatong and Tomoko to study orchard pollination, particularly the value of beehive pollen dispensers. Wertheim (1991) used the red-colored genotype *Malus* cv. Baskatong as a morphological marker of pollen spread in intensive apple orchards. They found the 'Baskatong' effect faded rapidly and was restricted to a distance of 5–10 m. The results obtained here for distances of pollen dispersal correspond to data found in previous publications. Similar patterns of pollen dispersal have been estimated previously by Kron et al. (2001b) for two different high-density commercial apple orchards, where pollen was dispersed 15 rows (73.5 m) and 18 rows (86 m). However, 44–80% of all dispersal occurred within three rows of the pollen donor. The authors described a significant effect of the fruit-bearing receptor cultivar on seeds sired by the pollen-dispenser, which was found to have the highest value in row one (76%) and the lowest value in row 18 (1%). Milutinovic et al. (1996) reported on pollen dispersal based on fruit set in monotypic blocks of apple cultivars and found that the pollination distance varied between 5 and 80 m, depending upon the fruiting cultivar. Kron et al. (2001a) reported differences in along- and across-row patterns of dispersal distance which was greater across rows (62.4 m) than along rows (13.7 m). This is controversial with respect to the widely accepted view that bees are more likely to move along than across rows (Mayer et al., 1989).

Red-colored plants have often been used as phenotypical markers in breeding research, because these markers facilitate the investigation of a large number of samples, since the pigmentation is controlled by a single dominant gene and a consistent expression is guaranteed (Zwintzscher, 1974). To confirm the monogenic, dominant inheritance of the red marker gene of TNR 31-35, the pollen-receptor cultivars were crossed with this hybrid and some of the seedlings showed unexpected green-colored leaves. Similar results were obtained by Lespinasse and Godicheau (1980) and Lespinasse et al. (1983). To produce haploid apple plants, different cultivars were crossbred with TNR 31-35 as a color-marker. It was assumed that every green seedling would be haploid and of maternal origin. After crossbreeding, some unexpected green-colored seedlings were detected which were not haploid. They gave no clear explanation for the origin of the green-colored seedlings.

Molecular markers provide a useful technique to evaluate the genetic similarity between the green-colored seedlings and the presumed parents. UrRahman et al. (1997) used RAPD markers additionally for a color-based selection to detect apomictic seedlings in *Malus* species. In the present experiments, the genetic status of the green-colored seedlings obtained from pollen-receptor cultivars after hand-pollination was verified by SSR-analysis. With one exception, it was possible to exclude TNR 31-35 as a progenitor and to confirm the monogenic, dominant inheritance of the red-marker gene. One green-leaved seedling always showed one specific allele of the mother cultivar and one specific allele of TNR 31-35 after analysis with four different SSR-markers. The absence of the red pigmentation could be explained by a mutation of the red-color gene, but it is more likely that after further SSR-analysis a non-specific allele for TNR 31-35 would be detected. The origin of most of the green-colored seedlings could be explained by contamination of pollen by an unknown crossing partner, probably because of inappropriate pollination bags. The pollination bags protected the apple flower against bees, but probably due to an inappropriate mesh size, wind pollination was successful. Eight seedlings in the hand-pollination experiment appeared to originate from self-pollination. Although all apple species exhibit a self-incompatibility controlled by a series of polymorphic S-alleles which

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prevent fertilization (Broothaerts, 2003), self-fertilization is not impossible, and it does occur at low frequencies.

The molecular method was also applied to the phenotypic evaluation of seedlings from open pollination, to provide an opportunity for the application of a visual marker. Four seedlings without the specific allele of the pollen-dispenser TNR 31-35 were obtained. It is assumed that in this case, when 7.4% of red-colored seedlings were detected, a misinterpretation of the leaf color in the greenhouse environment had taken place. As stress conditions in a greenhouse (light, temperature) can cause development of anthocyanin in apple leaves and leaf veins, this may lead to a red color of some green seedlings. In the remaining red-colored seedlings, the red leaf marker was detected.

This study also focused on the question of whether apple pollen is transported by wind. Apple pollen was found in traps released from the pollen dispenser tree, which was isolated from external apple pollen sources, at a distance of 20 m. This conflicts with Janssen et al. (1995) who reported that the heavy apple pollen can be transported by wind a maximum of 5–6 m. It has to be noted that using pollen traps for pollen counts did not take into account pollen mortality, pollen competition, failure of pollen to land on the stigma, receptiveness of the stigma and/or the developmental failure of the ovary, which may result in an overestimation of the maximum distance traveled by viable pollen and of the fertilization potential of pollen. However, it gives some evidence that in an insect-pollinated species, such as apple, wind-transported pollen may also take part in fertilization events.

These results have important implications for the assessment of gene flow from genetically engineered apple trees that will be released into the environment. Despite insect pollination in apple, the results indicate that gene flow will occur mainly around the pollinator, at distances up to 10 m where less than 10% of seeds will be sired by the pollinator. At distances up to 100 m, the proportion of seeds sired will be less than 1%. Since the orchard design used for this experiment differs very much from commercial orchards, which are more uniform for the number of cultivars planted in blocks, these results are especially relevant for orchards with a number of various cultivars, such as repositories of breeding material and genetic resources.

MATERIALS AND METHODS

Plant material and experimental design

The field experiments were conducted in 2003 and 2004 in an orchard of the Institute for Fruit Breeding (Dresden) consisting of various fruit tree collections of breeding material, genetic resources and scion cultivars, especially in apple, pear, cherry and plum. The block used for this study was 0.26 ha of an apple scion repository of genetic resources, and contained 300 trees of 50 different scion cultivars, *i.e.* three rows with approx. 100 trees each (Fig. 4). The trees were planted between 1994 and 1996 and grafted on various rootstocks (M9, M26, MM106, A2), depending on the growth capacity of the scion, to obtain equal sized trees. At the time of the experiment, the trees were morphologically more or less identical, due to training as a spindle and were approximately 2.50 m high. Distances between trees along the row were 2.00 m and between rows 4.50 m. Next to the apple block, sour cherries were planted. The apple block described was selected for the experiment for three reasons: (1) high genetic variability among apple trees which could be used for sampling; (2) the rows contained the apple hybrid TNR 31-35, which could be used as a pollen-dispenser genotype, as it is characterized by a morphological marker; and (3) it was assumed that there were no other apple genotypes near the experiment that bear a red-leaf marker gene. Ten beehives were placed at the same place every year during the blooming period, 100 m away from the first apple row and into the cherry block, so as not to kill bees by application of fungicides in the apple block. The flowering period of the cherries was almost finished when the apples started to flower.

Sampling for gene flow

From the block described above, 60 trees of 38 different apple cultivars were selected for the experiment to serve as pollen-receptor trees. As pollen-dispenser, the hybrid TNR 31-35, a descendant from 'Topred Delicious' pollinated with a red leaved type derived from *Malus sieversii* var. *sieversii* f. *niedzwetzkyana* (Dieck) (botanical name according to Mansfeld's Encyclopedia of Agricultural and Horticultural Crops (Hanelt, 2006)) was used. Fifteen pollen-dispenser trees were growing at one site in row three of the experimental block. TNR 31-35 was chosen as pollen dispenser due to its homozygous dominant marker gene which causes red colored leaves, stems and fruit (Lespinasse and Godicheau, 1980b; Lespinasse et al., 1983b). After fertilization of a green-leaf apple cultivar with pollen of TNR 31-35, the descendants show red-colored tissues.

The method of sampling is described in Figure 3. Near the pollen-dispenser trees, the proportion of trees selected as pollen-receptors was higher than further away. In the next row across from the pollen dispenser, samples were

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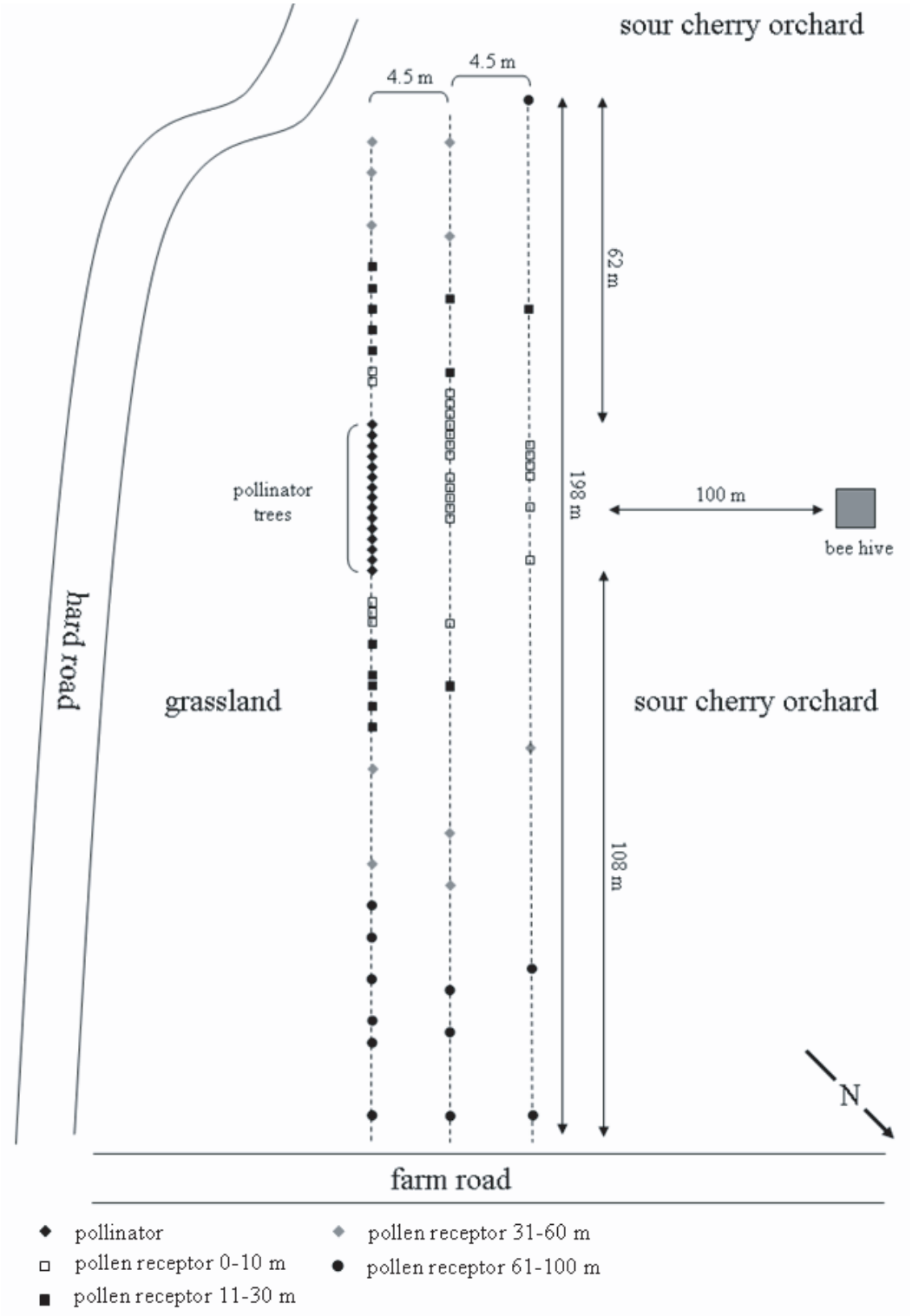

Figure 3. Map of the sampling area: the diagram indicates the location of the pollen-dispenser trees, TNR 31-35, and the location of trees from which fruit were sampled.

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obtained from 38% of all trees selected for the study. As it was assumed from literature that bees forage along the row, 47% of all trees examined were sampled next to the pollen dispenser in the same row.

The distances of selected trees in the neighboring rows were calculated using the Pythagoras' theorem $c = \sqrt{a^2 + b^2}$, where c is the distance between the tree selected and the pollen-dispenser, a is the distance between rows, and b is the distance from the tree in the neighboring row to the selected tree. For calculations, the nearest tree of TNR 31-35 to the selected tree was used.

Ripe apples were collected in September from trees of TNR 31-35, and from 60 selected trees in 2003 and 54 trees, respectively, in 2004. All fruit were selected randomly from different positions on the tree. The number of apples per tree was on average 18 in 2003, and 17 in 2004. On average, 115 seeds were obtained per tree in 2003, and 98 seeds per tree in 2004. The number of seeds obtained per tree was highly variable, as the fruit set of the genotypes was also variable, especially due to biennial bearing. Seeds were stored in moist sand in a refrigerator at 4 °C for 90 days until sown in a greenhouse. Seedlings were evaluated two weeks after sowing for their leaf coloration. All seedlings with red leaves or red leaf veins were classified as 'red'. Seedlings classified as 'green' had no sign of red coloration.

SSR analysis

Red-colored seedlings obtained from the selected trees after open pollination were evaluated using SSR markers. Extraction of DNA from leaf tissue was carried out using the DNAeasy-Plant Kit (Qiagen, Hilden, Germany). The PCR analysis was performed in a total volume of 10 µl containing 10 ng DNA, 1 µM IRD700- and IRD800-labeled forward SSR-primer, respectively, 2 µM reverse SSR-primer, 1× reaction buffer, 15 mM $MgCl_2$, 2 mM dNTPs and 0.5 U Taq Polymerase (Invitek, Berlin, Germany). The reaction conditions were as follows: denaturation at 94 °C for 5 min, then 33 cycles – denaturation at 94 °C for 60 s, annealing for 30 s at 58 to 60 °C (depending on the primer) and elongation of 90 s at 72 °C. After a final elongation for 5 min at 72 °C, the reaction mix was cooled to 4 °C. The PCR product was diluted in a ratio of 1:1 to 1:3 with loading buffer, containing 95% deionized formamide, 1 M EDTA and 80% (w/v) bromophenol blue. After a pre-run of 20 min at 1500 V, 34 W, 38 mA and 50 °C, 0.8 µl PCR product were separated on a 6.5% PAA gel (Long Ranger® Gel Solution, Cambrex Bio Science, Rockland, USA) in 1× TBE buffer for 2 h at 1400 V, 40 W, 40 mA and 45 °C. The fragment analysis was performed on a NEN Global IR^2 DNA Sequencer (Licor, Bad Homburg, Germany) using a 50–350 bp ladder (IRD-labeled) as size standard. The SSR markers which were used are described by Liebhard et al. (2002).

Identification of genotype-specific SSR markers for TNR 31-35

To identify genotype-specific SSR markers for the pollen-dispenser TNR 31-35, 13 different SSR primer combinations (Liebhard et al., 2002) were tested using DNA of the pollen-dispenser and 49 apple cultivars which were grown in the experimental block. Ten SSR primer combinations revealed patterns with five to 16 distinguishable alleles per locus (Tab. 3). Significantly different marker alleles compared to TNR 31-35 were obtained in 36 cultivars using the SSR marker CH05A05 and in 27 cultivars using the marker CH04E03 (Tab. 3). The SSR markers CH05C07, CH03D11, CH04A12, and CH04E02 showed a difference from the TNR 31-35 pattern in 21, 20, 14 and 6 apple cultivars, respectively. At least 23 apple cultivars showed polymorphic alleles in the SSR analysis for markers CH04E05, CH02C02b, CH03B10 and CH02F06. Based on these results, the pollen-dispenser genotype TNR 31-35 was clearly identified among all other cultivars of *M. domestica* investigated.

Cross-compatibility pollination treatments

Cross-compatibility was evaluated among genotypes of the apple block because differences in cross-compatibility may affect the proportion of seeds resulting from fertilization with pollen of TNR 31-35. Several branches were chosen from pollen-receptor tree and covered with pollination bags before any of the flowers had opened. At full bloom on each branch four to five inflorescences, *i.e.* around 100 flowers per tree, were hand pollinated with pollen of TNR 31-35 collected in advance. Flowers were not emasculated as self-fertilization is assumed to be irrelevant in apple. In 2003, a total of 27 receptor cultivars were tested. Two cultivars ('Piglos' and 'Starkrimson') were not included in the evaluation, as the pollination bags were destroyed. Nine cultivars ('Gloster', 'Greensleves', 'James Grieve', 'Jonathan', 'Priam', 'Realka', 'Releika', 'Remo' and 'Retina') were not evaluated because fruit setting was too low. In 2004, the hybridization experiment was repeated with 28 pollen-receptor cultivars. Due to low flower set, eight cultivars ('Carola', 'Cox Orangen

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Renette', 'Discovery', 'Idared', 'Jonadel', 'Jonagold', 'Piros' and 'Undine'), which were already tested in 2003, were not included into the second experiment. The number of fruit remaining after the June drop was recorded. Fruit set was calculated as the number of fruit observed in a treatment divided by the number of flowers pollinated. The compatibility of the crossbreed partners was estimated according to Fischer (1987) based on the percentage of fruit set obtained. Cross-compatibility is assumed to be high, if the proportion of pollinated flowers that set fruit is above 15%, moderate if the fruit set is between 15 and 8%, and low if the fruit set is less than 8%.

Pollen vitality and germination

Pollen of TNR 31-35 was collected and air-dried at room temperature for 2–5 days. The pollen vitality was evaluated after staining using 0.5% carmine-acetic-acid. After 10 min, 1000 pollen grains were examined based on their coloration. Non-vital pollen grains stayed uncolored, vital pollen grains changed to an orange-red color. To evaluate the germination capacity, a total of 2000 pollen grains was investigated as described by Filiti and Montalti (1982).

Sampling for wind-transported pollen

Experiments on wind transport of apple pollen were performed in 2005 and 2006. One potted apple tree (1.5 m height) of the cultivar 'Regia' (2005) and 'Lord Lambourne' (2006) in full bloom was used as a pollen dispenser. This tree was placed in the middle of a meadow (60 × 200 m) during blooming of *Taraxacum officinalis*. Contamination with pollen from external sources can be excluded. As pollen traps, microscope slides were covered with 2.5 × 2.5 cm of glycerine and fixed on 1.5 m sticks. The traps were positioned every 2 m from the potted tree up to a distance of 20 m in northern, eastern, western and southern directions from the pollen-dispenser tree. After six hours, the pollen traps were collected and stored at room temperature. The amount of apple pollen on traps was evaluated using a Zeiss Axioskop optical microscope.

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