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Identification and characterisation of sugarcane intergeneric hybrids, *Saccharum officinarum* × *Erianthus arundinaceus*, with molecular markers and DNA in situ hybridisation

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Abstract Molecular markers were used to characterise sugarcane intergeneric hybrids between *S. officinarum* and *E. arundinaceus*. Very simple diagnostic tools for hybrid identification among the progeny were derived from isozyme electrophoresis and a sequence-tagged PCR. Two enzyme systems (GOT and MDH B) and PCR amplification revealing spacer-size variation in the 5*s*-rDNA cluster were found most convenient. Specific characterisation of the two genomic components was possible using RFLP and in situ hybridisation. The strong molecular differentiation between *S. officinarum* and *E. arundinaceus* allows the identification of numerous *Erianthus*-specific RFLP bands in the hybrids. Genomic DNA in situ hybridisation allows for the differentiation of the chromosomes contributed by *S. officinarum* and *E. arundinaceus* in chromosome preparations of the hybrids. In situ hybridisation with the 18*s*-5.8*s*-25*s* rDNA probe highlights the basic chromosome numbers in the two parental species. The potential of these techniques to monitor the *Erianthus* genome during the introgression process is discussed.

Key words *Saccharum* · Intergeneric hybrids · Isozymes · RFLP · STS-PCR · In situ hybridization · rDNA

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Introduction

The genera *Saccharum*, *Erianthus* (=section *Ripidium*), *Miscanthus* (section *Diantra*), *Sclerostachya* and *Narenga* constitute a closely related interbreeding group, assumed to be involved in the origin of sugarcane and, thus, referred to as the "*Saccharum* complex".

Sugarcane belongs to the genus *Saccharum*, a complex genus which comprises six species, all characterised by a high ploidy level. Modern sugarcane varieties (*Saccharum* ssp., 2n=100–130) are derived from interspecific crosses, performed early this century, between the sugar-producing species *Saccharum officinarum* (2n=80) and wild relatives, mainly *S. spontaneum* (2n=40–128). Only a few parental clones were involved in these crosses (Arceneaux 1965; Price 1965). Thus the genetic base of modern varieties appears to be very narrow and could be the reason for the present slow progress in sugarcane breeding. To broaden this genetic base, interest has turned to the use of other genera from the *Saccharum* complex, mainly *Erianthus arundinaceus* section *Ripidium* (Berding and Roach 1987; Roach and Daniels 1987; Walker 1987). *E. arundinaceus* (2n=60) is a large grass with tall and thick stalks which has useful agronomic traits such as vigour, drought and waterlogging resistance, good ratooning ability, and disease resistance.

Despite several attempts, over many years, to introgress *E. arundinaceus* characters in sugarcane varieties, no conclusive success has been achieved. The first difficulty appears to be the identification of true hybrids using morphological traits. Two other factors suggested to be responsible for this lack of success are chromosome erosion during the successive back crossing and the lack of recombination between the chromosomes of the two genera.

In order to attempt to resolve these problems, isozyme analysis, sequence-tagged PCR, RFLP analysis and genomic in situ hybridization were applied to specifically detect the *E. arundinaceus* genome in intergeneric hybrids between *S. officinarum* and *E. arundinaceus*. Isozyme and sequence-tagged-site PCR markers are shown to be sim-

ple and powerful diagnostic tools to monitor true hybrids. RFLP and genomic in situ hybridization show great promise in following *Erianthus* chromosome segregation in introgressed material and to assess the occurrence of intergeneric recombination. The localisation of the rDNA sites provided information on the basic chromosome numbers of both parental species.

Materials and methods

Plant material

The material consisted of eight clones from the progeny of an intergeneric cross between BNS 3066 (*S. officinarum*, 2n=80) and IK 7648 (*E. arundinaceus*, 2n=60) and the two parental clones. This cross was performed at the West Indies Central Sugar Cane Breeding Station (WICSCBS) in Barbados and the progeny was grown and provided by CIRAD-Guadeloupe. In addition, an *E. arundinaceus* clone, IK 7624, provided by the Bureau of Sugar Experiment Stations (BSES), was used for the in situ hybridisation experiments.

Isozymes

Two enzyme systems were analysed, namely aminotransferase (glutamate oxaloacetate transaminase, GOT) and NADP-dependent malate dehydrogenase (MDH B). Extractions were performed from young fully expanded leaf blades, by grinding 200 mg of leaf tissue in 1 ml of cold 0.1 M sodium phosphate buffer, pH 7, with 0.01 dithioerythritol supplemented with 50 mg of polyvinylpyrrolidone. Filter-paper wicks (Whatman 3) were imbibed with the extract for use in 12% starch-gel electrophoresis. A mild centrifugation of the extract improved the banding quality. The starch-gel system involved a Tris-citrate buffer, pH 7, as described in Shaw and Prasad (1970). Enzyme staining followed standard procedures, as advised by Feldmann (1984).

PCR amplification of the 5*s* ribosomal DNA spacer

PCR reactions were performed on total DNA extracted from leaf tissues. Alternatively, to avoid DNA extraction, the PCR can be performed on small young leaf pieces following the protocol of Klimyuk et al. (1993). The sequences of the primers used were from Cox et al. (1992): P1: 5′-TGGGAAGTCCT(C/T)GTGTTGCA-3′; P2: 5′-(T/G)T(A/C)G(T/C)GCTGGTATGATCGCA-3′. The amplification reaction mix consisted of 5 ng of genomic DNA, 0.2 µM of each primer, 200 µM of dNTPs mix, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin) and one unit of *Taq* polymerase in a 25 µl final volume. The PCR was carried out for one 3-min cycle at 95°C, 30 cycles of 55 s at 93°C, 15 s at 55°C and 30 s at 72°C. The amplification products were separated by electrophoresis in 2% agarose gels in TAE buffer at 70 V/20 cm for 4.5 h.

RFLP

The probes BNL 16.06, BNL 12.06, UMC 39, UMC 44 and UMC 107 were obtained from Brookhaven National Laboratory (BNL probes) and from the University of Missouri, Columbia (UMC probes).

Total DNA was extracted from leaf tissues following the protocol of Hoisington (1992). DNA was digested with the *Hin*dIII restriction enzyme . The restriction fragments were separated by electrophoresis in an 0.8% agarose gel in TAE buffer. DNA was transferred onto nylon membrane (Hybond N+, Amersham). Probes were labelled with ^{32}P-αdCTP using the Amersham Megaprime commercial kit. Pre-hybridization, hybridization and washes were performed in a Hybaid oven, following the Hoisington (1992) protocol. The pre-hybridization buffer contained 5 × SSC, 0.2% SDS, 1 × Denhardts, 0.1 mg/ml of sheared salmon sperm DNA. The hybridization buffer was similar but supplemented with 10% dextran sulphate. Blots were washed 10 min in 2 × SSC, 0.5% SDS at room temperature and then four times for 30 min in 0.1 × SSC, 0.1% SDS at 65°C.

Chromosome preparation

Roots were harvested from plants cultivated in pots or from cuttings placed in wet paper at 30°C. Roots were treated in 0.04% hydroxyquinoline for 3 h, fixed for 72 h in 3:1 methanol:acetic acid and stored in 75% ethanol at 4°C. The fixed roots were rinsed twice in H_2O for 10 min, treated for 10 min in 0.25 N HCl, rinsed for 10 min in H_2O and then put in a digestion buffer (0.01 M citrate buffer, 0.075 M KCl) for 10 min. Root tips were cut and placed in an enzyme solution (5% cellulase Onozuka R-10, 1% pectolyase Y-23 in digestion buffer) in a microtube at 37°C for approximately 3 h (the time varying with the size of the roots). Root tips were then rinsed in H_2O and spread in a drop of 3:1 ethanol:acetic acid with a forceps. Slides were stored at –70°C.

Fluorescence in situ hybridisation

Slides were treated with RNAse (1 µg/ml) at 37°C for 45 min, denatured for 2.5 min in 70% formamide in 2 × SSC, at 70°C, then dehydrated through an ethanol series at –20°C. The hybridization mixture (30 µl per slide) consisted of 50% formamide, 10% dextran sulphate, 2 × SSC, 1% of SDS and the DNA probes. For genomic DNA in situ hybridisation, 200 ng of IK 7648 genomic DNA, labelled with biotin-14-dUTP, and 200 ng of BNS 3066 genomic DNA, labelled with digoxigenin-11-dUTP (Boehringer Mannheim), were used. For rDNA in situ hybridisation, 150 or 300 ng of the 18*s*-5.8*s*-25*s* probe, pTA 71 (Gerlach and Bedbrook 1979), labelled with biotin-14-dUTP or coumarin-4-dUTP (Fluoroblue, Amersham), respectively, were used with 1500 ng of sheared salmon sperm DNA. The hybridisation mixture was denatured for 10 min at 75°C. Hybridisation was performed overnight in a moist chamber at 37°C. The washes, detection of biotin with Texas Red and digoxigenin with FITC (fluorescein isothiocyanate), amplification, and counterstaining with DAPI (4′.6-diamidino-2-phenylindole) were all performed as described in Heslop-Harrison et al. (1991). The slides were mounted in vectashield antifade solution (Vector Lab) and photographed on Fujicolor 400 print film. When a second hybridisation was performed on the same slide, de-hybridisation was carried out as described in Heslop-Harrison et al. (1992).

Results

Isozymes

Electrophoretic polymorphism was revealed in the progeny (eight clones) and the two parental clones of the cross BNS 3066×IK 7648 for two enzyme systems: GOT (Fig. 1) and MDH B. In both cases, each parental clone displayed a single specific band in the most discriminant region of the zymograms. Among the eight putative clones, three gave a three-banded pattern, including the two parental bands and one additional band with an intermediate position; this is typical of a dimeric enzyme, with two ho-

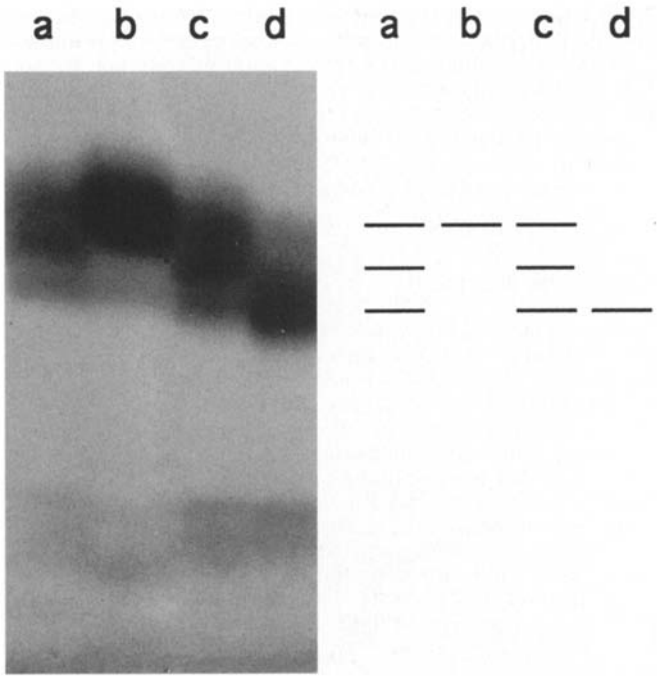


Fig. 1 Faster migratory region of GOT zymograms. *Lanes a and c*, intergeneric hybrids between BNS 3066 and IK 7648; *lane b*, BNS 3066 (*S. officinarum*); *lane d*, IK 7648 (*E. arundinaceus*). The intermediate bands on the hybrid zymograms correspond to heterodimeric enzymes combining subunits from both parents. Migration is from bottom to top. A diagrammatic representation is presented on the right

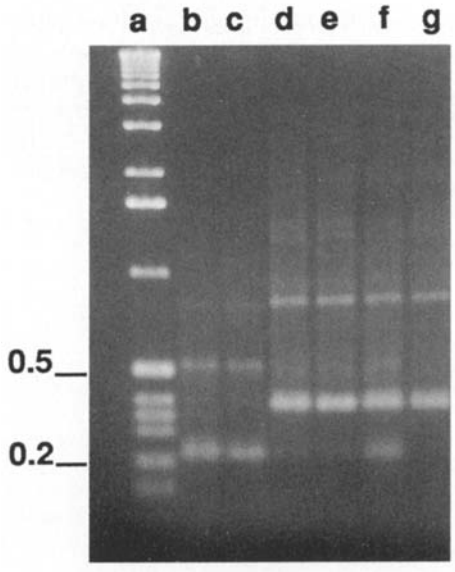


Fig. 2 Amplification of the 5*s* rRNA spacer on the parents and three clones from the cross BNS 3066 (*S. officinarum*) × IK 7648 (*E. arundinaceus*). *Lane a*, molecular-weight standard (the sizes are given in kilobase pairs); *lane b*, BNS 3066; *lane c*, one clone probably resulting from illegitimate pollination of BNS 3066; *lanes d and e*, two clones resulting from intergeneric hybridisation; *lane f*, control with a mix of both parents DNA; *lane g*, IK 7648

modimers and one heterodimer. The other five plants displayed only the bands of the female parent BNS 3066, shedding doubt on their intergeneric hybrid nature.

PCR amplification of the 5*s* ribosomal RNA spacer

The polymerase chain reaction was used to amplify the 5*s* rRNA spacer. A preliminary study addressed interspecific distinction on the basis of a sample of *S. officinarum, S. spontaneum* and *E. arundinaceus* clones (data not shown). Each clone produced one major fragment. No polymorphism has been detected among the seven *S. officinarum* clones analysed, which all showed a major band of approximately 230 bp. The five *S. spontaneum* clones surveyed displayed a major band close in size to the *S. officinarum* band, with however, some variation from approximately 190 to 230 bp. On the other hand, among the seven *E. arundinaceus* tested, six exhibited a major band of approximately 370 bp, and one showed a slightly smaller (about 360 bp) fragment. Amplification with the same primers was performed on the DNA of the two parents and the eight clones issuing from the intergeneric cross. A mix of both parents DNA was used as a control. Figure 2 shows the various PCR products obtained. The two parents displayed a different major band, in accordance with their taxonomic group. BNS 3066 gave a band of approximately 230 bp and IK 7648 a major band of approximately 370 bp. The control and three clones displayed two majors bands corresponding to the IK 7648 and BNS 3066 bands with, however, a weaker *S. officinarum* band on the latter three hybrid patterns. The presence of both parental bands identifies these three clones as intergeneric hybrids. The other five clones of the progeny exhibited exclusively the *S. officinarum* band and thus probably resulted from *S. officinarum* selfing. These five clones produced a zymotype identical to BNS 3066. Among the eight clones analysed, three were thus identified as intergeneric hybrids, whereas the other five were probably *S. officinarum* selfs.

RFLP

Hybridisations were performed with five single copy nuclear maize probes (BNL 16.06, BNL 12.06, UMC 39, UMC 44 and UMC 107) on total DNA restricted by *Hin*dIII. Eight clones of the intergeneric cross and the two parental clones were analysed. Figure 3 shows an example of the hybridisation pattern obtained with the maize probe BNL 16.06. With the five probes used, 49 bands were generated, from which 16 were specific to *Erianthus* IK 7648 and 33 were specific to *S. officinarum* BNS 3066. No single band was common to both parents. The three clones previously identified as hybrids with both isozymes and the 5*s* PCR marker displayed some IK 7648 (*E. arundinaceus*)-specific bands as well as some BNS 3066 (*S. officinarum*)-specific bands, while the five illegitimate individuals displayed only *S. officinarum* bands, thus appearing as most likely derived from self-pollination.

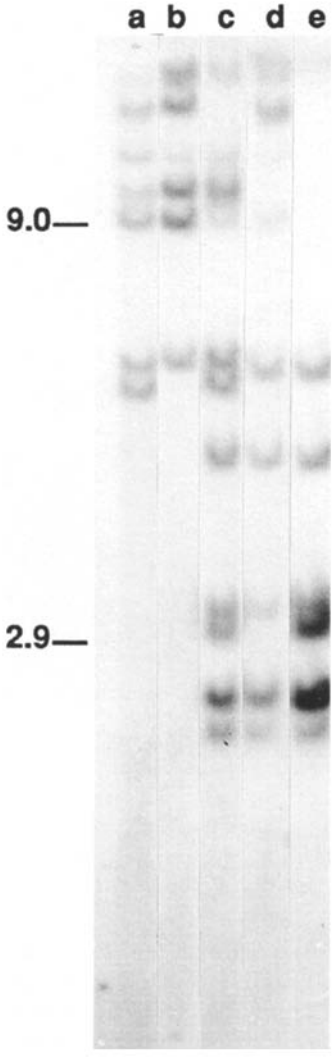


Fig. 3 Hybridisation pattern obtained with probe *BNL 16.06* on total DNA, digested by *Hin*dIII, from the parents and three clones of the progeny of the cross BNS 3066 (*S. officinarum*) × IK 7648 (*E. arundinaceus*). *Lane a*, BNS 3066; *lane b*, one clone probably resulting from self-pollination of BNS 3066; *lanes c and d*, two clones resulting from intergeneric hybridisation; *lane e*, IK 7648. The sizes are given in kilobase pairs

Localisation of the 18*s*-5.8*s*-25*s* ribosomal genes (rDNA)

Chromosome preparations of the *S. officinarum* parent, BNS3066, and an *E. arundinaceus* clone, IK 7624, were hybridised with the 18*s*-5.8*s*-25*s* rDNA probe (pTA 71) and detection was performed with Texas-red and counterstained with DAPI. DAPI bands were observed on one or both ends of most of the *E. arundinaceus* chromosomes; these bands correspond to AT-rich DNA regions and are generally indicative of the presence of highly repetitive families of DNA (Fig. 4a). Six rDNA sites were detected in terminal positions on six *E. arundinaceus* chromosomes (Fig. 4a) and eight rDNA sites were detected in terminal positions on eight *S. officinarum* chromosomes (Fig. 4b). The simplest interpretation was the presence of one locus in six copies for *E. arundinaceus* and eight copies for *S. officinarum* with a basic number of x=10 for both species. However, the observation of apparent size heterogeneity between chromosomes carrying an rDNA site may reflect a more complex situation.

Genomic in situ hybridisation

Genomic in situ hybridisation was carried out on a hybrid chromosome preparation using labelled IK 7648 (*E. arundinaceus*) total DNA as one probe and labelled BNS3066 (*S. officinarum*) total DNA as a second probe. The preparations were counterstained with DAPI (Fig. 5a). Sequences homologous to IK 7648 total DNA fluoresced red resulting from the detection of the IK 7648-labelled DNA with Texas red. Sequences homologous to BNS3066 total DNA fluoresced green resulting from the detection of the BNS3066-labelled DNA with FITC. A print of the preparation performed with a double exposure, one exposure using the fluorescein filter and one using the Texas red filter, is shown in Fig. 5b. In this picture, 39 chromosomes fluoresced green and thus were contributed by BNS3066 (*S. officinarum*) and 25 fluoresced red and thus were contributed by IK 7648 (*E. arundinaceus*). Of the ten presumed complete cells observed on two slides, 39 green chromosomes were constantly observed and 25 to 26 red chromosomes were found.

Some of the chromosomes showed a yellow terminal coloration. This coloration corresponds to superimposed green and red labelling and thus to sequences conserved between the two species. A second hybridisation was performed (data not shown) on the same slide using the 18*s*-5.8*s*-25*s* rRNA genes (pTA 71), labelled directly with a blue fluorochrome (Fluoroblue), as a probe. Blue spots were observed at the same location as the yellow ones detected in the first hybridisation, identifying these spots as rDNA sites. As can be observed in Fig. 5a, DAPI bands were visible at one or both ends of some of the chromosomes; these bands concerned almost exclusively chromosomes originating from *E. arundinaceus*.

Discussion

Intergeneric crosses between sugarcane and *E. arundinaceus* are difficult to perform. They often produce a large number of seedlings resulting from self pollination or pollen contamination. Previous experience has shown that hybrid identification through breeding cycles based on morphological traits can be very difficult.

Diversity within the *Saccharum* complex has been studied using isozyme variation and RFLPs (Glaszmann et al. 1989; Lu et al. 1994; and unpublished data). *Saccharum* species displayed complex banding patterns in relation to their high ploidy levels and their high polymorphism. *E. arundinaceus* clones displayed limited polymorphism.

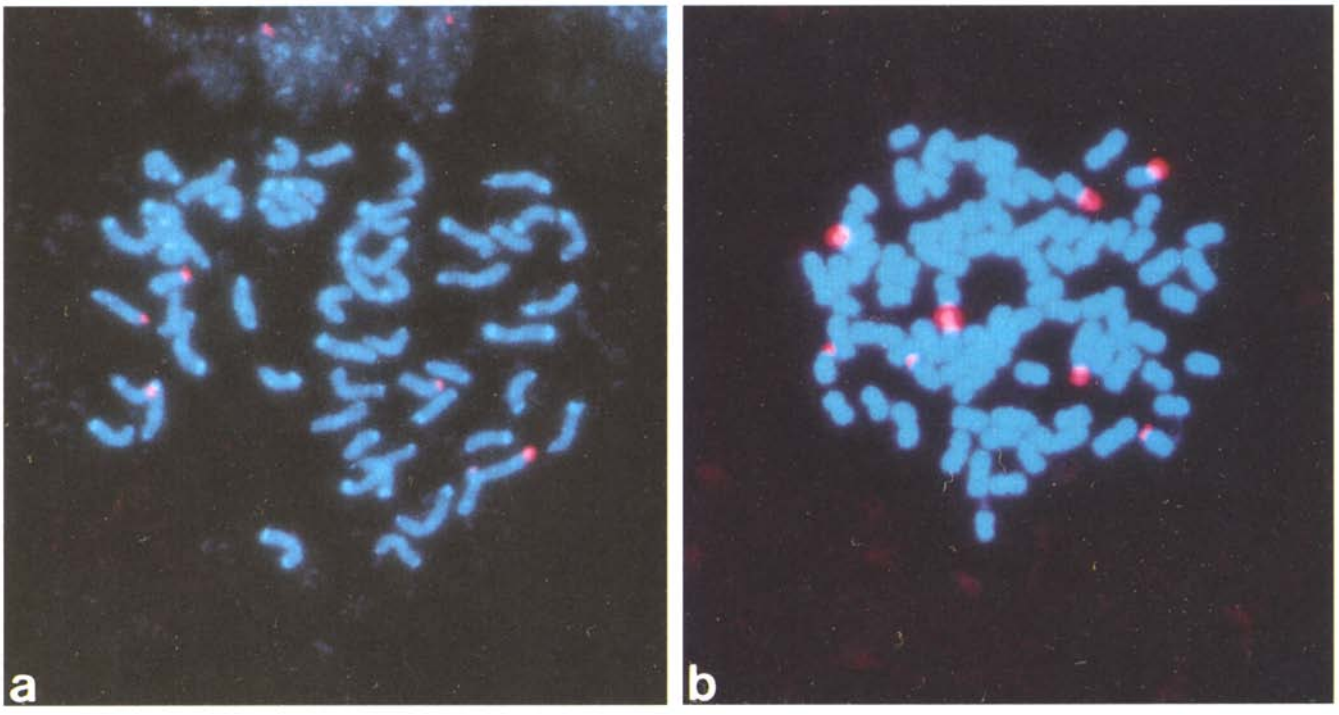
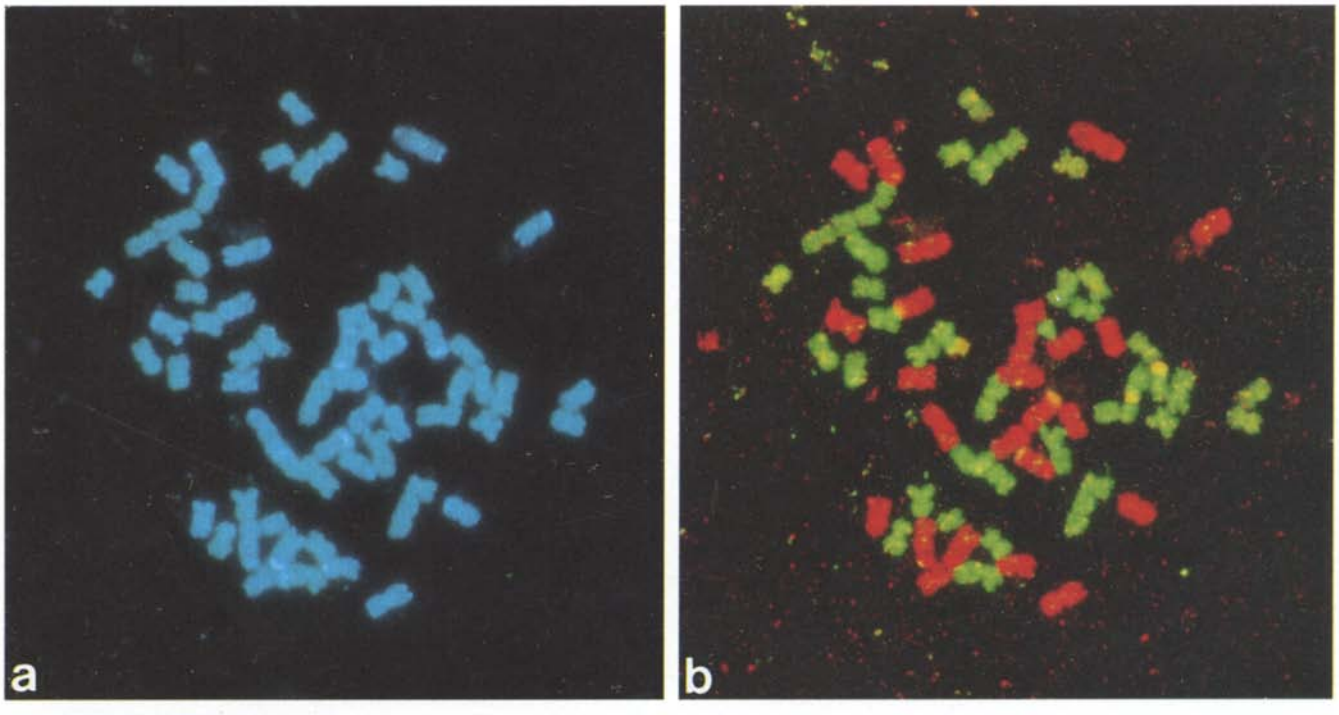


Fig. 4 Chromosome preparation of IK 7624 (*E. arundinaceus*) (**a**) and BNS 3066 (*S. officinarum*) (**b**) after in situ hybridization using the 18*s*-5.8*s*-25*s* rDNA probe (pTA 71) and detection with Texas Red. The chromosomes were counterstained with DAPI

Fig. 5a Chromosome preparation of an intergeneric hybrid between BNS 3066 (*S. officinarum*) and IK 7648 (*E. arundinaceus*) counterstained with DAPI. **b** Chromosome preparation after in situ hybridisation using labelled IK 7648 total DNA as one probe and labelled BNS 3066 total DNA as a second probe. The hybridisation sites of BNS 3066 DNA were detected by yellow-green fluorescence (FITC); the hybridization sites of IK 7648 DNA were detected by red fluorescence (Texas Red)

A strong molecular differentiation was detected between the *Saccharum* species and *E. arundinaceus*, allowing for the identification of *E. arundinaceus*-specific bands. We confirmed that, using these techniques, intergeneric hybrids can be efficiently identified by the presence of *Saccharum*- and *Erianthus*-specific isozyme or DNA markers.

The case of the 5*s* rDNA-derived markers is of particular interest. The 5*s* rRNA repeated genes are highly conserved, arranged in long tandem arrays, and separated by a spacer. The relatively rapid evolution of the spacer re-



gion has allowed this sequence to be widely exploited in phylogenetic studies (Dvorak et al. 1989; McIntyre et al. 1992; Moran et al 1992). The use of PCR to detect spacer size variations in the 5*s* rDNA cluster in *S. officinarum* and *E. arundinaceus* showed very little variation within each species. The sizes of the PCR products obtained in the two species were, however, notably different, about 230 and 370 base pairs, respectively. Intergeneric hybrids could thus be easily identified by the presence of both PCR products.

Isozyme and PCR analyses are simple to perform and can be applied quickly to a large number of samples. They require only a small amount of tissue and thus allow the use of young seedlings. Either method may be selected depending on the equipment and fresh-leaf availability. The capacity to determine true hybrids makes these two diagnostic tools very useful in sugarcane programmes.

The ability to detect numerous specific *E. arundinaceus* RFLP markers in the hybrids will facilitate the search for associations between particular RFLP markers and particular *E. arundinaceus* agronomic traits. The genome composition of modern varieties has already led us to focus attention on the specific contribution of a wild relative successfully used in breeding. Segregation analysis in a selfed progeny from one variety suggested that *S. spontaneum* chromosomes may be weakly affected by interspecific recombination in the different varieties (D'Hont et al. 1994). The same phenomenon is expected in intergeneric hybrids since the two parental species are distantly related (D'Hont et al. 1993; Sobral et al. 1994).

Basic chromosome numbers in the genus *Erianthus* and *Saccharum* have not been definitely established and the presence of several basic chromosome numbers in the *Saccharum* genera has been suggested (Sreenivasan et al. 1987). In the two species involved in the intergeneric cross studied here, the detection by in situ hybridisation of an rDNA site, at a terminal position on six chromosomes for *E. arundinaceus* and eight chromosomes for *S. officinarum* supports an identical basic chromosome number of x=10 for both species. The apparent size heterogeneity between chromosomes carrying an rDNA site requires specific attention in the future.

Classical cytological techniques allowed the quantification of chromosomes in putative intergeneric hybrids. A distinction between the chromosomes of the two genera has now become possible in crosses between *S. officinarum* and *E. arundinaceus* thanks to genomic DNA in situ hybridisation. This technique allows a detailed description of the genomic composition of the hybrids. In the particular hybrid analysed, 64 to 65 chromosomes were detected instead of the 70 expected. Thirty-nine chromosomes originated from *S. officinarum* and 25 to 26 chromosomes originated from *E. arundinaceus*. It thus seems that chromosome elimination has occurred.

Besides chromosome transmission during the production of H_1 intergeneric plants, genomic in situ hybridisation will enable us to precisely follow *E. arundinaceus* chromosome inheritance during successive subsequent backcrosses and to test the occurrence of recombination between the chromosomes of these two genera.

The different applications of molecular markers described here provide new tools which should help considerably in the exploitation of the *Erianthus* genome in sugarcane breeding.

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