Annals of Botany **107**: 699–707, 2011
doi:10.1093/aob/mcr018, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY Founded 1887

Searching for gene flow from cultivated to wild strawberries in Central Europe

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Received: 5 October 2010 Returned for revision: 16 November 2010 Accepted: 23 December 2010 Published electronically: 9 February 2011

• *Background and Aims* Experimental crosses between the diploid woodland strawberry (*Fragaria vesca* L.) and the octoploid garden strawberry (*F.* × *ananassa* Duch.) can lead to the formation of viable hybrids. However, the extent of such hybrid formation under natural conditions is unknown, but is of fundamental interest and importance in the light of the potential future cultivation of transgenic strawberries. A hybrid survey was therefore conducted in the surroundings of ten farms in Switzerland and southern Germany, where strawberries have been cultivated for at least 10 years and where wild strawberries occur in the close vicinity.

• *Methods* In 2007 and 2008, 370 wild *F. vesca* plants were sampled at natural populations around farms and analysed with microsatellite markers. In 2010, natural populations were revisited and morphological traits of 3050 *F. vesca* plants were inspected. DNA contents of cell nuclei of morphologically deviating plants were estimated by flow cytometry to identify hybrids. As controls, 50 hybrid plants from interspecific hand-crosses were analysed using microsatellite analysis and DNA contents of cell nuclei were estimated by flow cytometry.

• *Key Results* None of the wild samples collected in 2007 and 2008 contained *F.* × *ananassa* microsatellite markers, while all hybrids from hand-crosses clearly contained markers of both parent species. Morphological inspection of wild populations carried out in 2010 and subsequent flow cytometry of ten morphologically deviating plants revealed no hybrids.

• *Conclusions* Hybrid formation or hybrid establishment in natural populations in the survey area is at best a rare event.

Key words: *Fragaria vesca*, *Fragaria* × *ananassa*, hybridization, microsatellite markers, genetically modified organisms, gene flow.

INTRODUCTION

The genus *Fragaria* (Rosaceae) contains 23 reported herbaceous species, including well defined hybrids (Folta and Davis, 2006). The different species show various ploidy levels ranging from di- to octoploid. Today, the diploid woodland strawberry (*Fragaria vesca* L.) is the only *Fragaria* species that occurs throughout the northern hemisphere (Hancock, 1999). Tetraploid species are confined to East Asia and the hexaploid *F. moschata* L. to Europe. The octoploid species, which are generally interpreted as the phylogenetically most advanced, are distributed in the Americas.

Numerous experimental attempts to produce hybrids between species with the same or different ploidy levels within the genus *Fragaria* have been made to date to investigate the genetic compatibility of species and their phylogenetic relationship or to introduce novel traits into cultivars (Mangelsdorf and East, 1927; Yarnell, 1931*a*, *b*; Evans, 1974; Noguchi *et al.*, 2002; Marta *et al.*, 2004; Olbricht *et al.*, 2006). Generally, species with the same ploidy level can be crossed successfully and their progeny are fertile. Hybrids between species of different ploidy levels are far more difficult to breed. They show high mortality at early developmental stages and plants reaching maturity are usually highly sterile, but can be vigorous and vegetatively prolific. Gene flow between *Fragaria* species with the same ploidy level in the field has been reported repeatedly (Staudt *et al.*, 2003; Westman *et al.*, 2004). In addition, a tetraploid clone that originated either from autopolyploidization of *F. vesca* or from polyploidization of a *F. vesca* × *F. viridis* hybrid has been described from Finland (Ahokas, 1999). However, the only report of naturally occurring hybrids between *Fragaria* species of different ploidy levels that we are aware of comes from Bringhurst and Khan (1963). It describes two occurrences of pentaploid hybrids between octoploid *F. chiloensis* Mill. and diploid *F. vesca* in coastal California. These hybrids were described as infertile but competing well with their co-occurring parental species due to superior stolon productivity. Bringhurst and Khan (1963) assumed that interspecific hybrids arise fairly often in nature. Furthermore, they hypothesized that in the case of *F. chiloensis* × *F. vesca* hybrids the next fertile species level of decaploid hybrids may already have been reached by somatic chromosome doubling or the functioning of unreduced gametes.

Subsequently, Bringhurst and Senanayake (1966) continued the survey and reported >20 other pentaploid hybrid individuals as well as a nonaploid and a partially fertile hexaploid hybrid from seven sites in coastal California. These findings confirmed their assumption of widespread occurrences of hybrids.

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However, reports of hybrids in wild populations have not triggered any surveys on *Fragaria* populations in Europe, although surveys are increasingly important with a growing availability of genetically modified (GM) crop plants and the outlook for GM strawberries (Qin *et al.*, 2008). Besides the above-mentioned studies (Bringhurst and Khan, 1963; Bringhurst and Senanayake, 1966), we are only aware of one systematic survey on hybridization between *Fragaria* species. This is a survey on hybridization between the cultivated octoploid garden strawberry (*Fragaria* × *ananassa* Duch.) and one of its two wild parent species, the octoploid *F. virginiana* Mill. in south-eastern USA (Westman *et al.*, 2004). Not unexpectedly, this study showed substantial gene flow from cultivated strawberries to wild *F. virginiana*.

In Europe, cultivated *F.* × *ananassa* is the only octoploid species. It is grown widely as a high-value fruit crop. *Fragaria* × *ananassa* emerged from hybridization between the wild American species *F. chiloensis* and *F. virginiana*, and was first described by Duchesne in the 18th century from botanical gardens in Europe (Darrow, 1966). Wild *Fragaria* species present in Europe are *F. vesca*, *F. viridis* and *F. moschata. Fragaria vesca* is the most abundant species and is distributed all over the British Isles and continental Europe, including parts of Scandinavia and parts of the Iberian peninsula (Hancock, 1999). It has bisexual flowers, is self-compatible and generally reproduces vegetatively through formation of stolons. Commercial strawberry fields can often be found in the close vicinity of wild *F. vesca*. This is particularly the case in landscapes with small-scale structures such as hedges, groves and forest edges providing a suitable habitat for *F. vesca*. The main flowering times of *F.* × *ananassa* and *F. vesca* can overlap during April, May and June in Switzerland. Honey bees are the most important pollinators for cultivated strawberries in open fields (Hancock, 1999), but relatively little is known about pollinators of *F. vesca* (Knuth, 1898). A study on pollinator overlap between *F.* × *ananassa* and *F. vesca* from north-western Switzerland suggested that solitary wild bees are the most important pollinators for *F. vesca* in that area (Gross, 2009). Furthermore, solitary wild bees were also frequently pollinating *F.* × *ananassa* flowers. Only honey bees were more important pollinators of cultivated strawberries in that study, but they rarely visited wild strawberries.

Given the combined occurrence of both wild and cultivated strawberry species, flowering time overlap and pollinator overlap, we hypothesized that there is potential for gene flow between cultivated strawberries and wild *F. vesca* that might lead to stable hybrid populations.

To assess the extent of hybrid formation between cultivated *F.* × *ananassa* and wild *F. vesca* a hybrid survey in populations of *F. vesca* was designed. In 2007 and 2008 wild *F. vesca* plants in the vicinity of strawberry cultures at farm sites in Switzerland and Baden-Württemberg, southern Germany, were sampled and samples were tested at microsatellite loci for *F.* × *ananassa* alleles. As no hybrids were detected, all farm sites were revisited in 2010 and wild *F. vesca* populations were screened. Morphologically conspicuous plants from *F. vesca* populations were sampled and their ploidy levels estimated by flow cytometry.

We expected most first-generation hybrids to be pentaploid, but also hexaploid, nonaploid or even decaploid hybrids could result from pairing of unreduced and normally reduced gametes or two unreduced gametes. We assumed that back-crossing of F_1 hybrids with *F. vesca* would be a rare event, as pentaploid F_1 hybrids derived from crosses between diploids and octoploids are highly sterile (Mangelsdorf and East, 1927; Yarnell, 1931*a*; Bringhurst and Khan, 1963; Senanayake and Bringhurst, 1967; Olbricht *et al.*, 2006). Senanayake and Bringhurst (1967) estimated the amount of functional pollen as below 1 % for pentaploids and somewhat over 5 % for hexaploids of different interspecific *Fragaria* crosses.

At the same time, we performed experimental crosses between *F. vesca* and *F.* × *ananassa* and used these experimental hybrids to test the power of our molecular analysis and flow cytometry to detect hybrids. Furthermore, we estimated germination rates and survival of hybrids.

The aim of the present study was to assess the extent of hybridization between wild *F. vesca* and cultivated *F.* × *ananassa* under natural conditions, and thus to assess the risk of transgene escape associated with a potential future cultivation of transgenic strawberry cultivars.

MATERIALS AND METHODS

Plant reference samples

To identify *Fragaria vesca*- and *F.* × *ananassa*-specific alleles four *F. vesca* reference populations were sampled at forest sites in northern and north-western Switzerland (Fig. 1, Table 1). These populations were situated within an altitudinal range representative for *F.* × *ananassa* cultures in Switzerland and, as far as we know, never had immediate contact with *F.* × *ananassa* cultures. Ten plants were sampled in each population along

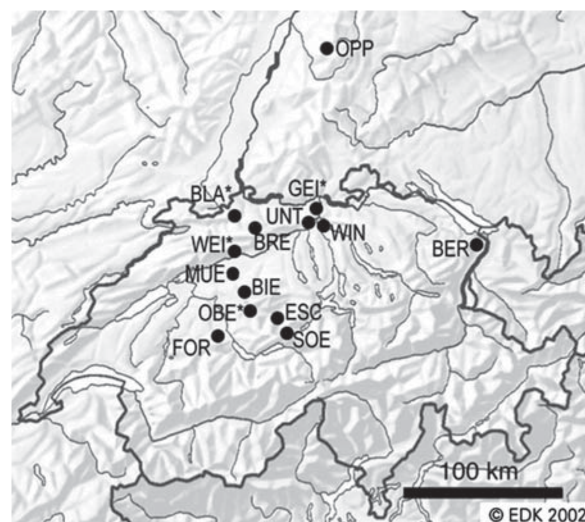

FIG. 1. Ten farm survey sites and four remote reference population sites (*) of woodland strawberries in Switzerland and southern Germany. BER, Berneck; BIE, Biembach; BLA, Blauen; BRE, Bretzwil; ESC, Escholzmatt; FOR, Forst; GEI, Geisberg; MUE, Muehledorf; OBE, Oberhueningen; OPP, Oppenau; SOE, Soerenberg; UNT, Unterboezberg; WEI, Weissenstein; WIN, Windisch.

TABLE 1. Fragaria vesca *reference populations, farm survey sites in Switzerland and Germany and information on sample sizes, genotype numbers and* F. ananassa *(*F. a.*) cultivation at survey sites*

Site name	Site type*	Co-ordinates North/East	Height a.s.l. (m)	Period of *F. a.* cultivation (years)	Acreage (ha)	Sampling distance from cultivation centre (m)	Acreage shift around cultivation centre (m)	Sample size for molecular/ morphological analyses	No. of genotypes found at sites
Blauen	1	47°26′48″/7°29′20″	600	–	–	–	–	10/–	10
Geisberg	1	47°31′51″/8°11′12″	680	–	–	–	–	10/–	9
Oberhueningen	1	46°52′05″/7°39′39″	980	–	–	–	–	10/–	10
Weissenstein	1	47°14′31″/7°30′25″	780	–	–	–	–	10/–	10
Berneck	2	47°25′29″/9°36′16″	460	51	0·6–1·2	70–300	Approx. 275	39/360	8
Biembach	2	47°00′19″/7°37′56″	600	30	Approx. 1	10–340	Approx. 250	38/242	11
Bretzwil	2	47°23′34″/7°38′55″	700	17	0·3–0·4	130–210	Approx. 200	34/185	8
Escholzmatt	2	46°56′24″/7°58′18″	850	28	0·1–0·3	140–170	Approx. 100	33/247	8
Forst	2	46°46′07″/7°31′33″	770	12	Approx. 0·4	80–90	Approx. 100	35/416	9
Muehledorf	2	47°08′18″/7°29′18″	610	37	0·4–3	110–420	Approx. 325	33/218	9
Oppenau (Germany)	2	48°30′26″/8°12′08″	630	38	Approx. 4	50–150	Approx. 175	37/315	8
Soerenberg	2	46°50′25″/8°01′01″	1080	20	0·5–1·5	40–250	Approx. 325	43/213	13
Unterboezberg	2	47°28′47″/8°10′14″	470	40	Approx. 0·05	80–120	Approx. 225	40/310	7
Windisch	2	47°28′05″/8°13′11″	430	40	1–1·5	200–350	Approx. 275	34/540	8

*1, reference population; 2, farm survey site.

forest tracks at spacings of 90–110 m. Additionally, single samples from a forest in Riehen, canton BS, and from the Morteratsch glacier forefield (2000 m a.s.l.), canton GR, were included in the analysis. Nineteen *F.* × *ananassa* cultivars that have been grown to a major extent at farm survey sites were obtained from nurseries and cultivar collections (Supplementary Data Table S1, available online). To reduce the possibility of confounding *F.* × *ananassa* with *F. moschata* or *F. viridis* alleles, two plants from two *F. moschata* populations in north-western Switzerland (Riehen and Dornach) as well as one *F. viridis* genotype (Niederau, Sachsen, DE) were included in the analysis (Supplementary Data Table S1).

DNA isolation, PCR conditions and analysis of PCR products

All samples consisted of young leaf tissue, and were stored in plastic bags with Silicagel Rubin (Sigma-Aldrich) for drying immediately after collection. Samples were kept in the dark at room temperature until analysis.

DNA was isolated using the Dneasy Plant Miniprep Kit (Qiagen) for identification of species-specific alleles (see below) and the Dneasy 96 Plant Kit (Qiagen) for screening of *F. vesca* samples from farm survey sites according to the manufacturer's protocol. Sample DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and were diluted with water to a DNA concentration of 3–12 ng μL^{-1}.

The M13(-21) method was used for labelling of PCR products (Schuelke, 2000). Forward primers of all primer pairs had an M13(-21) tail at their 5′ end. M13(-21) primer was labelled with fluorescent FAM, HEX or NED label (Applied Biosystems). PCR amplifications were carried out in 11 µL total volume of 1× PCR buffer (Colorless GoTaq Flexi Buffer; Promega), 2 mM MgCl, 0·2 mM of each of the four dNTPs, 0·05 µM of M13(-21) forward primer, 0·2 µM reverse primer, 0·2 µM M13(-21) primer$^{(FAM,\ HEX\ or\ NED)}$, 2 U of Go-Taq Flexi DNA Polymerase (Promega) and 3–12 ng of template DNA.

The following PCR conditions were used: an initial denaturation step of 94 °C (3 min), then 30 cycles of 94 °C (30 s), 60 °C annealing temperature (30 s) and 72 °C (30 s), followed by eight cycles of 94 °C (30 s), 52 °C (30 s) and 72 °C (30 s), and a final elongation step of 72 °C (5 min).

Fragments were separated by electrophoresis on an ABI PRISM 3130 × 1 Genetic Analyzer (Applied Biosystems). GeneScan-500 LIZ was used as internal size standard in each run. Data were analysed with Genemapper 3·7 software (Applied Biosystems).

For identification of species-specific alleles (see below), fragment length analysis was carried out for PCR products of every primer pair and every sample separately. For fragment length analysis of samples from survey sites, two to three differently labelled PCR products of the seven primer pairs that differed in fragment length range were grouped and analysed together (Table 2).

Ten per cent of *F. vesca* samples from survey sites were re-amplified with markers ARSFL 22, EMFv 27, EMFvi 108, EMFvi 109 and EMFvi 136 (Table 2), and the allele scoring error rate was calculated. No re-amplifications were made with markers ARSFL 27 and ARSFL 31 that were monomorphic for all *F. vesca* samples from survey sites.

Microsatellite primers and identification of F. vesca- *and* F. ananassa-*specific alleles*

A microsatellite marker analysis of sampled plants was conducted. Primers for microsatellite loci are highly specific, therefore microsatellite analysis is less prone to erroneous results caused by accidental DNA contamination of samples than other techniques such as, for example, amplified fragment length polymorphism (AFLP; Selkoe and Toonen, 2006).

Many microsatellite markers are available for the genus *Fragaria* and they show high transferability between species

TABLE 2. *Overview of microsatellite markers and corresponding alleles in* F. vesca (F. v.) *reference samples and* F. ananassa (F. a.) *cultivars*

Name	Source genome	Repeat motif*	Total no. of alleles		Allele size range (bp)		Alleles in *F. a.* cultivars (average)	Source†
			F. v.	*F. a.*	*F. v.*	*F. a.*		
EMFvi 108	Genomic library of *F. viridis*	$(ag)_n$	15	7	202–258	178–213	1–4 (3)	1
EMFvi 109	Genomic library of *F. viridis*	$(tc)_n$	11	6	293–317	281–291	1–3 (2)	1
EMFvi 136	Genomic library of *F. viridis*	$(tc)_n$	2	15	177–179	140–183	3–6 (5)	1
EMFv 27	Genomic library of *F. v.*	Compound trinucleotide	2	2	260–265	248–253	1–2 (1)	2
ARSFL 22	Genomic library of *F. a.*	$(ga)_{11}$	30	17	200–286	150–206	3–8 (5)	3
ARSFL 27	Genomic library of *F. a.*	$(ct)_{45-1}$	1	11	181	159–230	2–6 (4)	3
ARSFL 31	GenBank sequence of *F. a.*	$(ag)_{10}$	1	11	223	187–246	2–7 (4)	3

* Subscript number n–1 means the repeat was not perfect with either a base pair missing or a base pair substitution.
† 1, Sargent *et al.* (2003); 2, Hadonou *et al.* (2004); 3, Lewers *et al.* (2005).

(Sargent *et al.*, 2003; Hadonou *et al.*, 2004; Lewers *et al.*, 2005; Davis *et al.*, 2006). Microsatellite markers were selected on the basis of a published linkage map for diploid *Fragaria* that contains seven linkage groups (LGs) (Sargent *et al.*, 2006). The linkage map for diploid *Fragaria* can be used as a reference map for the octoploid *F.* × *ananassa* (Sargent *et al.*, 2006) as diploid and octoploid species share a common genetic basis (Hancock, 1999). Transferability of the diploid reference map to *F.* × *ananassa* has been confirmed by a study of comparative genetic mapping between *F.* × *ananassa* and its diploid relatives, which showed that high levels of conserved macrosynteny and colinearity exist between octoploid homoeologous LGs and their corresponding LGs in the diploids (Rousseau-Gueutin *et al.*, 2008).

Eighteen microsatellite primers were tested in a subset of *F.* × *ananassa* cultivars, *F. vesca* samples from reference populations and on the *F. moschata* plants described above. Out of these, seven microsatellite loci with species-specific fragment lengths were chosen (Table 2). Because we wanted to use physically unlinked microsatellite loci with an even distribution throughout the genome, all seven loci were chosen from different linkage groups (Sargent *et al.*, 2006). Among them, four loci were monomorphic or diallelic for reference *F. vesca* samples, one of them being also diallelic for *F.* × *ananassa* (Table 2). The other three loci showed moderate to high variability, and the size ranges for the majority of species-specific alleles were different. The three variable loci regularly showed stutter peaks in *F. vesca* with peaks separated by 2–3 bp. This made scoring of alleles for these loci unreliable within a size range of $\pm 2–3$ bp in *F. vesca*. Nevertheless, we included these loci in our study as they provided additional information to the low-variability loci, and stutter peak alleles did not overlap with the size range of the majority of well defined *F.* × *ananassa* alleles (Table 2).

Farm survey sites and sampling procedure

In summer and autumn of 2007 and 2008 we located strawberry producers in Switzerland and Baden-Württemberg, Germany. About 90 producers were interviewed with regard to the duration and acreage of strawberry cultures and the vegetation surrounding the strawberry fields. Based on these interviews, ten farms were selected where (*a*) strawberries were grown for at least 10 years; (*b*) strawberry cultures were shifted within a relatively narrow range; and (*c*) wild *F. vesca* plants were growing in the close vicinity of strawberry cultures (Table 1). These farms are located in north-western, central and eastern Switzerland and one in Baden-Württemberg, Germany (Fig. 1). Information on the time span of strawberry cultivation, present and former acreage and location of strawberry cultures was obtained from farmers (Table 1). Furthermore, farmers provided lists of major strawberry cultivars that were used throughout the period of strawberry cultivation. The centre of strawberry cultivation was estimated as the centre of the shifting acreages used for strawberry cultures for each survey site. This centre of cultivation served as the reference point for calculations of mean distances between *F. vesca* sites and strawberry cultures (Table 1). We assume that all *F. vesca* sites were

established when strawberry cultivation was started at the respective farms.

In autumn 2007 and 2008 leaves of wild *F. vesca* were sampled at the farms. A transect was laid through each site of *F. vesca*, and plants closest to 1 m spaced markings on the transect line were sampled. End points of transects and the sampled plants were marked with wooden pegs, and co-ordinates of *F. vesca* sites were recorded with GPS in case a re-examination of individual plants would be necessary. Furthermore, we searched for *Fragaria* plants with morphological traits differing from common *F. vesca* traits. Attention was paid to sampling all *F. vesca* occurrences close to strawberry cultures. At each strawberry farm, 33–43 *F. vesca* individuals from 3–6 different sites were sampled. Altogether 370 plants were sampled.

In summer 2010 all *F. vesca* sites at all ten farms were revisited. Each site was screened for morphologically conspicuous plants, and the total numbers of *F. vesca* plants were counted, or estimated where plant density was very high. Many of the morphological traits of *F.* × *ananassa* cultivars are intermediate to those of their parent species. *Fragaria virginiana* leaves are relatively thick, medium to dark green and their shape is obovate to oblong, while *F. chiloensis* leaves are very thick and leathery, usually glossy, dark green and broadly obovate (Darrow, 1966). Leaves of different *F.* × *ananassa* cultivars exhibit a mixture of these characters. *Fragaria vesca* leaves, in contrast, are thin and light green and relatively narrow cuneate–ovate to rhombic–ovate (Darrow, 1966). It was observed that leaves of all vigorous *F. vesca* × *F.* × *ananassa* F_1 hybrids that originated from hand-crosses (see below) were either intermediate between the parental species with regard to thickness, colour and leave shape or showed a dominance of *F.* × *ananassa* traits. Consequently, we screened *F. vesca* sites for *Fragaria* plants differing from common *F. vesca* plants in one or more of the following characters: leaf thickness, leaf colour, leaf shape and plant size. At each farm, 190–540 *F. vesca* plants were inspected, resulting in a total of 3050 plants.

Flow cytometry

Samples of ten morphologically conspicuous *Fragaria* plants collected from farm sites in 2010 were analysed by flow cytometry. Fresh young leaves of sampled plants were chopped together with leaves of *F.* × *ananassa* 'Calypso' as internal standard with a sharp razor blade in a Petri dish containing 0·8 mL of nuclei isolation buffer (Galbraith *et al.*, 1983) supplemented with 1 % polyvinylpyrrolidone K90. After 2 min of incubation the solution was filtrated through a 50 µm CellTrics filter (Partec) and 1·6 mL of 4′,6-diamidino-2-phenylindole (DAPI) staining solution (Cystain UV Precise P, Partec) was added. After 2 min of staining, fluorescence intensities of nuclei were measured with a CyFlow Ploidy Analyzer (Partec) equipped with a UV-LED of 365 nm emission wavelength.

As reference samples *F. vesca* × *F.* × *ananassa* hybrid plants from hand-crosses (see below) and their parental lines were used. Three experimentally produced hybrid individuals each of type *F. vesca* × *F.* × *ananassa* 'Calypso' and *F. vesca* × *F.* × *ananassa* AN93.231.53 were analysed. All measurements of reference samples were repeated three times.

Cloning and sequencing of overlapping alleles

Alleles of two *F. vesca* samples from survey sites and one *F.* × *ananassa* cultivar with overlapping fragment length (see below) were cloned and sequenced. A 1·5 µL aliquot of each PCR product was ligated into the pJET1 vector using the GeneJet-PCR cloning kit (Fermentas). A 5 µL aliquot of ligation product was transformed into 50 µL of chemically competent *Escherichia coli* cells (SURE, Stratagene). *Escherichia coli* cells were grown on LB-ampicillin plates. Twenty-four clones from each *F. vesca* sample and 48 clones from the *F.* × *ananassa* sample were used as template for colony PCR with pJET1 vector primers. PCR products with the correct length were identified on agarose gels and 5 µL thereof purified with 10 U of exonuclease I (Fermentas) and 1 U of shrimp alkaline phosphatase (Promega) at 37 °C for 15 min. Inserts were cycle sequenced using BigDye Terminator v3·1 chemistry (Applied Biosystems) in combination with the pJET1 forward sequencing primer. Products were filtered through a Durapore filter plate (Millipore MSHVN4510) loaded with Sephadex-G50 (GE Healthcare) to remove unincorporated dyes, and resolved on an ABI PRISM 3130 × l Genetic Analyzer (Applied Biosystems).

Sequences were edited with the software Sequence Navigator 1·0 (Applied Biosystems). Sequences were collapsed with the software Collapse 1·2 (Posada, 2006) and the sequences flanking the microsatellite repeats were compared with one another. Haplotypes that were found only once and that differed from more common haplotypes by a single base pair substitution/indel were considered likely PCR or cloning artefacts and were discarded.

Defined crosses between F. vesca *and* F. ananassa

Crosses were carried out in one direction with *F. vesca* plants from four different field sites as mother plants. As pollen donors two different *F.* × *ananassa* lines were used, *F.* × *ananassa* 'Calypso' and *F.* × *ananassa* AN93.231.53 (provided by B. Mezzetti, Marche Polytechnic University, Italy). From April to September 2008, hybrid seeds were generated by 100 controlled hand pollinations. Pollen was collected from closed *F.* × *ananassa* flowers. Anthers were placed in 2 mL tubes and were dried during 2–4 d in an exsiccator filled with Silicagel Rubin (Sigma-Aldrich). Pollen was used immediately after drying, or was stored in a fridge at 5 °C for up to 5 weeks prior to use. *Fragaria vesca* flowers were emasculated 2–3 d before opening. Anthers, sepals and petals were removed with a circular cut through the receptacle using a scalpel. This cutting treatment can be performed more quickly than removal of anthers with forceps and seems not to affect the following development of fruits negatively (pers. comm. from breeders). Furthermore, mechanical contact with anthers can be reduced. Prior to emasculation all redundant flowers were cut off and plants were rinsed with water to wash off pollen adhering to the plants. After emasculation plants were isolated in a polyester mesh tent to avoid accidental pollinations by insects.

Depending on the availability of suitable flowers, 1–4 flowers per *F. vesca* plant were pollinated. Flowers were pollinated twice, on day 1 or 2 and on day 3 or 4 after emasculation. Pollen was applied to flowers with a marten-hair brush that was washed with 96 % ethanol before and after pollination. After pollination plants were again isolated in a polyester mesh tent for 14 d. A total of 100 crosses of the type *F. vesca* × *F.* × *ananassa* were carried out. Sixty crosses were made with pollen from *F.* × *ananassa* 'Calypso' and 40 with pollen from *F.* × *ananassa* AN93.231.53. Ripe strawberries were cut in half and dried on blotting paper.

For germination, dishes (10·5 × 13 cm) were used; seeds were put on a moist 1:1 mixture of quartz sand and soil for germination (Ricoter, Aarberg, Switzerland) and were covered with a thin layer of quartz sand. This seed bed was covered with moist blotting paper and wrapped with plastic foil to avoid drying out. Seeds were then kept in the dark at 5 °C in a cold storage room for 2 weeks. Thereafter, dishes were placed in a greenhouse and all germinated seedlings were recorded for a period of 7 weeks. Seedlings that germinated within this period were transplanted to small pots after they reached the one- or two-leaf stage. All seedlings were treated with fungicide Previcur N (Bayer CropScience AG) after transplantation.

We could raise 67 and 55 seedlings from *F. vesca* × *F.* × *ananassa* 'Calypso' and *F. vesca* × *F.* × *ananassa* AN93.231.53 hybrid seeds, respectively (see below). From both crossing types 25 plants together with their parental lines were randomly sampled. Molecular analysis of these samples was performed with the same methods as described above for *F. vesca* samples from farm survey sites.

RESULTS

Fragaria vesca *sampling at farm survey sites in 2007 and 2008*

A total of 368 *F. vesca* plants were sampled from transects through *F. vesca* sites. None of them showed any morphological indications of hybrid identity. In addition, two morphologically conspicuous plants were sampled at Berneck at the margin of a former strawberry field that is now an apple orchard. These had thick, leathery leaves typical for *F.* × *ananassa* but were otherwise small and deformed. These two plants had specific *F.* × *ananassa* alleles at all seven loci and lacked any of the specific *F. vesca* alleles at the monomorphic and diallelic loci for *F. vesca*. This clearly identified them as feral *F.* × *ananassa* plants. Furthermore, the two individuals had more than two alleles at four loci, which indicated their polyploid status. None of the 368 *F. vesca* samples had an allele that was specific for *F.* × *ananassa* at any of the four loci that were either monomorphic or diallelic for *F. vesca* (Table 2). In fact, we only found alleles already known from the *F. vesca* reference populations for these four loci at all survey sites.

At the three loci that showed high variability, many new alleles were found for survey site samples. Two plants from site Forst and 12 plants from site Unterboezberg had allele fragment lengths that matched *F.* × *ananassa* alleles at the highly variable locus ARSFL 22 and were not present in reference populations. These overlapping alleles were cloned and sequenced (see below). Due to the absence of characteristic *F.* × *ananassa* alleles at all seven loci the remaining 354 plants were classified as genetically pure *F. vesca* plants. At all loci we never found more than two alleles per sampled *F. vesca* individual. The number of multilocus genotypes in survey site samples was estimated (Table 1). These results are based on some markers with high allele scoring error rates (see below) and are likely to overestimate genotype numbers. However, genotype numbers show that *F. vesca* is highly clonal and consists of a limited number of genets in survey site populations.

Ten per cent of *F. vesca* samples were re-amplified. No re-amplifications were made with markers ARSFL 27 and ARSFL 31 that were monomorphic for all samples from survey sites. The allele scoring error rate for EMFv 27 and EMFvi 136 was 0 %. The allele scoring error rate for loci that yielded stutter peaks in *F. vesca* were 20, 21 and 29 % for EMFvi 108, EMFvi 109 and ARSFL022, respectively, but re-scoring errors did not exceed a range of ±1–3 bp.

TABLE 3. *Relative DNA contents of cell nuclei of* F. vesca *(*F. v.*)* × F. ananassa *(*F. a.*) hybrids, their* F. vesca *mothers and ten* F. vesca *field samples*

	Relative DNA content	s.d.
F. v. 1	0·354	0·012
F. v. 2	0·361	0·009
F. v. 3	0·351	0·018
F. v. 4	0·362	0·003
F. v. 5	0·342	0·019
F. v. 6	0·352	0·001
F. v. 1 × *F. a.* 'Calypso'	0·692	0·013
F. v. 2 × *F. a.* 'Calypso'	0·666	0·007
F. v. 3 × *F. a.* 'Calypso'	0·672	0·008
F. v. 4 × *F. a.* AN93.231.53	0·684	0·006
F. v. 5 × *F. a.* AN93.231.53	0·678	0·002
F. v. 6 × *F. a.* AN93.231.53	0·708	0·031
F. v. field samples (10)	0·357–0·395	–

Relative DNA contents were calculated with *F.* × *ananassa* 'Calypso' as standard. Samples of hybrids and their mother plants were measured three times and field samples once.

Fragaria vesca *sampling at farm survey sites in 2010*

All *F. vesca* sites were revisited and a total of 3050 plants were inspected. Ten plants that had one or more conspicuous traits (i.e. unusually thick leaves, broad leaflets of rather obovate shape and extraordinary size of plants) were sampled. The size of cell nuclei of all sampled plants matched nuclei sizes of *F. vesca* reference plants (Table 3).

Cloning and sequencing of overlapping alleles

Two plants from site Forst and 12 plants from site Unterboezberg that were sampled in 2007 and 2008 had allele fragment lengths that matched *F.* × *ananassa* alleles at the highly variable locus ARSFL 22. The allele sizes were 192 and 206 bp for plants from Forst and Unterboezberg, respectively, and were not present in reference populations. To ascertain whether overlapping alleles were derived from

TABLE 4. *Haplotypes of two* F. vesca *plants and* F. ananassa *'Hummi grande' that have alleles of overlapping length at microsatellite marker ARSFL 22*

Position (bp)	1	6	7	27	36	41	44	60 Indel	61 Indel	63 Indel	69	86	Frequency
F. vesca F.1	A	C	C	T	A	A	T	TA	AA	–	T	C	9
F. vesca F.2	.	.	.	C	.	.	.	.	.	.	.	.	4
F. vesca U.1	.	T	.	.	.	.	.	.	.	.	.	.	4
F. vesca U.2	.	.	.	N	.	.	A	.	.	.	.	.	2
F. × *ananassa* 1	.	.	A	.	C	.	.	.	.	.	–	.	2
F. × *ananassa* 2	G	T	.	.	C	.	.	.	–	.	–	T	1
F. × *ananassa* 3	.	A	.	.	C	.	.	.	–	.	–	T	2
F. × *ananassa* 4	.	.	.	.	C	T	.	.	.	.	.	.	3
F. × *ananassa* 5	.	A	.	.	C	.	.	.	.	GGTC	–	.	8
F. × *ananassa* 6	.	T	.	.	C	.	.	–	–	.	–	.	5
F. × *ananassa* 7	.	A	.	.	C	.	.	–	–	.	–	.	2
F. × *ananassa* 8*	.	.	.	.	C	.	.	.	–	TGTC	.	.	–

Haplotypes of the sequences flanking the microsatellite repeats were collapsed from 22, 12 and 37 sequences for individuals *F. vesca* F, *F. vesca* U and *F.* × *ananassa*, respectively. *Fragraraia vesca* F and *F. vesca* U were sampled at sites Forst and Unterboezberg, respectively. The microsatellite repeat sequence is flanked by positions 62 and 63. The total length of the flanking sequences is 103 bp.

*Consensus haplotype based on four single haplotypes that differed in one nucleotide from one another at position 6 or 7.

F. × *ananassa* or not, we cloned and sequenced PCR products of one plant each from sites Forst (*F. vesca* F) and Unterboezberg (*F. vesca* U) and of *F.* × *ananassa* 'Hummi grande' that contained both the 192 bp and the 206 bp allele. A total of 37, 22 and 12 sequences were obtained for *F.* × *ananassa* 'Hummi grande', *F. vesca* F and *F. vesca* U, respectively. Collapsing of haplotypes resulted in eight, two and two haplotypes for *F.* × *ananassa* 'Hummi grande', *F. vesca* F and *F. vesca* U, respectively (Table 4). In one case a group of four single *F.* × *ananassa* haplotypes that all differed in one nucleotide from one another at positions 6 or 7 were collapsed to a consensus haplotype. A single *F.* × *ananassa* haplotype was classified as a recombinant of *F.* × *ananassa* haplotypes 4 and 5 and therefore discarded. Microsatellite repeats were variable in sequences of cloned alleles, which made it impracticable to assign specific sequences to overlapping alleles based on sequence length. *Fragaria* × *ananassa* and *F. vesca* haplotypes were clearly different (Table 4).

Defined crosses between F. vesca *and* F. ananassa

Fruits from hand pollinations yielded a total of 2999 and 2987 seeds for crosses of the type *F. vesca* × *F.* × *ananassa* 'Calypso' and *F. vesca* × *F.* × *ananassa* AN93.231.53, respectively. From these, 67 and 55 seedlings of crossings *F. vesca* × *F.* × *ananassa* 'Calypso' and *F. vesca* × *F.* × *ananassa* AN93.231.53, respectively, were raised. Molecular analysis of 25 randomly selected plants of each crossing type showed that seven of them had only alleles of the *F. vesca* mother at all seven loci and never had more than two alleles per locus. Five of them were collected from the same fruit. They were classified as pure *F. vesca* plants that resulted from accidental pollination with *F. vesca* pollen, e.g. by pollen shattering during emasculation. Of the remaining 43 plants, 42 had specific *F.* × *ananassa* alleles at all seven loci. One plant had *F.* × *ananassa* alleles at six of the seven loci. Forty plants had *F. vesca* alleles at all seven loci and three plants had *F. vesca* alleles at six loci. Furthermore, all plants had more than two alleles at three loci, which suggests a polyploid status; average allele numbers were 4 (range 3–6), 3 (range 2–4) and 4 (range 3–5) for microsatellite markers ARSFL 22, ARSFL 31 and EMFvi 136, respectively. Based upon these results all 43 plants were classified as true hybrids. All *F.* × *ananassa* alleles scored in hybrids matched exactly the known alleles in the parental line. It was therefore concluded that alleles are inherited in unchanged size by hybrids. Similarly, alleles of parental *F. vesca* lines were inherited unchanged, and variations of scored fragment length sizes for the variable loci yielding stutter peaks never exceeded $\pm 1–3$ bp. Only in one plant was an allele scored that differed from all known alleles in parental lines.

By extrapolating the results from our sub-sample, we estimated the proportion of true hybrids among our 122 seedlings to be 86 %. This results in an average germination and survival rate of hybrids of 1·8 % until the seedling stage.

DISCUSSION

No hybrids were found at any of the ten survey sites, although some of the oldest commercial strawberry farms in Switzerland were included in the survey.

Alleles for some *F. vesca* and *F.* × *ananassa* samples at sites Forst and Unterboezberg overlapped at the highly variable marker ARSFL 22. Overlapping alleles were cloned and sequenced, and it was found that *F. vesca* and *F.* × *ananassa* alleles were clearly different (Table 4). As the results of our analyses of hybrids from controlled crosses clearly showed that microsatellite marker analysis and flow cytometry both have high power to detect F_1 hybrid plants, we are confident that we did not sample any F_1 hybrids at our survey sites. The reliability of our method was furthermore confirmed by definite classification of two conspicuous plants from site Berneck as feral *F.* × *ananassa*.

The finding of two feral *F.* × *ananassa* plants raises the question about the potential of strawberries to become feral, which is of special interest regarding future GM cultivars. In central Europe, feral *F.* × *ananassa* do occasionally occur on and beside former strawberry fields or in the vicinity of

garden waste dumpsites (pers. comm. from breeders and farmers). Such plants probably establish from runners. Seedlings often germinate in agricultural fields, but seedling establishment beyond the favourable conditions of agricultural fields seems to be an unlikely event. At least we are not aware of any such reports. Although there is occasional establishment of feral *F.* × *ananassa*, the species appears neither in the FloraWeb database of the Federal Agency for Nature Conservation of Germany that lists about 500 local or country-wide established neophytes (Bundesamt für Naturschutz, 2010) nor in the inventory of alien species in Switzerland containing >300 plants (Wittenberg *et al.*, 2005). Unless transgenes will enhance the fitness of cultivars under non-agricultural conditions the occurrence of feral *F.* × *ananassa* would probably remain a sporadic event in our survey area. Nevertheless, there are geographical regions in which *F.* × *ananassa* escape is more likely, e.g. the mid-western and southern USA (Rosskopf, 1999).

We assume that our sample size was large enough to rule out a widespread occurrence of *F. vesca* × *F.* × *ananassa* hybrids in our restricted survey area. It seems that hybrid formation or hybrid establishment under natural conditions is a rather rare event. Differing ploidy levels of the two species are the major obstacles for establishment of hybrids (Darrow, 1966; Evans, 1974). Nevertheless, our sample size was not big enough to rule out completely the possibility of hybrid establishment under natural conditions. Vigorous hybrids between *F. vesca* and octoploid *Fragaria* species have been reported from experimental crossings and from field sites (Mangelsdorf and East, 1927; Yarnell, 1931*a*; Bringhurst and Khan, 1963; Olbricht *et al.*, 2006), and our own observations of *F. vesca* × *F.* × *ananassa* hybrids confirm these reports. We therefore warn against an uncritical use of our results for promoting the cultivation of transgenic strawberries.

It still is unclear to what extent pollen flow between the species occurs and whether pollinator behaviour contributes to hybridization in the field. Little is known about pollinators and pollinator overlap of *Fragaria* species and even less about the frequency of pollinators visiting two populations of differing *Fragaria* species during the same period. Another complication for assessing the probability of pollen flow from *F.* × *ananassa* to *F. vesca* is the self-compatible nature of the latter. *Fragaria vesca* seems to be a predominantly selfing species (Arulsekar and Bringhurst, 1981). This reduces the chances of interspecific outbreeding in comparison with dioecious species such as *F. chiloensis* that can hybridize naturally with *F. vesca* as reported in Bringhurst and Khan (1963).

Our results from germinating hybrid seeds showed, in good accordance with results from previous workers (Mangelsdorf and East, 1927; Yarnell, 1931*a*; Marta *et al.*, 2004; Ulrich *et al.*, 2007), that germination and survival rate of hybrids between *Fragaria* species with differing ploidy levels is low (approx. 1–2 %). As a comparison, the germination rate of *F. vesca* achenes collected at four different field sites on field soil in the greenhouse was 46 % after 8 weeks (unpubl. res.). This rate is 25 times higher than the germination rate of our hybrid seeds, although *F. vesca* seeds were exposed to less favourable germination conditions. Assuming that some pollen flow occurs, we do not know whether under natural conditions it is the relatively low germination rate of hybrid seeds that is a major obstacle for establishment of *F. vesca* × *F.* × *ananassa* hybrids or if natural selection selects against later developmental stages of hybrids, that are not fit enough to compete with co-occurring plants. Further experiments with pollinators as well as competition experiments between hybrids and *F. vesca* plants are underway and will clarify whether the small probability of hybridization between *F.* × *ananassa* and *F. vesca* can be explained by pollinator preferences for any of the species and/or hybrids lacking fitness.

SUPPLEMENTARY DATA

Supplementary data are available online at ww.aob.oxfordjournals.org and consist of the following. Table S1: Allele numbers and ranges of allele lengths at seven microsatellite loci of major *F.* × *ananassa* cultivars grown at farm survey sites and reference samples of *F. moschata* and *F. viridis* accessions.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation (grant number 405940-115642 to A. E. and P. S.). We thank Claudia Michel and Aria Minder for assistance during laboratory work and Klaus Olbricht for helpful discussions and supply of plant samples. We are grateful to two anonymous referees for valuable suggestions to this manuscript.

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