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DNA-based determination of suitable pollinating cultivars for the pear cultivar 'Carola' *(Pyrus communis)*

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Summary: Pollen-limited fruit set has long been suspected in some relatively low-yielding orchards with the Swedish pear cultivar 'Carola'. Fruit was therefore harvested on 23 'Carola' trees in a commercial pear orchard. The seeds were germinated and five seedlings from each tree were sampled to determine which of the surrounding cultivars had been the most successful pollinators. Leaves of 'Carola', the 7 putative pollinating cultivars and the 115 seedlings were analysed with 6 RAPD primers. By comparison of the band patterns, paternity could be ascertained for 74 seedlings. The by far most successful pollinator was 'Clara Frijs' which had sired approx. half of the seedlings, followed by 'Herzogin Elsa', 'Skånskt Sockerpäron', 'Alexandre Lucas', 'Colorée de Juillet' and 'Doyenné du Comice'. The latter is the maternal parent of 'Carola', and these two cultivars must therefore share one S-allele and hence can only be semi-compatible. In addition, 6% of the seedlings were in all likelihood derived from selfing since they showed no bands that did not occur also in 'Carola'. Maximum distance between 'Carola' trees and suitable pollinators should not exceed 15–20 m. Longer distances may produce a serious dearth of compatible pollen as evidenced by the large percentage of seedlings derived either from selfing (25%) or from long-distance (> 40 m) pollen transfer (25%) when 'Carola' trees were surrounded by non-preferred pollinators.

Key words: Molecular marker, pear, pollination, self-incompatibility, RAPD

Introduction

A high fruit set is necessary to ensure high yields and thus a profitable business for pear orchards. Some cultivars are inherently unproductive due to e.g. insufficent flower production or poor tree structure, and others may be difficult to grow in certain areas due to e.g. sensitivity to freeze damage during flowering. In yet other cultivars, fruit yields may be very high in some orchards while considerably lower in neighbouring orchards. In these cases, pollination problems may be suspected. Availability of suitable pollinating insects must be ensured, and is often handled by placing bee hives in the orchard (*Stern* et al., 2004). Although a few pear cultivars are partially self-compatible (*Moriya* et al., 2005), most are self-incompatible and different cultivars must therefore be planted sufficiently close to enable pollen transfer between compatible genotypes.

In many rosaceous crops, self-incompatibility is controlled by an S-locus which encodes for the different S-RNases that determine the S-alleles of different genotypes. Apple and pear cultivars that share one S-allele have reduced compatibility and do not achieve their potential yield capacity (*Schneider* et al., 2005b; *Zisovich* et al., 2005). If both S-alleles are shared, the cultivars are usually incompatible and do not yield at all except for occasional fruits obtained by bypassing the self-incompatibility system through pollination between genetically incompatible genotypes or through selfing.

The pear cultivar 'Carola' was developed at Balsgård from a cross performed in 1946 between the local Danish cultivar 'Johantorp' and the internationally well-known 'Doyenné du Comice'. 'Carola' was registered in 1983 and it has very good fruit size and texture, shows field resistance against pear scab and is well adapted to the relatively harsh climate in Scandinavia. Inconsistent yields have, however, been a major problem with 'Carola', leading to a reduced acreage compared to the potential of this cultivar.

DNA-based identification of the S-allele composition is now being undertaken for a growing number of European pear cultivars (*Zuccherelli* et al., 2002; *Zisovich* et al., 2004; *Sanzol* et al., 2006; *Takasaki* et al., 2006; *Moriya* et al., 2007). Unfortunately such information has, however, been published for only a few of the cultivars grown in Scandinavia like 'Doyenné du Comice' (S4S5, *Sanzol* et al., 2006; or SaSb, *Zuccherelli* et al., 2002; *Takasaki* et al., 2006), 'Colorée de Juillet' (SeSm, Moriya et al., 2007) and 'Conference' (SdSh, *Zuccherelli* et al., 2002, or SdSr, *Takasaki* et al., 2006). Since 'Doyenné du Comice' is one of the parents of 'Carola', the latter should have one of the alleles reported for 'Doyenné du Comice'. It is also evident that 'Carola' must be at least semi-compatible with both 'Colorée de Juillet' and 'Conference' since these two cultivars can share a maximum of one S-allele with 'Carola'.

Some information regarding cross-compatibility can also be obtained from the pollination experiments involving 'Carola' that were conducted at Balsgård in the 1970s and

1980s. Pollination with 'Conference' was carried out on a total of 417 'Carola' flowers yielding 42 fruits and 187 viable seeds. Corresponding data for the pollinations with 'Herzogin Elsa' were 188 flowers, 41 fruits and 153 seeds. In addition, 'Carola' was successfully used as a pollinator for e.g. 'Colorée de Juillet', 'Conference', 'Doyenné du Comice' and 'Herzogin Elsa'. By contrast, no fruit was obtained when 'Carola' pollen was used on 'Clara Frijs'. Although experimental pollinations in general are not sufficiently sensitive to discriminate between fully compatible (no S-alleles in common for the two genotypes) and semi-compatible (one S-allele in common) (*Schneider* et al., 2005b), they do at least indicate that complete incompatibility (both S-alleles in common) does not exist between 'Carola' on the one hand, and 'Colorée de Juillet', 'Conference', 'Doyenné du Comice' or 'Herzogin Elsa' on the other hand.

Two studies have recently been performed in 'Carola' orchards to determine the pollination success of various other pear cultivars. One of these studies was carried out at a research station in Kivik (Mattisson et al., manuscript submitted) whereas the other study was conducted in a commercial pear orchard. In this latter study, reported here, seedlings obtained from fruit harvested on 23 'Carola' trees were analysed using RAPD (Random Amplified Polymorphic DNA) to determine the relative importance of the other cultivars as pollinators for 'Carola'.

Materials and methods

Plant material

The study was undertaken in the commercial fruit tree orchard 'Nordanvik', situated in NE Skåne, the southernmost province of Sweden. Although specialised in apples and sweet cherries, this orchard also contains 5–6 hectares of pear trees. The study was carried out in a field with pear trees grafted on Adam's quince rootstock and planted in 1994 (Fig. 1). The trees were planted two m apart in the rows, with four m between rows. The main cultivars in this field were 'Carola', 'Alexandre Lucas' and 'Conference'. On the other side of a small field road, 'Clara Frijs' was planted as well as a few young and small trees of 'Concorde' and one large tree of the old summer-fruiting cultivar 'Skånskt Sockerpäron' (probably identical with 'Kleine Gelbe Frühbirne' and 'Jaune Précoce', *Dahl*, 1929), which is sometimes grown as a pollinator. Some trees of various other pear cultivars (e.g.,'Colorée de Juillet' and 'Herzogin Elsa') occurred farther away on the farm. For the present study, 23 trees of 'Carola' were chosen and ripe fruit was harvested in 2003. Seeds were extracted, stratified and germinated. Leaves were collected from five seedlings from each harvested tree (a total of 115 seedlings) for future DNA extraction. Leaves were also collected from 'Carola' and 7 putative pollinators ('Alexandre Lucas', 'Clara Frijs', 'Coloreée de Juillet', 'Conference', 'Doyenné du Comice', 'Herzogin Elsa' and 'Skånskt Sockerpäron').

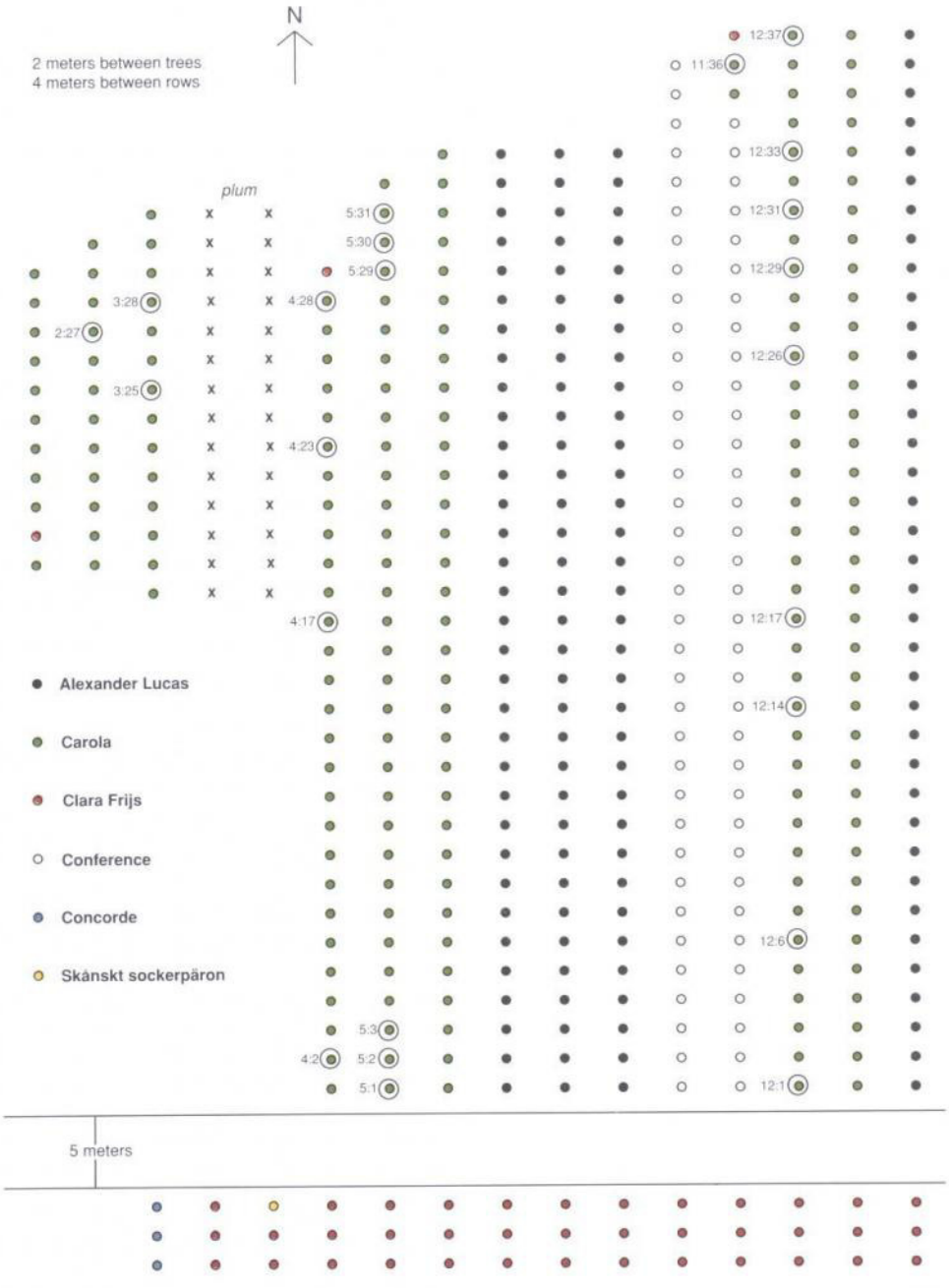

Figure 1. Experimental orchard and experimental setup in the commercial fruit tree orchard 'Nordanvik', Sweden.

DNA extraction

The collected leaves were stored in –80°C until DNA extraction. Total genomic DNA was extracted using the Qiagen DNeasy™ Plant Mini Kit for some seedlings and e.Z.N.A SP Plant DNA Miniprep Kit (200) from Omega Bio-Tek for other seedlings. The following changes were made to the manufacturer's protocol; approx. 100 mg of leaf tissue was ground using a disposable plastic pestle in 200 µl of Buffer AP1/Buffer SP1 in 1.5 ml eppendorf tubes. An additional 200 µl of Buffer AP1/Buffer SP1 and 4 µl of RNase A (100 mg ml^{-1}) was added to the ground leaf tissue.

RAPD

A total of 226 primers (Operon Biotechnologies, Inc.) were screened for their ability to amplify bands that were specific for one (or at least only a few) of the putative pollinators and that did not occur in 'Carola'. Six informative primers (OPA08, OPA11, OPA19, OPF03, OPF07 and OPG09 were subsequently used for screening all 115 seedlings. The reactions contained 1 unit of *Taq* polymerase (ABgene), 2.5 µl of the supplied buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.6 µM primer and 5 ng DNA in a total volume of 25 µl. The amplifications were performed in a Px2 Thermal Cycler (Thermo Hybaid) programmed as follows: 1 cycle of 60 s at 96C; 35 cycles of 30 s at 94C, 30 s at 53C and 60 s at 72C; 1 cycle of 420 s at 72C. The PCR products were separated by agarose gel electrophoresis (1.8% agarose), and then visually analysed.

Results and discussion

Choice of method

Fruit set in commercial fruit tree orchards is dependent on the availability of compatible cultivars with closely overlapping flowering time as well as the presence of pollinating insects that move the pollen between cultivars. While cross-compatibility is a prerequisite for successful pollination, many other, insufficiently known factors like flower shape, amount of pollen and nectar composition may affect the flight patterns of pollinating insects (Free, 1993).

Contrary to previous studies of bee movement, pollen tube growth or hand-pollination experiments, paternity assignment of seedlings derived through open pollination in the orchard allow us to estimate true pollination success. Paternity of apple and pear seedlings has thus been ascertained using e.g. allozymes (*Kron* et al., 2001a; 2001b; *Routley* et al., 2004), RFLP with minisatellite DNA probes (*Nybom & Schaal*, 1990) and S-alleles (*Schneider* et al., 2001a; 2001b; 2005b; *Zisovich* et al., 2005). Amount of selfing (usually very low) versus cross-pollination has thus been estimated, and pollen transfer within and between rows has been monitored.

In the above-mentioned studies, usually only 2–3 cultivars had to be disciminated and then allozymes as well as S-alleles may be sufficient. When more cultivars are involved, multi-locus methods like RAPD or multiple single-locus markers like SSR (Simple Sequence Repeats) are usually the markers of choice (*Weising* et al., 2005). In our study, clearly scorable and completely reproducible pollinator-specific bands were few, and in spite of screening a total of 226 primers, we succeeded in finding such bands only for 'Clara Frijs', 'Herzogin Elsa' and 'Skånskt Sockerpäron'. Most of these bands were apparently heterozygous, and showed up in only 21 of the seedlings; 'Clara Frijs' had sired 13, 'Herzogin Elsa' five, and 'Skånskt Sockerpäron' three seedlings. However, by comparing the band pattern of each seedling with its known parent, i.e. 'Carola', and its putative parent, i.e. the set of 7 possible pollinators, we were able to ascertain paternity for another 53 seedlings, resulting in a total of 74 seedlings with known paternity *(Tables 1 and 2)*.

Pollinator success

In our study, the by far most successful pollinator was 'Clara Frijs' which had sired 44.3% of the 115 seedlings, followed by 'Herzogin Elsa' with 4.3%, 'Skånskt Sockerpäron' with 3.5%, 'Alexandre Lucas' with 2.6%, 'Colorée de Juillet' with 1.7% and 'Doyenné du Comice' with 0.9%. The latter is a parent of 'Carola', and these two cultivars must therefore share one S-allele and hence can only be semi-compatible. In addition, 6.1% of the seedlings may have been derived from selfing since they showed no bands that did not occur also in 'Carola'.

Unfortunately, it was not possible to ascertain paternity for the remaining 41 seedlings. For each of these, possible

Table 1. RAPD bands (identified by Operon primer number and approximate size in kilobase pairs) used for assaigning paternity in pear seedlings, by analysis of bands not present in 'Carola' but instead present in 1–4 of the putative pollinators. Bold signs represent cultivar-specific DNA bands.

Primer	Colorée de Juillet	Alexandre Lucas	Skånskt Socker-päron	Conference	Clara Frijs	Doyenné du Comice	Herzogin Elsa
A08-1250	-	-	-	-	+	-	-
A08-550	+	+	-	+	+	-	-
A19-350	-	-	+	-	+	-	-
A19-200	-	-	+	-	-	-	-
F03-285	-	-	-	-	-	-	+
A11-1040	+	-	-	+	+	+	-
A11-370	-	+	-	-	-	+	+
G09-2170	-	+	+	-	-	+	+
G09-1240	-	+	+	-	+	-	+
F07-1210	-	-	-	-	-	-	+
F07-530	+	+	-	-	+	+	+

Table 2 RAPD-derived paternity of the five analysed pear seedlings for each of 23 sampled 'Carola' trees. For positions of the 'Carola' trees in the field, see Fig. 1.

Tree no.	Father
2:27	5 'Clara Frijs'
3:25	1 'Alexandre Lucas', 3 'Clara Frijs', 1 unknown
3:28	4 'Clara Frijs', 1 unknown
4:2	3 'Clara Frijs', 1 'Skånskt Sockerpäron', 1 unknown
4:17	1 'Carola' (selfing), 1 'Clara Frijs', 2 'Herzogin Elsa', 1 'Skånskt Sockerpäron'
4:23	1 'Clara Frijs', 1 'Colorée de Juillet', 3 unknown
4:28	2 'Clara Frijs', 1 'Alexandre Lucas', 2 unknown
5:1	2 'Clara Frijs', 1 'Colorée de Juillet', 1 'Herzogin Elsa', 1 'Skånskt Sockerpäron'
5:2	4 'Clara Frijs', 1 unkown
5:3	2 'Clara Frijs', 1 'Skånskt Sockerpäron', 2 unknown
5:29	3 'Clara Frijs', 2 unkown
5:30	3 'Clara Frijs', 2 unkown
5:31	1 'Carola', 2 'Clara Frijs', 2 unkown
11:36	1 'Alexandre Lucas', 2 'Clara Frijs', 2 unkown
12:1	2 'Clara Frijs', 2 'Herzogin Elsa', 1 unkown
12:6	1 'Carola', 4 unkown
12:14	1 'Carola', 4 unkown
12:17	1 'Carola', 2 'Clara Frijs', 2 unkown
12:26	1 'Clara Frijs', 1 'Doyenné du Comice', 3 unknown
12:29	5 'Clara Frijs'
12:31	2 'Carola', 1 'Clara Frijs', 2 unkown
12:33	2 'Clara Frijs', 3 unkown
12:37	2 'Clara Frijs', 3 unkown

fathers according to the band patterns were listed (data not shown), i.e. pollinators that together with 'Carola' would account for all the bands found in the individual seedling. Not surprisingly, 'Clara Frijs' again came out as the probably most efficient pollinator as it could actually have sired all of these 41 seedlings. High numbers were noted also for 'Colorée de Juillet', with 37 possible sirings, and 'Conference' with 34 possible sirings. Lower numbers were found for 'Doyenné du Comice' with 17 possible sirings, 'Alexandre Lucas' with 14, 'Herzogin Elsa' with three and 'Skånskt Sockerpäron' with one.

According to the owner of Nordanvik pear orchard, 'Skånskt Sockerpäron' and 'Clara Frijs' appear to be efficient pollinators for 'Carola'. While 'Clara Frijs' certainly was very efficient, 'Skånskt Sockerpäron' had only sired a few of the seedlings and is probably overrated as a pollinator. The inability of 'Carola' pollen to fertilize 'Clara Frijs' in a previous hand-pollination experiment at Balsgård was probably due to environmental circumstances only.

Orchard design

Fruit tree orchards need to be planted in such a way that pollen movement between compatible cultivars is facilitated. Pear flowers secrete very little nectar but they produce an abundance of pollen grains and are therefore relatively attractive to honeybees. Honeybees are thus important for pollination in pear trees, and many growers keep bee hives in their orchards during flowering. Honeybees have been reported to fly rather short distances, and to restrict much of their mobility to single rows (reviewed in *Kron* et al., 2001a). When siring success among apple cultivars was estimated by allozyme profiling of seedlings, pollination was shown to occur mainly within rows or between adjacent rows (*Kron* et al., 2001a). However, in another apple study, average pollen dispersal distance appeared to be only 5.8 m along rows but 17.4 m across rows (*Kron* et al., 2001b). Obviously honeybee flight patterns can be quite complex, and are possibly affected by many different factors including the relative attractiveness of the available cultivars at a certain point in time.

At the study site Nordanvik, 'Carola', 'Alexandre Lucas' and 'Conference' had been planted in a typical orchard design, with three rows of each cultivar. However, our DNA analyses showed that 'Alexandre Lucas' had only sired a minor part of the seedlings and 'Conference' none except possibly some of the 'father unknown'-seedlings.

By contrast, the two single trees of 'Clara Frijs' in the northern end of the field had been very important for the pollination and hence fruit set in the studied 'Carola' trees. Out of the 42 seedlings with ascertained paternity originating from 13 'Carola' trees in the northern part of the field and with less than 18 m to a 'Clara Frijs' tree, i.e. trees 2:27, 3:25, 3:28, 4:23, 4:28, 5:29, 5:30, 5:31, 11:36, 12:26, 12:29, 12:31, 12:33 and 12:37, 35 seedlings had 'Clara Frijs' as a father, while three had 'Alexandre Lucas', one had 'Colorée de Juillet' and three were apparently obtained by selfing.

At the other end of the field, cultivars planted south of the adjacent field road were obviously crucial for fruit set in the 'Carola' trees. Among the 20 seedlings with ascertained paternity produced by trees 4:2, 5:1–5:3 and 12:1, 13 had been sired by 'Clara Frijs', three each by 'Herzogin Elsa' and 'Skånskt Sockerpäron', and one by 'Colorée de Juillet'. Five of the studied trees had more intermediate positions in the field, 4:17, 12:6, 12:14, 12:17 and 12:26. Twelve of the seedlings from these trees had ascertained paternity, with four sired by 'Clara Frijs', two by 'Herzogin Elsa', and one each by 'Doyenné du Comice' and 'Skånskt Sockerpäron'. In addition, four seedlings were apparently derived by selfing. Probably these 'Carola' trees were suffering from lack of suitable pollen and therefore run the risk of experiencing a pollen-limited fruit set.

According to the previously mentioned pollination experiments with 'Carola' at Balsgård, several of the here analysed putative pollinators should be cross-compatible with 'Carola'. Honeybees have, however, been reported to prefer some cultivars to others based on differences in flower colour, scent and the number of flowers per tree (*Free*, 1993). Thus, differences between two apple cultivars in size of flowers and spread of filaments had a significant effect on the ability to attract honeybees (*Schneider* et al., 2005a). Probably some of the pear cultivars in our study were similarly more attractive to honeybees than others. In addition, some of the cultivars may have been only semi-compatible with 'Carola'. This must certainly be true for 'Doyenné du Comice' which is a parent of 'Carola'.

In another pollinator study, 'Carola' was grown together with four putative pollinators, 'Clara Frijs', 'Seigneur d'Espéren', 'Clapp's Favourite' and 'Skånskt Sockerpäron'

(Mattisson et al., manuscript submitted). One or two trees had been planted of each putative pollinator cultivar, surrounded by trees of 'Carola'. The ten closest within-row 'Carola' trees were sampled for each putative pollinator, and paternity was assigned for three seedlings from each 'Carola' tree. Number of sired offspring per pollinator tree was 39% for 'Clapp's Favourite', 31% for 'Seigneur d'Espéren', 28% for 'Clara Frijs' and only 2% for 'Skånskt Sockerpäron'. Fruit set in trees growing at a 4–10 m distance from a same-row pollinator was only 59% of the fruit set in trees immediately adjacent (2 m) to the same pollinator. Evidence for a pollen-limited fruit set in this experiment was provided also by the fact that the more distant trees (4–10 m) had only 86% of the seed set found in closer trees (2 m).

Conclusions and recommendations

Suitable cultivars for pollinating 'Carola' are apparently 'Clapp's Favourite', 'Clara Frijs' and 'Seigneur d'Espéren' as shown in the present study as well as in a field study in Kivik (Mattisson et al., manuscript submitted). The remaining cultivars involved in these two pollination studies sired considerably fewer seedlings. Although popular among growers, 'Skånskt Sockerpäron' appears to be heavily overrated as a pollinator.

Maximum distance between 'Carola' trees and suitable pollinators should not exceed 15–20 m. Longer distances may produce a serious dearth of pollen as evidenced by the large percentage of seedlings derived either from selfing (25%) or from long-distance (> 40 m) pollen transfer (25%) when 'Carola' trees were surrounded by non-preferred pollinators.

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