Theor Appl Genet (1999) 99:962–968

K. Alix · F. Paulet · J.-C. Glaszmann · A. D'Hont

Inter-*Alu*-like species-specific sequences in the *Saccharum* complex

Received: 7 March 1999 / Accepted: 25 March 1999

Abstract *Alu* sequences constitute the most abundant family of short interspersed nuclear elements, SINEs, in the primate genome. The *Alu*-PCR method, which consists of amplification between *Alu* sequences, is usually applied in human genetics to provide polymorphic markers. Here we report the presence of *Alu*-like sequences in sugarcane and related species by applying the *Alu*-PCR-like method. Amplifications using a PCR primer defined in conserved regions of *Alu* human sequences lead to specific complex multiband profiles in all the *Saccharum* and related genera clones surveyed. The isolation and characterisation of the amplified genus-specific inter-*Alu*-like fragments allowed us to isolate repeated sequences that are specific for different genera of the *Saccharum* complex: MsCIR2 from *Miscanthus*, EaCIR6 and EaCIR7 from *Erianthus*, and SrCIR2 from *Saccharum*. Two PCR diagnostic tests were developed from the inter-*Alu*-like sequences MsCIR2 and EaCIR6, and proved efficient in identifying intergeneric hybrids *Saccharum*×*Miscanthus* or *Saccharum*×*Erianthus*, respectively. The present study illustrates how the *Alu*-PCR-like method could help investigating the origin of amphiploid species and monitoring introgression in plants.

Key words *Alu*-like sequences · Repeated DNA · Species-specific sequences · *Saccharum* complex · Surgarcane

Introduction

Alu sequences represent the most abundant family of short interspersed elements (SINEs) in human and primate genomes (Deininger 1989). Historically, they have been identified as a fraction of renatured repeated DNA which was specifically restricted by the endonuclease *Alu*I (Houck et al. 1979). They were first considered as existing only in primates, and their evolution was thus studied within the primate group; they are supposed to have first appeared 65 million years ago (Deininger and Daniels 1986). The consensus *Alu* sequences are approximately 280-bp long, and are repeated more than 500 000 times in the haploid human genome (Rinehart et al. 1981), covering about 5–10% of this genome (Kass et al. 1996). In human genetics, one important application of the *Alu* sequences is in chromosome banding using in situ hybridisation (Korenberg and Rykowski 1988; Baldini and Ward 1991; Muller et al. 1996). Another major application is the *Alu*-PCR method (Nelson et al. 1989) which involves amplification between *Alu* sequences. This method provides polymorphic markers (Charlieu et al. 1992; for review see Kass et al. 1996) for human genome mapping, population diversity analysis, and filiation studies such as paternity assessment (Novick et al. 1995; Kass et al. 1996).

Alu-like sequences showing partial homology with human *Alu* sequences have been characterised in other mammals (in cattle, Richardson et al. 1986) and applied to chromosome banding (in pig, Yasue et al. 1991; in bovine and ovine, Racjan-Sepavoric and Sabour 1993). *Alu*-related sequences have rarely been reported in plant genomes (Blin et al. 1983), and the *Alu*-PCR method was applied for the first time in plants for banana genome studies (Baurens et al. 1998). The possibility of developing a range of applications in plants led us to explore the presence of *Alu*-PCR-derived markers in sugarcane and to apply them to particular matters of interest in its genetics.

Sugarcane is a member of the *Andropogoneae* tribe of the *Gramineae* and belongs to the genus *Saccharum*. *Saccharum* is associated with four other genera, *Erianthus* (sect. *Ripidium*), *Miscanthus* (sect. *Diantra*), *Sclerostachya* and *Narenga*, to constitute the "*Saccharum* complex", a closely related interbreeding group suggested to be involved in the origin of sugarcane (Daniels and

Communicated by R. Hagemann

K. Alix · F. Paulet · J.-C. Glaszmann · A. D'Hont (✉)
CIRAD, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, avenue Agropolis, BP 5035, F-34032 Montpellier cedex 1, France
Fax: +33-4-67-61-56-05; e-mail: dhont@cirad.fr

Roach 1987). Introgression plays a major role in the improvement of this crop, involving principally the wild species *Saccharum spontaneum*, in order to transfer useful agronomic characters such as profuse tillering, strong ratooning ability, wide adaptation and disease resistance. Recently, new introgression programmes have been initiated involving the two genera *Erianthus* and *Miscanthus*. Molecular markers appeared valuable to identify true hybrids and monitor introgression programmes, because of a lack of diagnostic morphological traits (D'Hont et al. 1995; Alix et al. 1998). Moreover, species-specific markers can help to better understand the complex phylogeny of sugarcane and its relatives.

The present study reveals the existence of *Alu*-like sequences in the *Saccharum* complex, and describes the efficiency of the *Alu*-PCR-like method for genome profiling in *Saccharum* and related genera, and isolating species-specific repeated sequences.

Materials and methods

Plant material and DNA extraction

Thirty three clones, representative of the main genera belonging to the *Saccharum* complex, and nine sugarcane cultivars were analysed. The different species and clones are listed in Table 1. Two intergeneric hybrids, Raiatia (*Saccharum*×*Miscanthus*) and WI 89 704 (*Saccharum*×*Erianthus*), were also surveyed. They were provided by CIRAD-Guadeloupe, the West Indies Central Sugar Cane Breeding Station (WICSCBS) in Barbados, and the Bureau of Sugar Experiment Station (BSES) in Australia. Total DNA was extracted from freeze-dried leaf tissue according to Hoisington (1992).

Alu-PCR-like amplifications

The *Alu* primer AGMI 41 was previously defined by Baurens et al. (1998) in conserved regions of human *Alu* sequences. Amplification reactions were performed in a final volume of 50 µl in the presence of 100 ng of total genomic DNA, 400 nM of AGMI 41 and 200 µM of dNTP (Pharmacia) in a buffer containing 67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20 (v/v), 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerase (EurobioTaq polymerase DNA, Eurobio). The PCR was carried out using a PTC 100 thermocycler (MJ Research). The programme used by Baurens et al. (1998) was applied to generate polymorphic multiband profiles. In order to isolate species-specific sequences, the following programme, based on a 'touch-up' PCR programme, was used: 94°C for 2 min, 11 cycles of [92°C for 2 min, 45°C+(1°C/cycle) for 45 s, 70°C for 30°s], 26 cycles of (94°C for 1 min, 55°C for 45 s, 70°C for 30 s), and a final step at 70°C for 5 min. These conditions generated intense amplified bands which simplified cloning procedures. All amplification products were analysed on 2.5% agarose gels (w/v) in TBE buffer (89 mM Tris-Borate, 2 mM EDTA, pH 8.0) run at 6.25 V/cm.

Cloning of species-specific sequences

In order to clone several species-specific bands of the inter-*Alu* PCR-like profiles, crude PCR products were purified using the Qiaquick procedure with columns (Qiaquick PCR purification kit, Qiagen), then ligated into pGEM-T easy vector (according to manufacturers instructions, Promega), and transformed in *Escherichia coli* strain DH5α. The recombinants were screened according to the size of their insert, and verified by PCR amplification using the universal pUC/M13 forward and reverse primers. The clones showing the appropriate insert size were used as probes in Southern-blot hybridisation experiments to check their putative species specificity.

Southern-blot hybridisation experiments

Ten micrograms of total DNA were restricted with an endonuclease, either *Alu*I, *Bam*HI, *Eco*RV, *Eco*RI, *Hae*III or *Hin*dIII (Gibco BRL). The restriction fragments were separated by electrophoresis on 2.5% agarose gels (w/v) in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) at 5.5 V/cm for 5 h, and then transferred to nylon membranes (Hybond N+, Amersham). Labelling of probes and Southern-blot hybridisations were performed as described by Alix et al. (1998).

Specific inter-*Alu*-like sequence-tagged PCR

For two genus-specific inter-*Alu*-like sequences isolated in this study, specific PCR primer sets were defined using Oligo™ software: a *Miscanthus*-specific PCR primer pair AGRP 78=5´-TGA-CTC-CTG-CTG-TGA-CTC-CCC-CTA-A-3´/AGRP 79=5´-GGA-TCC-AAA-AGG-CAA-AGC-GAC-AAA-G-3´, and an *Erianthus*-specific PCR primer pair AGRP 80=5´-GGG-TTG-TCY-TTG-CCA-TCA-TA-3´/AGRP 81=5´-GAG-YAG-CRC-AGA-GGT-TAC-GA-3´. The different genus-specific sequence-tagged PCR experiments were carried out using the same reaction mix as described above but with 15 ng of total DNA as a template, using the two following programmes: 94°C for 4 min, 35 cycles of (94°C for 30 s, 67°C/60°C for 30 s, 72°C for 45 s), and a final cycle of 72°C for 4 min, for the *Miscanthus/Erianthus*-specific PCR test, respectively. The amplification products were analysed on 1.5% agarose gels (w/v) in TBE at 10 V/cm.

Results

Germplasm profiling survey

Alu-PCR-like amplifications were performed on representative clones of the *Saccharum* complex: multiband profiles were obtained, with bands ranging from 250 bp to 3 kb (Fig. 1). The profiles were highly polymorphic, and each clone could be identified by one specific inter-*Alu* PCR-like profile. Fewer bands were amplified in the *Miscanthus* (14 discrete bands) and *Erianthus* (12–20 discrete bands) clones, leading to more readable patterns than those obtained for the *Saccharum* clones (about 20–25 discrete bands). Within one given genus, only few bands were common to all the clones surveyed: this was the case for the 500-bp amplified band in *Saccharum* and the 250-, 320-, 395- and 700-bp bands in *Erianthus*. Within *Saccharum*, the *S. officinarum* clones showed less diverse inter-*Alu*-like profiles with several bands in common (in particular the 320-bp one). By contrast, the *S. spontaneum* clones showed a great level of variability in their patterns.

Four series of *Alu*-PCR-like amplifications were carried out under the same experimental conditions on a sample of nine sugarcane cultivars. Two to four different individuals of each variety were surveyed. The inter-*Alu* PCR-like profiles obtained were consistent between the different experiments (Fig. 2). Approximately 21 discrete bands were identified, 18 of them being polymorphic between cultivars. All the sugarcane varieties could be identified by a specific inter-*Alu* PCR-like profile.

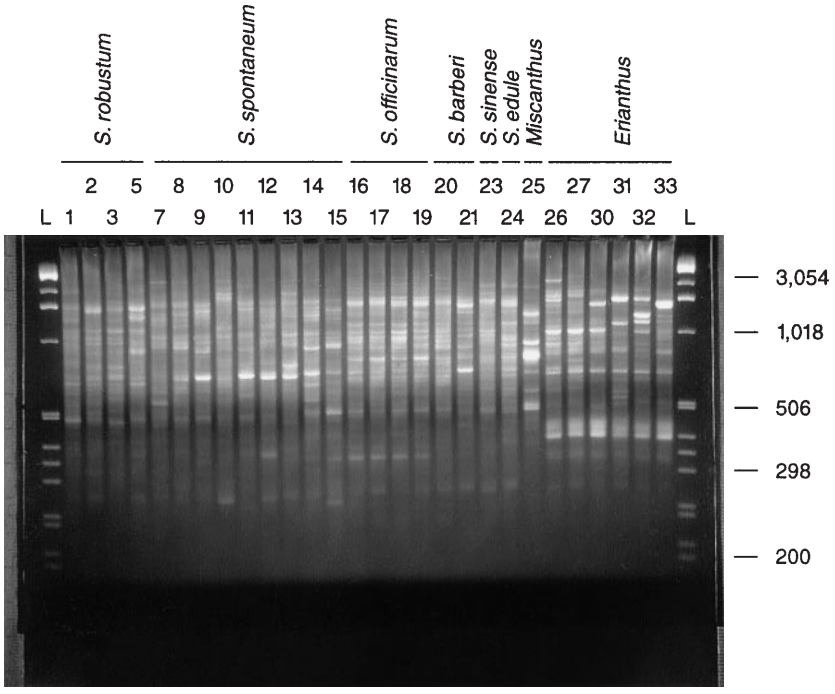

Fig. 1 Inter *Alu*-like PCR profiles of 28 clones of the *Saccharum* complex. *Lane numbers* correspond to the clones as listed in Table 1. *L*: 1-kb ladder, sizes (bp) are given on the right of the figure

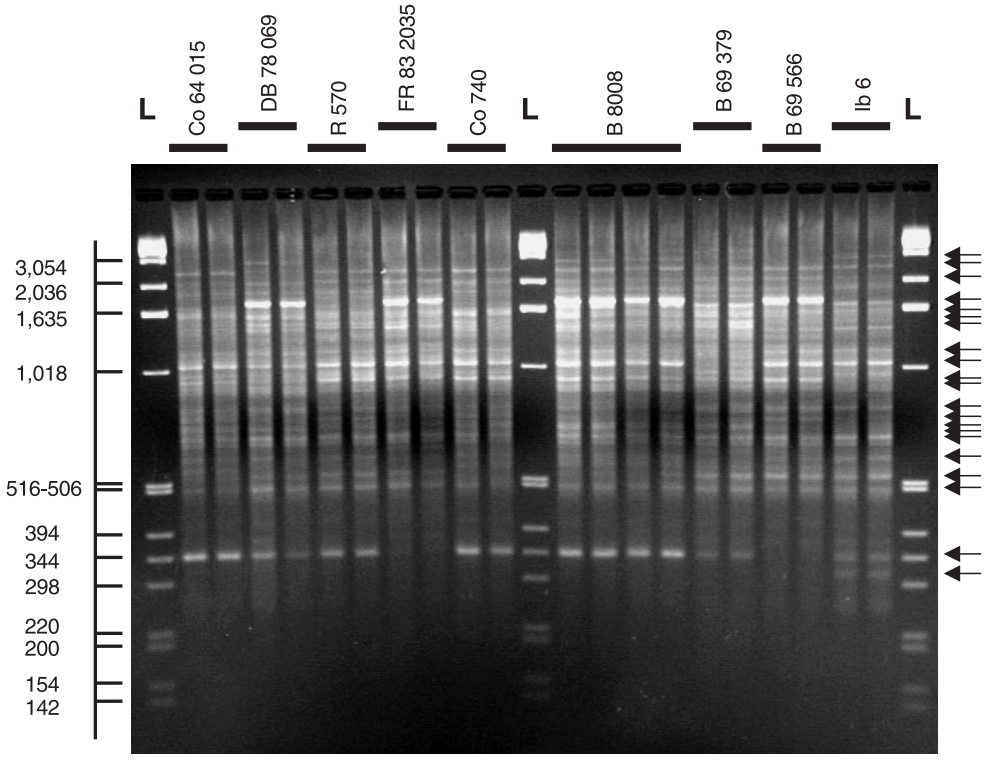

Fig. 2 Inter-*Alu*-like PCR profiles of nine sugarcane cultivars. *L*: 1-kb ladder, sizes (bp) are given on the left of the figure

Isolation of genus-specific sequences

Genus-specific amplified fragments were cloned and sequenced. The insert sizes are 381 bp for MsCIR2 isolated from *M. sinensis* (NG 7722), 395 bp for EaCIR6 isolated from *E. arundinaceus* (IK 7648), 253 bp for EaCIR7 isolated from *E. elephantinus* (SES 305), and 496 bp for SrCIR2 isolated from *S. robustum* (IM 76234) (EMBL accession numbers: Y17576, Y17578, Y17579, and Y17577 respectively). These sequences were compared to entries in the EMBL data bank using the BLAST algorithm (Altschul et al. 1990) and they showed no significant homology with other registered plant or animal nucleotide sequences.

Table 1 Clones of the *Saccharum* complex surveyed in this study

No.	Clone	Chromosome number[a]	Origin
Saccharum robustum			Indonesia New Guinea
1	IM 76234	60	
2	NG 77230	80	
3	MOL 4503	60	
4	NG 28251	?	
5	NG 7754	96	
Saccharum spontaneum			
6	Coimbatore	64	India
7	SES 14	64	India
8	SES 106B	48	India
9	NG 51-2	80	New Guinea
10	Glagah 1286	112	Indonesia
11	Tainan 96	96	Taiwan
12	IK 7667	?	Indonesia
13	IK 7686	?	Indonesia
14	MOL 5801	80	Molokaï
15	MOL 5904	80	Molokaï
Saccharum officinarum		80	Indonesia New Guinea
16	Lousier		
17	Crystallina		
18	Black Cheribon		
19	BNS 3066		
Saccharum barberi			
20	Chunnee	90–91	India
21	Pathri	82	India
22	Paunra	82	India
Saccharum sinense			
23	Oshima	112	Japan
Saccharum edule			
24	NG 28201	?	New Guinea
Miscanthus sinensis			
25	NG 7722	38[b]	New Guinea
Erianthus arundinaceus			
26	IK 7625	60[b]	Indonesia
27	IK 7648	60[b]	Indonesia
28	IS 76176	60[b]	Indonesia
29	NG 28-7	60[b]	New Guinea
30	SES 300	40[b]	India
Erianthus elephantinus			
31	SES 305	20[b]	India
Erianthus ravennae			
32	US 67-8-1	20[b]	–
33	*Erianthus sarpet*	30[b]	–
Sugarcane cultivars			
Co 64 015		–	
DB 78 069		–	
R 570		–	
FR 83 2035		–	
Co 740		–	
B 80 08		–	
B 69 379		–	
B 69 566		–	
Ib 6		–	

[a] Lu et al. 1994; Mohan and Sreenivasan 1983; Panje and Babu 1960
[b] Personal observations

The distribution of these sequences in the *Saccharum* complex was surveyed by Southern-blot hybridisation experiments on representatives of this complex. MsCIR2 and EaCIR6–7 probes produced hybridisation signals exclusively on *Miscanthus* or *Erianthus* DNA, respectively, after only 3 h of exposure time (Fig. 3A and B). These sequences were therefore considered as *Miscanthus*-specific and *Erianthus*-specific repeated sequences. After 3-days exposure, all the *Saccharum* clones displayed multiband profiles with MsCIR2 while they did not produce any hybridisation signal with the two *Erianthus* probes (data not shown). After 14-days exposure, the *Erianthus* probe EaCIR6 produced weak but detectable signals on all the *Saccharum* clones and no signal on the *Miscanthus* one, while EaCIR7 hybridised exclusively to the *Erianthus* clones; the *Miscanthus* probe did not hybridise to the *Erianthus* clones at all. The *Saccharum* probe, SrCIR2, gave strong hybridisation signals with all the *Saccharum* clones after 3-days exposure, and the profiles were highly polymorphic (Fig. 3C). This sequence was thus considered as a *Saccharum*-specific moderately repeated sequence. By comparison, single-copy homologous probes generally give good hybridisation signals after 7-days exposure. The *Saccharum* probe also produced very faint but detectable signals with the *Miscanthus* and *Erianthus* clones surveyed (Fig. 3C).

The genomic distribution of the sequences MsCIR2, EaCIR6 and SrCIR2 was investigated by Southern hybridisation. MsCIR2 was used as a probe on genomic DNA from *M. sinensis* restricted with *Eco*RI, *Eco*RV and *Hin*dIII. Characteristic patterns of dispersed repeats (Kiefer-Meyer et al. 1995) were observed, consisting of an irregular pattern superimposed on a background smear (Fig. 3A). Similarly, irregular ladder patterns, with bands ranging from approximately 200–700 bp superimposed on a background smear, were obtained after hybridisation experiments using EaCIR6 as a probe on *Erianthus* genomic DNA restricted by *Hae*III. This indicates an organisation in small clusters (Fig. 3B), probably dispersed throughout the genome. SrCIR2, used as a probe on genomic DNA from several *Saccharum* clones restricted with *Eco*RI, *Eco*RV and *Hin*dIII, produced patterns similar to the ones obtained with single-copy sequences (Fig. 3C), suggesting that this moderately repeated sequence is not repeated in tandem arrays and is present at only a few loci in the genome.

Specific inter-*Alu*-like sequence-tagged PCR

In order to obtain *Miscanthus*- and *Erianthus*-specific PCR markers, two PCR primer pairs, AGRP 78/79 and AGRP 80/81, were defined in MsCIR2 and EaCIR6 respectively. The different primer sets were used to amplify total genomic DNAs of various clones: NG 28251 (*S. robustum*), Black Cheribon (*S. officinarum*), NG 7722 (*M. sinensis*), Raiatia (a putative *Saccharum*×*Miscanthus* hybrid), IK 7648 (*E. arundinaceus*), and a *S. officinarum*×*E. arundinaceus* hybrid, WI 89 704.

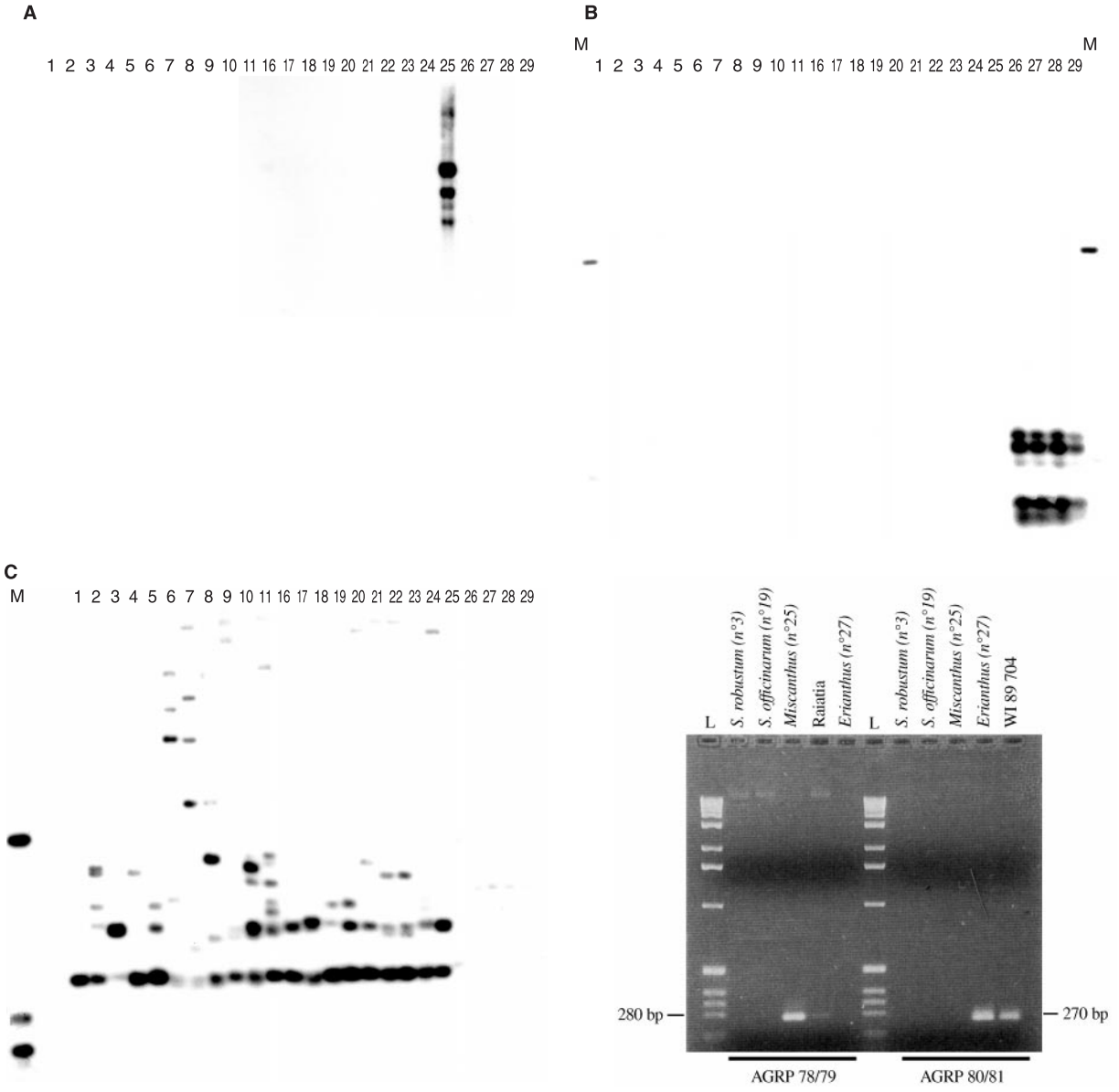

Fig. 3A–C Hybridisation patterns obtained on total DNA of 25 clones representative of the *Saccharum* complex **A** restricted by *Eco*RV and hybridised with MsCIR2 (3-h exposure); **B** restricted by *Hae*III and hybridised with EaCR6 (3-h exposure); **C** restricted by *Eco*RI and hybridised with SrCIR2 (3-days exposure). *Lane numbers* correspond to the clones as listed in Table 1. *M*: size marker

Fig. 4 Amplified products obtained on total DNA of different clones, using the *Miscanthus* (AGRP 78/79) and *Erianthus* (AGRP 80/81) specific PCR primer pairs. *L*: 1-kb ladder

The *Miscanthus* primer pair produced a major band of the expected size (about 280 bp according to the positions of the primers defined in MsCIR2) in the *Miscanthus* clone NG 7722 and also in the clone Raiatia (Fig. 4). Hybridisation of the probe MsCIR2 on genomic DNA of Raiatia, also produced a very strong signal after a 3-h exposure (data not shown).

The *Erianthus* primer pair produced a band of approximately 270 bp in the *Erianthus* clone and the intergeneric hybrid WI 89 704 (Fig. 4).

Discussion

Assessment of the existence of *Alu*-like sequences in plants

In the different species of the *Saccharum* complex analysed, the use of one single PCR primer, defined in terms of conserved human *Alu* sequences (AGMI 41), generates a typical multiband profile, similar to the one generated with human DNA (Baurens et al. 1998). Similar results were obtained in a range of plant species: oil palm, coconut tree, cocoa tree, rubber tree, citrus, sorghum, rice, *Arabidopsis* (data not shown) and banana (Baurens et al. 1998). These results suggest that plant genomes harbour inverted repeated sequences, dispersed through-

out the genome, which show conserved regions homologous to human *Alu* sequences: these sequences can be assessed as *Alu*-like sequences.

Cloning *Alu*-like elements in plants – through PCR walking (Devic et al. 1997) or Vectorette PCR (Kilian and Kleinhofs 1992) – would be interesting to estimate their evolution among the plant kingdom and to compare them to primate *Alu* sequences. This would give an insight into the evolution of *Alu* sequences, and help to understand the functions, still contentious, of such sequences in the eukaryotic genome (for a review see Mighell et al. 1997).

Application of the *Alu*-PCR-like method for sugarcane profiling

In a sample of clones representative of the *Saccharum* complex, *Alu*-PCR-like amplifications generated highly polymorphic discriminating profiles. The polymorphism observed is in agreement with previous diversity studies based on RFLP analyses where *S. spontaneum* appeared the most variable species and *S. officinarum* the least variable within the *Saccharum* genus (Lu et al. 1994; Burnquist et al. 1995). Furthermore, *Erianthus* displayed the lowest level of variability among the *Saccharum* complex genera (Besse et al. 1997). However, *Erianthus* clones can be differentiated from one another by the *Alu*-PCR-like method (examining in particular the amplified fragments greater than 1 kb). Baurens et al. (1998) showed that in banana, genetic diversity revealed with the *Alu*-PCR-like method led to results in accordance with RFLP analysis using single-copy probes. The application of this PCR-based method on various sugarcane varieties is also conclusive: all the cultivars surveyed can be differentiated from one another.

The *Alu*-PCR-like method for isolating species-specific sequences

The *Alu*-PCR-like method allowed us to isolate *Miscanthus*- and *Erianthus*-specific highly repeated sequences and a *Saccharum*-specific moderately repeated sequence. A survey of the distribution of these sequences in appropriate material could help to determine phylogenetic relationships among the *Saccharum* complex. We can already note that the distribution of the sequences MsCIR2 and EaCIR6–7 revealed by Southern hybridisation places *Miscanthus* more closely related to the *Saccharum* genus than *Erianthus* is, as previously suggested by Sobral et al. (1994) and Alix et al. (1998). Indeed, MsCIR2 produced signals after 3-days exposure on the genomic DNA of several *Saccharum* clones, while EaCIR6 or EaCIR7 produced very faint signals, or no signal, respectively, after 2-weeks exposure.

The genus-specific inter-*Alu*-like sequence primers designed in the present study could be helpful in resolving the identity of unknown clones and also the pedigree of putative *Saccharum* intergeneric hybrids involving *Miscanthus* or *Erianthus*. They would be useful as a complement to the specific PCR markers already available: the 5S ribosomal RNA gene marker (D'Hont et al. 1995), and specific satellite DNAs (Alix et al. 1998), which are present at one locus and in subtelomeric positions of most chromosomes, respectively. The RFLP profiles obtained with the different *Miscanthus*- and *Erianthus*-specific inter-*Alu*-like sequences suggest a dispersed distribution; molecular markers with such a genomic distribution are particularly appropriate to detect small introgressed fragments in later generations of introgression. The specific genomic distribution of these sequences should be assessed by in situ hybridisation. The experiments we conducted were not conclusive, probably due to insufficient sensitivity of our protocol (Alix et al. 1998) which requires further refinement.

The present work reveals the existence of *Alu*-like sequences in sugarcane, which are of particular interest for their ability to provide species-specific sequences. Specific molecular markers represent appropriate tools to investigate the origin of amphiploid species and to monitor introgression from wild species to cultivated clones. The *Alu*-PCR-like method should therefore be of particular value for those crops where several species or genomes are involved.

Acknowledgements We particularly thank F.-C. Baurens and P.J.L. Lagoda for providing the AGMI 41 primer and for valuable discussions, and G. Piperidis for correcting the English of the manuscript. This research was supported by a grant from the French 'Ministère de l'Enseignement Supérieur et de la Recherche'.

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