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DNA "fingerprints" applied to paternity analysis in apples (*Malus* × *domestica*)

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Summary. Analysis of minisatellite DNA sequences, yielding so called DNA "fingerprints", has proven useful in paternity analysis for several different organisms. Here 64 apple seedlings, grown from seeds collected in an orchard with three cultivars, were analyzed using the M13 "fingerprint" probe. Paternity could be determined for 56 of the seedlings, 2 of which were derived through selfing. The analysis was facilitated by the occurrence of a multiallelic locus. The five different fragments determined by this locus migrated to similar positions, whether digesting the DNA with restriction enzymes TaqI or RsaI.

Key words: DNA fingerprint – M13 probe – RFLP – Paternity analysis – Apple

Introduction

DNA "fingerprints" obtained from restriction fragment length polymorphism (RFLP) analysis of minisatellite DNA sequences (Vassart et al. 1987; Dallas 1988; Rogstad et al. 1988; Ryskov et al. 1988; Nybom et al. 1989; Nybom and Schaal 1990) have proven useful in paternity analysis in humans and animals (Gill et al. 1985; Jeffreys et al. 1985a, b; Burke and Bruford 1987; Wetton et al. 1987; Burke et al. 1989; Hillel et al. 1989), utilizing the human minisatellite probes. In plants, paternity testing has many potential applications, one of them being the determination of parentage for economically important cultivars of unknown origin. Such information can then be utilized in subsequent plant breeding work. Since the M13 probe has proven successful in distinguishing between different apple cultivars (Nybom et al. 1990), a study was undertaken to determine whether this probe could be used for paternity analysis of apple seedlings.

Materials and methods

Seeds of the commercially grown apple cultivars Golden Delicious and Jonathan were obtained at an orchard near Grafton/IL, USA. The trees from which the seeds were collected had been planted together with Red Delicious trees in adjacent rows in a field with no other cultivars close by. Leaves were collected from these three cultivars, and only one tree of each cultivar was used in the subsequent analysis, since no variation had previously been detected within any of these cultivars in material collected from the same field (Nybom et al. 1990).

The seeds were stratified for 2 months at +4 °C and then sown in pots in a greenhouse at room temperature. Leaves from the three putative parental cultivars and from 64 seedlings were ground to a fine powder in liquid nitrogen and stored at −80 °C until used. DNA was extracted according to a modified version of Saghai-Maroof et al. (1984). Separate aliquots of 8–10 µg were digested with TaqI (cultivars and all 64 seedlings) and RsaI (cultivars and 31 of the seedlings), respectively. Digested DNA was electrophoresed in submersed 0.9% agarose gels. The gels were Southern blotted onto Micron Separations hybridization transfer nylon. Hybridizations were carried out utilizing a 780-bp sequence derived from the M13 bacteriophage as a probe, and hexamer-labeled with ^{32}P-dCTP (Rogstad et al. 1988). Filters were hybridized and washed according to Westneat et al. (1988), except that the final wash was carried out at 65 °C for 45 min. Autoradiographs were developed at −80 °C for 5–10 days with intensifying screens.

Results

All 64 seedlings were analyzed with TaqI (Tables 1 and 2) and about half with RsaI (data not shown). A large number of fragments were visible on the autoradiographs

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Table 1. Seedling offspring of Golden Delicious (GD), digested with TaqI. Offspring nos. 1–5 appear to have Jonathan (JO) as pollen parent, and nos. 6–29 Red Delicious (RD), whereas no. 30 appears to have arisen by pollination from a source not included in this study. For each fragment the cultivars that have this fragment are shown to the left (GD, JO, RD). Diagnostic fragments, i.e., fragments shared between a seedling and either JO or RD but not both, are denoted in bold print

Fragment no.		Seedlings																												
		1	2	3	4	5	6	7	8[a]	9[b]	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24[c]	25[d]	26[a]	27[d]	28[d]	29[e]	30[f]
GD	1													1									1		1	1					
RD	2						**2**	**2**			**2**	**2**	**2**	**2**		**2**		**2**	**2**	**2**	**2**	**2**	**2**	**2**							
GD, JO	3	3	3	3	3	3			3	3					3		3								3	3	3	3	3	3	3
JO	4	**4**	**4**			**4**																									
GD, RD	I		I	I	I		I	I	I		I	I	I	I	I	I	I	I	I	I	I	I			I	I	I	I	I		
JO	II	**II**	**II**																												
JO	III			**III**	**III**	**III**																									
GD	IV	IV				IV		IV	IV	IV?	IV	IV	IV										IV	IV		IV	IV	IV	IV	IV?	IV
RD	V									**V**?				**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**						**V**?	**V**
JO, RD	5	5	5		5	5		5			5			5				5	5		5	5	5	5	5	5		5	5		
JO	6	**6**		**6**		**6**																									

[a] Paternity could be determined only with RsaI
[b] Both fragments IV and V are probably present but difficult to evaluate; paternity was instead determined with RsaI
[c] Presence of fragment 5 rules out selfing; if fragments I–V are presumed to be alleles, this seedling should be homozygous for I, with one of the alleles derived from RD
[d] Presence of fragment 5 rules out selfing; if fragments I–V are presumed to be alleles, fragment I should be derived from RD, since IV must come from GD
[e] Both fragments IV and V are probably present but difficult to evaluate, however, additional fragments not otherwise included in this study were shared between this seedling and RD
[f] Additional fragments, not present in any of the putative parents, were found

Table 2. Seedling offspring of Jonathan (JO), digested with TaqI. Offspring nos. 31–39 appear to have Golden Delicious (GD) as pollen parent, nos. 40–55 Red Delicious (RD), nos. 56 and 57 appear to have been derived through selfing, nos. 58–62 have probably resulted from pollination by either GD or RD, whereas nos. 63 and 64 appear to have arisen by pollination from a source not included in this study. For each fragment, the cultivars that have this fragment are shown to the left (GD, JO, RD). Diagnostic fragments, i.e., fragments shared between a seedling and either GD or RD but not both, are denoted in bold print

Fragment no.		Seedlings																																
		31	32	33	34	35	36	37	38	39	40[a]	41[a]	42[a]	43[a]	44[a]	45	46	47	48	49	50	51	52	53	54	55	56[b]	57[b]	58[c]	59[c]	60[c]	61[c]	62[c]	63[d]	64[d]
GD	1	**1**		**1**		**1**	**1**	**1**	**1**																										
RD	2																		**2**	**2**	**2**		**2**											2	2
GD, JO	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3				3		3	3	3	3	3	3	3	3	3		
JO	4		4	4				4					4			4				4	4		4	4		4	4					4		4
GD, RD	I	I				I	I	I	I		I	I	I	I	I					I	I			4				I	I	I	I	I		I
JO	II	II	II	II		II				II	II					II	II					II	II				II	II					II	
JO	III				III		III	III	III			III	III	III	III			III	III	III	III			III	III	III		III	III	III	III	III		III
GD	IV		**IV**	**IV**	**IV**					**IV**																								
RD	V															**V**	**V**	**V**	**V**			**V**	**V**	**V**	**V**	**V**								
JO, RD	5	5	5		5	5	5		5	5		5	5	5	5		5		5	5	5	5	5	5	5	5	5	5	5		5	5	5		
JO	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

[a] Paternity could be determined only with RsaI
[b] All fragments present (also the ones not included in this table) were found in JO, suggesting that this seedling was derived through selfing
[c] Presence of fragment I rules out selfing; however, either GD or RD could be the pollen parent
[d] Additional fragments, not present in any of the putative parents, were found

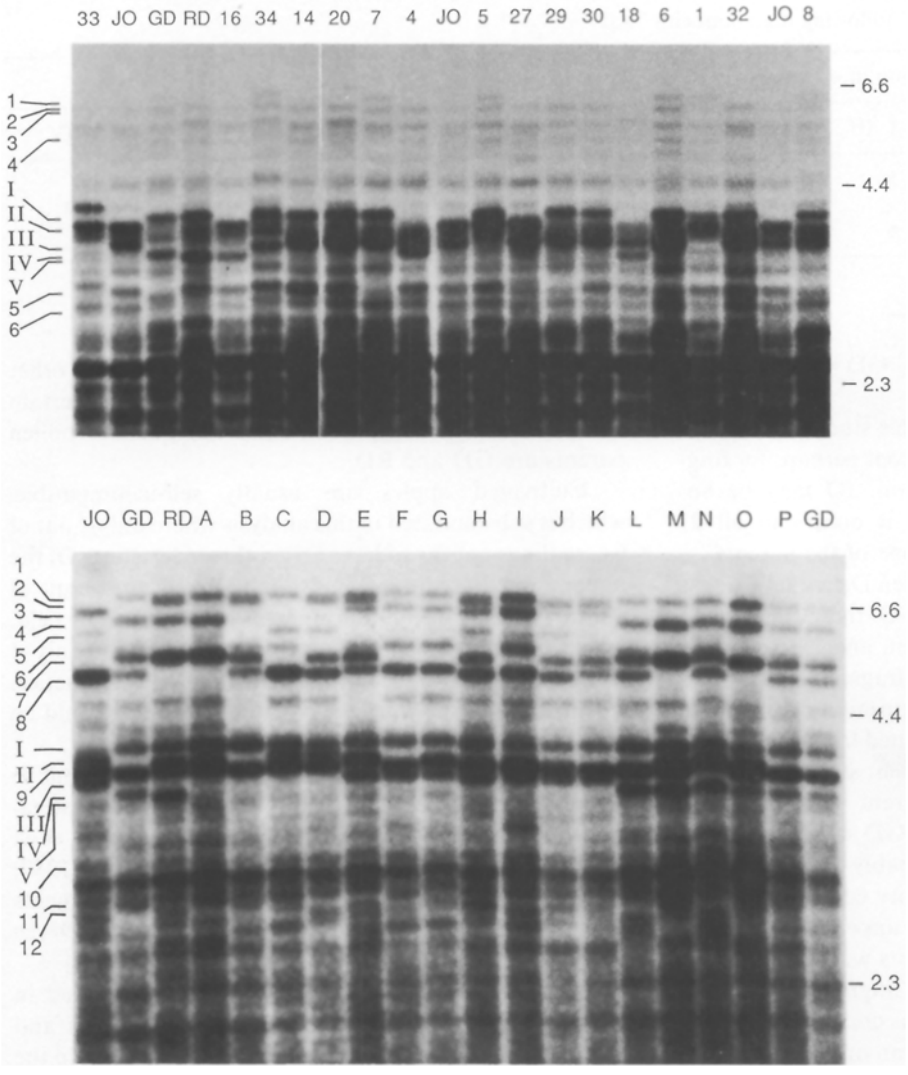

Fig. 1. DNA, digested with TaqI and hybridized with the M13 minisatellite probe, of apple cultivars GD = Golden Delicious, JO = Jonathan, RD = Red Delicious, and 16 seedlings that have JO as seed parent (for evaluation of fragment patterns, see Table 2). Fragments utilized in this study are denoted to the *left*, size markers (kb) obtained by digesting λ DNA with HindIII to the *right*. Several autoradiographs of varying intensity were utilized in the evaluation

Fig. 2. DNA, digested with RsaI and hybridized with the M13 minisatellite probe, of apple cultivars GD = Golden Delicious, JO = Jonathan, RD = Red Delicious, seven seedlings (A, B, L–P) that have GD as seed parent, and nine seedlings (C–K) that have JO as seed parent. Fragments utilized in this study are denoted to the *left*, size markers (kb) obtained by digesting λ DNA with HindIII to the *right*. Several autoradiographs of varying intensity were utilized in the evaluation

(Figs. 1 and 2), and the parental cultivars as well as all seedlings exhibiting individual-specific fragment patterns. However, only fragments present in one or two, but not in all three, of the putative parental cultivars were used in this analysis. Also, only fragments that could be reliably and consistently evaluated in each sample were included. These fragments were between 2.5 and 6.5 kb. Smaller DNA fragments could not be easily resolved, to a large extent probably due to co-migration. Altogether, 11 fragments were utilized in the TaqI-gels and 16 in the RsaI-gels.

The fragments denoted I in Golden Delicious (GD) and Red Delicious (RD), II and III in Jonathan (JO), IV in GD, and V in RD are similar in size, ranging between 3.2 and 3.9 kb, and hybridize very strongly to the M13 probe. They seem to constitute alleles of the same locus since two of them, or rarely one, occur in all offspring. Those cases where only one fragment was found could be explained either by homozygosity (two of the GD offspring appear to be homozygous for fragment I) or by pollen from an outside source (one of the JO offspring). Moreover, these fragments occur with similar intensity in the autoradiographs as expected from alleles at the same locus, since these would have the same core sequence and thus also the same degree of homology with the probe.

Fragments I–V are the only ones that could be matched between the TaqI and RsaI digestions. No inconsistencies in scoring of these fragments were found when results from the two digestions were compared. The fragments are distributed in proportions that suggest that the parental cultivars are heterozygous for all alleles involved (Table 3). However, the combination of fragments in the individual seedlings appears to deviate somewhat from a 1 : 1 : 1 : 1 distribution, with a deficiency in seedlings homozygous for fragment I. Due to low numbers, a chi-square analysis could be calculated only for

Table 3. Distribution of fragments I–V following digestion with TaqI

Parents	Fragment combination in offspring									
	I:I	I:II	I:III	I:IV	I:V	II:IV	II:V	III:IV	III:V	IV:V
GD × JO		3	5			4		2		
GD × RD	2			9	9					4
JO × RD		1	6				4		5	

the largest group of offspring, i.e., GD × RD, yielding $X^2 = 6.34$, $df = 3$, $0.1 > p > 0.05$.

All other fragments utilized in this study also appear to occur in a heterozygous state, except perhaps for fragment 8 following the RsaI digestion. JO may be homozygous for this fragment since it occurs in all 21 seedlings that have JO as at least one of the parents.

A total of 30 offspring with Golden Delicious (GD) as seed parent were analyzed (Table 1). Of these 5 appear to have Jonathan (JO) as pollen parent, and 24 Red Delicious (RD). One seedling has some fragments not found in any of these cultivars, and thus appears to either have undergone mutation or to have resulted from pollination by a source not included in the present study. A total of 34 offspring with JO as seed parent were analyzed (Table 2). Of these, 9 appear to have GD as pollen parent, and 16 RD. Another 5 seedlings probably have either GD or RD as pollen parent, but paternity could not be unequivocally assigned. Two seedlings appear to be the result of selfing, since all their fragments were found in JO only. Another 2 seedlings have some fragments not found in any of the three cultivars, and thus could be the result of either mutation or pollination by an outside source.

Paternity was thus established with a reasonable certainty for 56 seedlings, 2 of these resulting from selfing in JO, and the remaining 54 from intercultivar pollinations. For 49 of these, paternity was determined solely by use of diagnostic fragments, i.e., fragments not present in the seed parent but instead in the seedling, as well as in one of the two putative pollen parents. These are designated in bold type in Tables 1 and 2. For another 5 seedlings, paternity could be determined by assuming that fragments I, II, III, IV, and V belong to the same locus as justified above.

Discussion

The putative parental cultivars are readily distinguishable with any of five restriction enzymes investigated (Nybom et al. 1990). However, GD and RD are much more similar to each other than to JO after digestion with any one of these enzymes. Paternity can be determined for all offspring that have GD as a seed parent, since the choice is between the dissimilar JO and RD. On the other hand, paternity is considerably more difficult to ascertain when JO is the seed parent, since the putative pollen parents are GD and RD.

Cultivated apples are usually self-incompatible, which is substantiated in this analysis where only 2 out of 64 seedlings appear to have arisen through selfing. Of the remaining 62 seedlings, 59 are most likely the result of pollination from neighbouring trees within the same field. Long distance pollen dispersal is suggested for only 3 seedlings, in accordance with general cultivation practice, which states that 1 out of every 10 or 20 trees should be of a cross-compatible variety.

In the present analysis, almost all fragments appear to be heterozygous, as found also in minisatellite investigations of other cross-fertilizing species (Jeffreys et al. 1985b; Wong et al. 1986; Jeffreys and Morton 1987; Wetton et al. 1987). On the other hand, most fragments appear to be homozygous in cultivars of rice, *Oryza sativa*, which are self-fertilized (Dallas 1988).

Heterozygous fragments are usually transmitted in a 1 : 1 distribution (Jeffreys et al. 1985b; Jeffreys and Morton 1987; Dallas 1988; Gyllensten et al. 1989). In the present analysis, a 1 : 1 distribution was found for most but not all fragments. The cultivated apple normally has $2n = 18$ chromosomes and is most likely of polyploid origin. Thus, a high number of duplicate loci has been reported in isozyme investigations (Weeden and Lamb 1987). However, most isozyme alleles show disomic inheritance, suggesting either an ancient alloploid origin or extensive diploidization (Bournival and Korban 1987; Weeden and Lamb 1987). Still, tetrasomic inheritance cannot be ruled out as a cause for the skewed distribution found for some of the minisatellite fragments.

Whereas hybridization with single-locus VNTR (variable number of tandem repeat) probes may yield large numbers of fragments belonging to the same locus (Wong et al. 1986; Nakamura et al. 1987), utilization of the common minisatellite probes usually results in only one fragment being found per locus (Jeffreys and Morton 1987; Hillel et al. 1989). This may be due in part to large size differences between alleles, with short alleles either run off of the gel or present in the complex and poorly resolved, low-molecular-weight region. However, one hypervari-

able locus with several alleles has been reported in mice (Jeffreys et al. 1987). Fragments of similar size were obtained with different enzymes, suggesting the occurrence of a restriction site-deficient minisatellite flanked by normal DNA containing multiple restriction endonuclease cleavage sites. In the present study, the existence of a locus comprising at least five alleles, all yielding fragments of similar size and detectable with both TaqI and RsaI, is suggested. These alleles appear to be heterozygous in the parental cultivars, with the resulting fragments transmitted in a 1 : 1 distribution. However, the allelic combinations may deviate somewhat from expected frequencies, with a possible deficiency of plants homozygous for the allele causing fragment I. Such deviations can result from the locus being linked to other genes, for which homozygosity is selectively disadvantageous. Selection could have occurred before or just after germination, since approximately 25% of the seedlings died before attaining sampling size. A highly useful locus-specific probe could probably be isolated from this particular locus in the manner described by Wong et al. (1986) and Gyllensten et al. (1989).

Low levels of genetic linkage between loci are usually reported in minisatellite investigations (Burke and Bruford 1987; Jeffreys and Morton 1987; Jeffreys et al. 1987; Dallas 1988; Burke et al. 1989). However, five large-sized fragments consistently co-occurred in domestic dogs (Jeffreys and Morton 1987), possibly representing one very large minisatellite with five internal HinfI restriction sites. Similarly, ten fragments showed complete linkage in mice, producing a "haplotype" of multiple cosegregating fragments (Jeffreys et al. 1987). In poultry, 0–3 possible cases of linked loci were detected (Hillel et al. 1989), the uncertainty due to low sample sizes. In the present RsaI analysis (data not shown), fragment 3 occurs together with fragment III in 13 of the 20 JO offspring. The remaining 7 JO offspring, which lack fragment 3, have instead fragment II. Thus, the loci for fragment 3 and for the I–V fragments appear to be linked. Assuming that fragments III and 3 instead were part of the same minisatellite, one would expect this to have not only an internal RsaI cleavage site but also a TaqI site very close, since fragment III has approximately the same size after digestion with any of these enzymes. However, no equivalent to fragment 3 is found after digestion with TaqI. Also, in the RsaI analysis fragment 3 is considerably weaker then fragment III, suggesting that their core sequences are dissimilar. It thus seems more plausible that fragments 3 and III are caused by two different, although probably rather closely located, minisatellites. More information is needed, however, before final conclusions can be drawn.

In summary, minisatellite DNA investigation utilizing the M13 probe seems to be a promising method for determining parentage in plants. Many commercially important apple cultivars are of unknown origin, such as the three included in this study. Thus, a DNA analysis of the cultivars in question together with that of putative parents may be an important step in future plant breeding.

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