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Stability and potential use of RAPD markers in a sugarcane genealogy

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Summary

A complete ancestral history of the recently developed and closely related South African commercial sugarcane varieties N11 and NCo376, which differ markedly in their response to sugarcane mosaic virus (SCMV), was elucidated from archival records. The genealogy spans seven generations, starting with early intraspecific crosses between varieties of *Saccharum officinarum* and interspecific crosses between *S. officinarum* and either *S. spontaneum* or *S. barberi*. In total, the genealogy comprises 38 different varieties. Nineteen of these, representing all seven ancestral generations, were found to be available in local germplasm collections. Genomic DNA samples from N11 and NCo376 respectively were screened for polymorphisms using the PCR-RAPD technique. Ten polymorphic fragments ranging in molecular size from 317 to 1263bp were identified from a total of 1159 loci amplified with 100 random decamer primers. Two of the 10 polymorphic fragments were shown to be consistently present in N11 (resistant) and absent in NCo376 (susceptible), while 8 showed the reverse occurrence. The primers producing the polymorphisms were used to screen genomic DNA samples from all 19 varieties representing the genealogy. Results have indicated that (1) specific PCR-RAPD generated polymorphic fragments can indeed be identified across the seven generations; (2) certain fragments are sufficiently definitive to be used as markers to trace parentage; (3) the validity of documented crosses and/or the authenticity of germplasm material may be questioned using this technique, and (4) there is the potential to subject the markers to linkage analysis once a full and accurate assessment of the SCMV resistance phenotype is obtained.

Introduction

DNA genetic markers form the basis of most current strategies for genome analysis, gene mapping and germplasm identification. In addition, it has been argued that DNA markers with significant linkage to phenotypic characters could be useful in breeding programmes since they would facilitate accurate, rapid and early screening of progeny independently of environmental or ontogenic factors. A number of markers linked to dominant genes in important crops have been characterised (Martin et al., 1991; Haley et al., 1993; Adam-Blondin et al., 1994). DNA genetic markers include restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980; Beckmann & Soller, 1983) and random amplified polymorphic DNA fragments (RAPDs) (Welsh & McClelland, 1990; Williams et al., 1990). The advantages of RAPDs have been well documented (Welsh et al., 1991; Tingey & del Tufo, 1993).

Within the field of plant breeding, RAPD markers have been used extensively in population genetics for the identification of cultivars and clones (Hu & Quiros, 1991; Castiglione et al., 1993; Demeke et al., 1993; Yang & Quiros, 1993; Iqbal & Rayburn, 1994), for the detection and analysis of genetic diversity (Mosseler et al., 1992; Haley et al., 1994b; Marmey et al., 1994; Orozco-Castillo et al., 1994) and for the estimation of outcrossing rates (Fritsch & Reisberg, 1992). They have also proved useful for high density genetic mapping (Chaparro et al., 1992; Reiter et al., 1992), even in polyploid species such as *Saccharum spontaneum* (Al-Janabi et al., 1993; Sobral & Honeycutt, 1993). To a more limited extent they have been applied to

problems of phylogeny and pedigree: inheritance in F_1 hybrids of corn (Heun & Helentjaris, 1993), detection of interspecific gene introgression in coffee (Orozco-Castillo et al., 1994) and apple (Durham & Korban, 1994), characterisation and confirmation of somatic hybrids in potato (Baird et al., 1992; Takemori et al., 1994) and determination of parentage in maize (Welsh et al., 1991), apple (Harada et al., 1993) and grapevine (Büscher et al., 1994).

Sugarcane cultivation and breeding has a long international history. Detailed records of most of the crosses contributing to present day varieties do exist, making it possible to trace the ancestry of interesting varieties back six or seven generations to the original interspecific *Saccharum* crosses made in the previous century. Moreover, germplasm is available in international collections which represents a comprehensive historical range of parental genotypes. Because of this, sugarcane offers fascinating opportunities for genealogical studies. Precise knowledge of ancestry is an attractive prospect in sugarcane breeding because an extensive past genealogy holds potential for establishing cosegregation between putative genetic markers and phenotype. However, knowledge of the parents is subject to some uncertainty due to the method of crossing which has the capacity to result in illegitimate pollination.

In this report we describe the stability of RAPD markers in screening across several generations of a specific genealogy in sugarcane and the use of screening data in paternity analysis directed towards the identification of incorrectly documented crosses. In addition we discuss the potential application of genealogical analysis in the identification of genetic markers.

Materials and methods

Plant material. All 19 sugarcane species and hybrid varieties from the genealogy available in South Africa (Fig. 1) were harvested from the South African Sugar Association Experiment Station's permanent germplasm collection at Mount Edgecombe.

Genomic DNA isolation. DNA was extracted from freshly harvested field grown leaf roll using a modification of the method of Honeycutt et al. (1992). Young leaf roll tissue from three individual plants (clones) was pooled (6–10 g in total) and homogenised for 2 minutes (Ultra-Turrax T25) in 40 ml ice cold homogenisation buffer (50 mM Tris-HCl, 5 mM EDTA, 0.5 mM spermidine, 1% (w/v) polyethylene glycol (PEG) (8000), 0.1% (v/v) 2-mercaptoethanol, 0.35 M sucrose, pH 8.0). The homogenate was filtered through two layers of mutton cloth and the filtrate centrifuged at 5000 × g for 20 min at 4° C. The supernatant was discarded and the cell pellet resuspended in 10 ml cold wash buffer (same as homogenisation buffer but containing 25 mM EDTA and lacking PEG) and placed on ice. To this suspension were added, sequentially, 5 M NaCl to a final concentration of 0.7 M, 10% (w/v) sodium dodecyl sulphate (SDS) to a final concentration of 0.7% (w/v) and 10% (w/v) cetyltrimethylammonium bromide (CTAB) to a final concentration of 0.9% (w/v). The resulting mixture was incubated at 60° C for 30–40 min then cooled at room temperature for 15 min. Proteins were removed by gentle mixing with an equal volume of chloroform:isoamyl alcohol (24:1), centrifugation of the emulsion at 3500 × g for 10 min at 4° C, collection of the aqueous phase and repetition of the extraction. After final collection of the aqueous phase, DNA was precipitated by the addition of an equal volume of isopropanol, spooled out with a glass hook, drained and dissolved in 1.0 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was quantified and its quality assessed both spectrophotometrically (260/280 nm) and by agarose gel electrophoresis using uncut Lambda DNA (Boehringer Mannheim) as standard. DNA stock solutions were stored at - 20° C.

PCR-RAPD analysis. Stock genomic DNA solutions of the various species and varieties were diluted in TE buffer to give working solutions with final concentrations of 3 ng/μl and 0.3 ng/μl respectively which were stored at 4° C and used for up to 4 weeks. Random decamer primers (Operon Technologies, series A, B, C, D and E) were respectively diluted in TE buffer to a final concentration of 6 μM and stored at - 20° C. In the PCR-RAPD procedure, two reactions were set up for each genomic DNA using template (DNA) concentrations differing by an order of magnitude: each reaction volume was 24 μl and contained 10 mM Tris-HCl pH 8.3, 10 mM KCl, 4 mM $MgCl_2$, 2 μg acetylated bovine serum albumin, 0.22 μM primer, 0.1 mM of each deoxynucleoside triphosphate (dNTP), 1 U Ampli Taq Stoffel fragment (Perkin-Elmer) and 24 ng or 2.4 ng template. Reaction mixtures were overlaid with 30 μl mineral oil before thermal cycling. The following thermal profile was used: 1 cycle of 94° C/3 min, 35° C/1 min and 72° C/2 min with a 2.4° Cs^{-1} ramp, 40 cycles of 94° C/1 min, 35° C/1 min and 72° C/2 min with a 2.4° Cs^{-1} ramp and a final elongation step of

Table 1. Summary of PCR-RAPD analysis of sugarcane varieties N11 and NCo376

Number of primers used	100
Number of loci characterised	1159
Number of polymorphisms identified	10
Polymorphic fragments characteristic of N11	2
Polymorphic fragments characteristic of NCo376	8
Frequency of polymorphism	0.86%

72° C for 7 min. The PCR amplification products were separated on 2% (w/v) agarose gels run at 5.6 V/cm in 0.5 × TBE buffer (45 mM Tris, 44 mM boric acid, 1 mM EDTA, pH 8.0) and visualised by staining in ethidium bromide solution (1 μg/ml TBE buffer) for 45 min followed by destaining in TBE buffer for 30 min, both with constant shaking.

Results and discussion

Choice and construction of the genealogy. Ancestral lines leading to the current South African commercial varieties N11 and NCo376 were identified by consulting sugarcane breeders, local archival records and various lists of sugarcane clones and their parents published by the curators of world sugarcane germplasm collections, in particular those from the United States of America (Clones in the World Collection of Sugarcane and Related Grasses, 1988) and China (The Parents of Sugarcane Varieties of the World, 1992). The varieties N11 and NCo376 were selected as the focus of the genealogy because they combine close genetic relationship with extreme differences in a phenotype of great importance to the sugar industry. Variety N11 is highly resistant to sugarcane mosaic virus (SCMV), one of the most widely distributed and destructive pathogens of sugarcane worldwide, while, by contrast, NCo376 is highly susceptible (Bailey et al., 1994). The full genealogy is presented in Fig. 1.

Incidence of polymorphisms between N11 and NCo376. One hundred random decamer primers were used for comparative PCR-RAPD analysis of genomic DNA extracted from the varieties N11 and NCo376. Loci were considered polymorphic only when differences were observed to be unambiguous

Table 2. Decamer primers of arbitrary sequence found to identify polymorphic loci between sugarcane varieties N11 and NCo376

Primer designation (Operon technologies)	Sequence	Polymorphic fragments generated	
OB09	5′-TGGGGGACTC-3′	$OB09_{710}$[a]	(NCo376)
OC07	5′-GTCCCGACGA- 3′	$OC07_{846}$	(NCo376)
		$OC07_{818}$	(N11)
		$OC07_{550}$	(N11)
		$OC07_{504}$	(NCo376)
OC16	5′-CACACTCCAG-3′	$OC16_{904}$	(NCo376)
OD19	5′-CTGGGGACTT-3′	$OD19_{317}$	(NCo376)
OE06	5′-AAGACCCCTC-3′	$OE06_{856}$	(NCo376)
		$OE06_{560}$	(NCo376)
OE08	5′-TCACCACGGT-3′	$OE08_{1263}$	(NCo376)

[a] Polymorphic RAPD loci were given designations using the nomenclature of Michelmore et al. (1991) and Miklas et al. (1993) in which the subscript indicates the size (bp) of the fragment generated.

i.e. when a fragment was present at both concentrations in the one variety and absent at both concentrations in the other variety (Fig. 2). The results are summarised in Table 1. A total of 1159 loci were amplified, an average of 11.6 loci per primer. Ten of the loci were polymorphic, signifying a frequency of variation of 0.86%. Two of the polymorphisms were expressed as fragments characteristic of N11, while eight were fragments characteristic of NCo376. The ten polymorphic fragments were generated by six primers and ranged in size from 317 to 1263 bp (Table 2). The low incidence of polymorphism between N11 and NCo376, indicating 99.14% sequence similarity, is in keeping with their particularly close relationship (Fig. 1) and the general lack of genetic diversity which appears to be detected among current commercial varieties of sugarcane when the PCR-RAPD technique is applied (Harvey et al., 1994). Similarity values calculated from RAPD data are somewhat higher than those based on specific PCR using telomere and microsatellite sequences (M. Harvey, personal communication) or those derived from RFLP data (Lu et al., 1994). For the wider purpose of this study, the level of variation was interpreted simply as demonstrably low, and was regarded as a positive feature in that it increased the likelihood of any one of the polymorphisms identified being linked to the major phenotypic difference of interest between the two varieties: that of resistance/susceptibility to sugarcane mosaic virus.

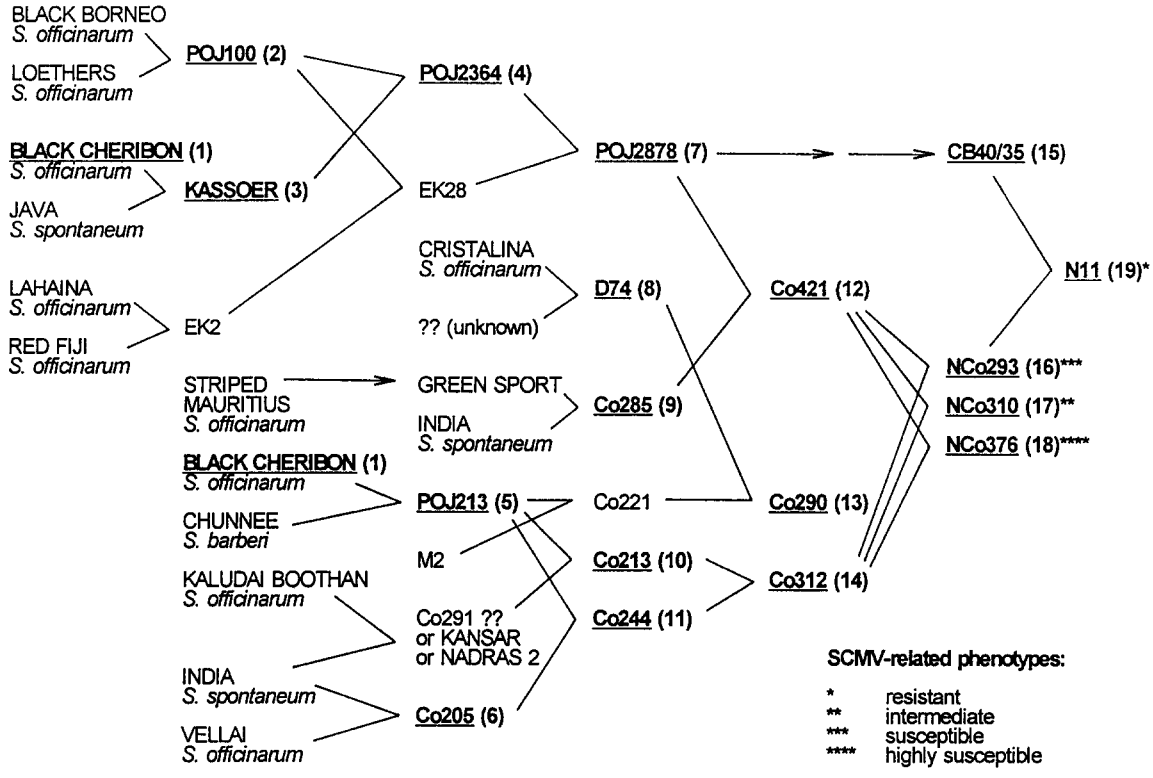

Fig. 1. Genealogy of the closely related sugarcane varieties N11 and NCo376 which differ markedly in their response to sugarcane mosaic virus (SCMV). The 19 *Saccharum* species and hybrid varieties shown in bold and underlined are those for which germplasm was available. Leaf roll tissue from these sources was harvested for genomic DNA extraction and subsequent PCR-RAPD analysis.

Table 3. Expression of polymorphic RAPD fragments across the genealogy: variation in fidelity

Fragment	Number of varieties out of the 19 analysed in which the fragment is unambiguously present or absent	% Fidelity
$OC16_{904}$	19	100
$OE06_{856}$	19	100
$OE06_{560}$	18	95
$OD19_{317}$	17	89
$OE08_{1263}$	17	89
$OC07_{818}$	16	84
$OC07_{846}$	14	74
$OB09_{710}$	14	74
$OC07_{550}$	highly variable	low
$OC07_{504}$	highly variable	low

Note: In the case of the polymorphisms of intermediate fidelity (74–89%) the presence or absence of the band was consistent but bore no particular relation to ancestral position.

Evaluation of polymorphism fidelity across the genealogy. The six primers generating polymorphisms between N11 and NCo376 (Table 2) were used to screen all 19 varieties in the genealogy for which germplasm was available. The varieties were then scored for the presence or absence of each polymorphic fragment. Results showed that individual polymorphism expression differed in both clarity and stability across the genealogy. Fragments $OC16_{904}$ and $OE06_{856}$ were found to be clearly and unambiguously present or absent in all of the varieties screened, independently of the template concentration variation (Fig. 3A). Other polymorphic fragments showed similar reliability in only a proportion of the genealogy; for example, $OE06_{560}$ could be scored unambiguously in 18 of the 19 varieties, $OD19_{317}$ and $OE08_{1263}$ in 17, $OC07_{818}$ in 16 and $OC07_{846}$ and $OB09_{710}$ in 14 varieties (Fig. 3B; Table 3). In such cases the ambiguities usually consisted of amplification of the fragment at only one of the two template concentrations (Fig. 3B), although in some instances it was the result of a faint or blurred signal in both of the relevant lanes. Yet other fragments ($OC07_{550}$ and $OC07_{504}$) proved

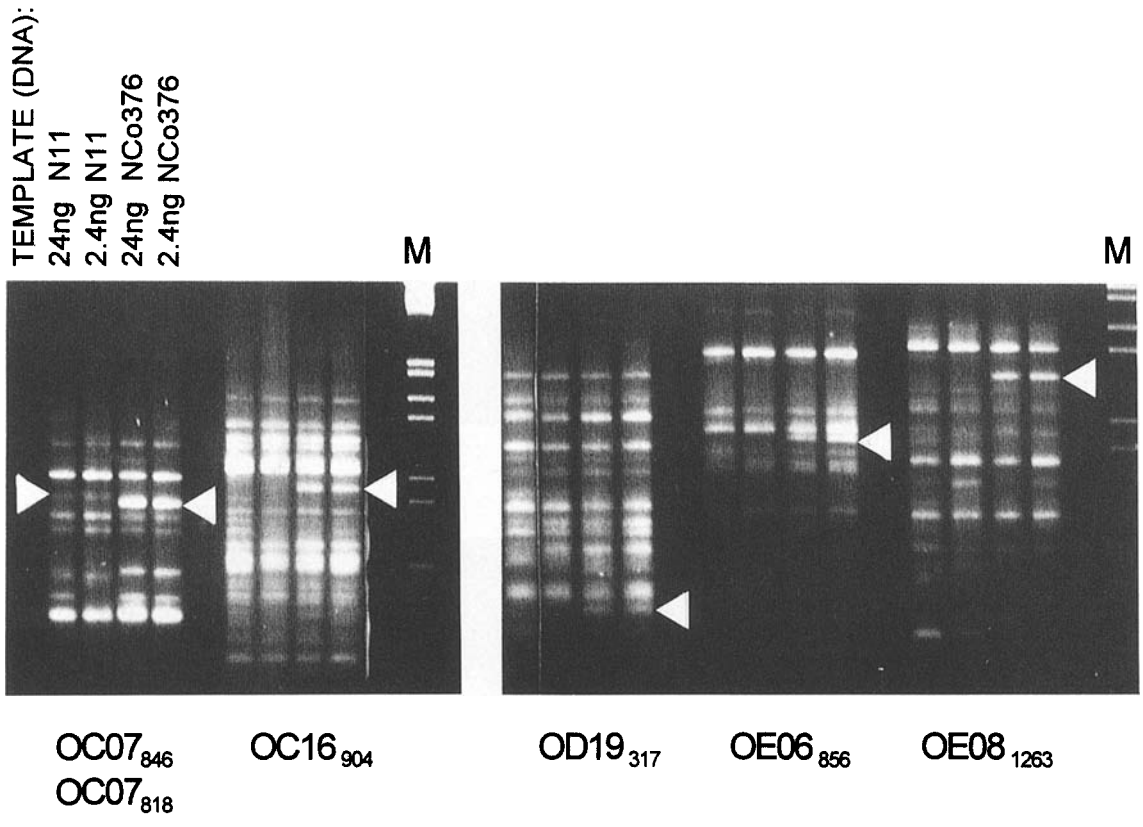

Fig. 2. Examples of genetic loci shown by PCR-RAPD analysis to be polymorphic between sugarcane varieties N11 and NCo376. For each primer/template combination, two PCR-RAPD reactions were conducted at template concentrations differing by an order of magnitude: 24 and 2.4 ng DNA per reaction respectively. Loci were considered polymorphic only when a fragment was present at both template concentrations in the one variety and absent at both concentrations in the other variety, through four or more repetitions. M = λ - *Eco* RI + *Hind* III molecular weight markers. All DNA fragment sizes were determined using WinCam 2.1 gel image analysis software (Cybertech).

unsuitable for screening as they could be visualised in only a few varieties and the amplification products were very variable (examples not shown; Table 3). These varying degrees of infidelity could be ascribed to the nature of either the primers or the template. All the primers used are the same size (10 nucleotides) and have similar G + C contents of 60–70% and are unlikely, therefore, to be responsible for the observed effect. A more likely explanation lies in inherent variability in either the genomic target sequence or its physical accessibility, factors which could account for spurious or masked expression in the manner of the 'epistatic' effects of genetic background proposed by Heun & Helentjaris (1993). Extremely stable polymorphisms such as $OC16_{904}$ and $OE06_{856}$ probably represent parts of the genome which are both conserved over several generations of breeding and relatively uncomplexed with proteins or nucleic acids in a typical DNA extract. In discussing the stability and scope of usefulness of RAPD fragments linked to specific genes, Haley & coworkers (1994a) have suggested that they might be limited to use within certain gene pools or within certain races or market classes of the same gene pool. However, those authors observed that one particular RAPD marker in bean ($OK14_{620}$) and its linkage to a rust resistance gene (*Ur-3*) appeared to have remained intact during various introgression and meiotic events, supporting the concept that certain RAPD fragments do behave as extremely stable markers beyond the bounds of gene pool or race specificity. The utility of markers for routine screening procedures and map-based cloning is clearly dependent on universal reliability of locus identification. It is for this reason that the development of sequence-characterised amplified regions (SCARs) as stable replacements for RAPD markers has been proposed as essential for these purposes (Paran & Michelmore, 1993). However, for specific investigations such as genealogical and parental analysis,

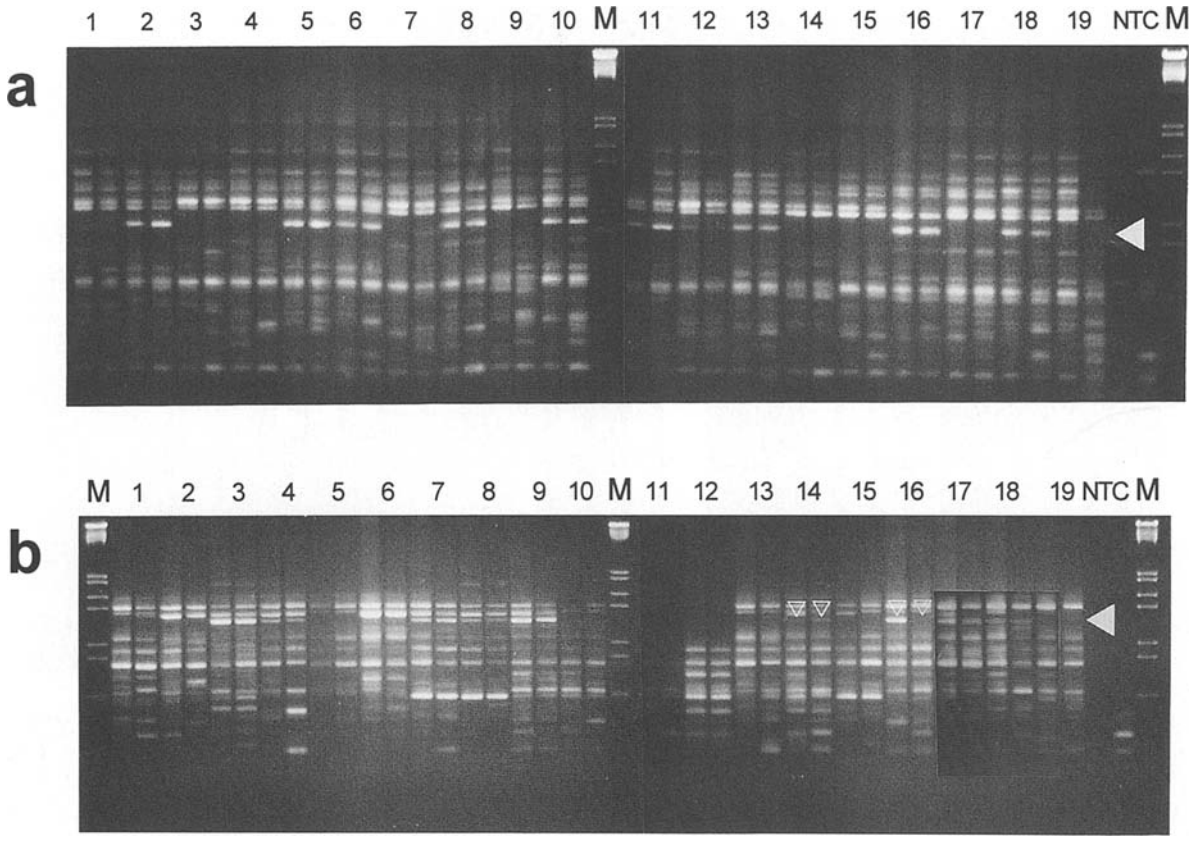

Fig. 3. Expression of polymorphic RAPD fragments across the genealogy: result of screening for $OC16_{904}$ (a) and $OE08_{1263}$ (b). For screening with a particular primer, each variety (1–19, Fig. 1) was represented by two PCR-RAPD reactions conducted using template concentrations differing by an order of magnitude, 24 and 2.4 ng DNA per reaction, and the respective reaction products run in adjacent gel lanes. The varieties were then scored for the presence or absence of each polymorphic fragment. Some fragments, for example $OC16_{904}$ (a) were found to be clearly and unambiguously present or absent in all of the varieties screened, independently of the template concentration variation. Other polymorphic fragments showed similar reliability in only a proportion of the genealogy; for example $OE08_{1263}$ (b) could be scored unambiguously in 17 of the 19 varieties, while in 2 of the 19 varieties (vertical arrows) variations related to template concentration were evident, confounding simple interpretation. M = λ - *Eco* RI + *Hind* III molecular weight markers. NTC = no template control.

it is clearly possible to make selective use of those RAPD markers that are transmitted and expressed stably across a number of generations. Furthermore, by implication, such RAPD markers, if linked to a trait of interest, could be used directly in plant breeding selection programmes.

Inheritance of polymorphic fragments and interpretations concerning parentage. Although the documented genealogy was represented by an incomplete set of varietal DNA samples (19 out of 38), eight crosses within the genealogy from the second to the seventh generation could be reconstructed and examined in terms of RAPD fragment transmission and parental contribution to the offspring, using screening data provided by the more informative polymorphisms. The results demonstrated that, consistent with expectations, presence of a RAPD fragment in any one variety was generally matched in one or both of the parents (Table 4). Clearly, this does not provide absolute confirmation of the documented parentage, since the analysis is based on the transmission patterns of only five stable markers. However, the converse situation did occur, where presence of one or more RAPD fragments in a particular variety was not matched in either of the parents, for example in the crosses Co213 × Co244 (1 transmission inconsistency) and POJ2878 × Co285 (2 inconsistencies). It is possible that such incongruous fragment behaviour could arise as a result of recombination, but is not likely where more than

Table 4. Questionable crosses within the genealogy as identified by incongruous transmission of stable RAPD markers

Female parent	Male parent	Progeny	RAPD marker $OC16_{904}$	RAPD marker $E06_{856}$	RAPD marker $OE06_{560}$	RAPD marker $OE08_{1263}$	RAPD marker $OD19_{317}$
POJ100	× Kassoer	POJ2364	C	C	C	C	C
POJ213	× Co205	Co244	C	C	C	C	C
Co213	× **Co244**	Co312	C	C	C	**NC**	C
POJ2878	× **Co285**	Co421	**NC**	C	**NC**	C	C
Co421	× Co312	NCo293	C	C	C	C	C
Co421	× Co312	NCo310	C	C	C	C	C
Co421	× Co312	NCo376	C	C	C	C	C
CB40/35	× NCo293	N11	C	C	C	C	C

Presence of a RAPD marker in progeny was matched in one or both of the parents i.e. was consistent with expected (Mendelian) patterns of transmission (C) or was not matched in either of the parents i.e. was not consistent with expected patterns of transmission (**NC**). The identity of the germplasm shown in bold (pollen parents underlined) must be treated as uncertain.

one primer binding site is implicated. In both these cases paternal identity should perhaps be questioned. An alternative potential reason for the disparity is misidentity of germplasm; hence efforts would have to be made to obtain DNA samples of the germplasm in question from other sources, in order to investigate this possibility, before making any final conclusions about the pollen parents. RAPD markers have been shown to be useful in several inheritance studies to date, in spite of earlier doubts based on inconsistencies in their behaviour as dominant characters with expected Mendelian segregation (Echt et al., 1992; Reiter et al., 1992). The work of Heun & Helentjaris (1993), designed specifically to address this problem, led to the conclusion that those RAPD fragments classed as 'unambiguous polymorphisms' (simple presence or absence of a specific fragment without variation in intensity) behaved predictably (95.2%) as dominant markers in F_1 segregating populations. Working with grapevine varieties, Büscher & coworkers (1994) found that most (97.2%) RAPD fragments followed Mendelian segregation patterns, bands occurring in the progeny being derived from either of the two parents. Using an approach similar to that used in the present work, those authors were able to exclude at least one of the previously assumed parental varieties as actual progenitor of an important grapevine cultivar, and concluded that cross-fertilisation by an unknown pollen grain was the most likely cause of the discrepant records. As in the present study, their analyses allowed the identification of a false cross but not the absolute confirmation of a correct cross: they were not able to pinpoint the true parents with certainty. However, making selective use of RAPDs generated by five long random primers (19-mer to 29-mer) in conjunction with RFLP evaluation of the PCR products, Harada & coworkers (1993) found that paternity analysis, including positive identification of the true male parent from six putative candidates, was quite feasible in *Malus* species. Their studies, like those of Büscher and colleagues, were limited to only one retrospective generation. The present work on sugarcane suggests that, as long as RAPD polymorphisms have been characterised as stable and unambiguous in the germplasm of interest, they may be used as markers for parentage analysis in varieties of both historical and current importance, as well as for wider genealogical investigations.

Potential use of genealogy to identify genetic markers. Using field-based assessments of phenotype currently available for the recent commercial varieties, it has been possible to make a preliminary examination of linkage of N11/NCo376 RAPD polymorphisms to the SCMV resistance/susceptibility trait within the genealogy. However, results to date (not shown) are very limited in scope and hence extremely speculative. Information on viral resistance for the early to intermediate ancestors in the genealogy is completely lacking and, until specific phenotypic data relating to SCMV response have been generated, it will not be possible to continue with linkage analysis. For this reason the

19 species and varieties for which germplasm is available in the genealogy (Fig. 1) are being assessed for SCMV resistance/susceptibility in several ways. Both field trials (in one of the localities heavily infested with SCMV) and glasshouse pot trials (using artificial inoculation with the virus in the absence of the aphid vector) have been established. In addition it is intended to develop an *in vitro* test for varietal response to SCMV. Linkage of RFLP and RAPD polymorphisms to genetic regions of interest has been determined most successfully using pairs of backcross-derived near-isogenic lines (NILs) (Paran et al., 1991; Martin et al., 1991), the principle being the identification of markers located in the linkage block surrounding the introgressed gene (Melchinger, 1990). A major problem encountered in many crop species is that the development of NILs for economically important genes is time consuming and costly, and few are available (Haley et al., 1993). For this reason the alternative strategy proposed by Michelmore & his group (1991), bulk segregant analysis, has aroused considerable interest and has been used fairly widely, sometimes in combination with backcross introgression (Miklas et al., 1993). In sugarcane, the high ploidy level, constraints on backcrossing and lack of NILs has necessitated the modelling of the bulk segregant analysis approach without the benefit of backcrossed lines (Msomi & Botha, 1994), and other routes to marker identification are being considered and investigated. Having established, in the present work, that the potential exists to track RAPD polymorphisms unambiguously across several generations in sugarcane, the use of genealogy as a base for preliminary identification of marker linkage in this crop appears to be an attractive alternative strategy. As for other approaches, ultimate validation of putative markers would have to be achieved through rigorous assessment of the progeny of test crosses.

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