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Microsatellites and RFLP probes from maize are efficient sources of molecular markers for the biomass energy crop *Miscanthus*

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Abstract A survey of Gramineae markers was carried out with the aim of developing cost-effective methods for the molecular analysis of *Miscanthus* species. Ten out of twenty Gramineae RFLP probes from "anchor" sets hybridized well to *Miscanthus* DNA while all 15 maize probes tested cross-hybridized successfully, showing similar patterns in both species. Cross-taxa amplification of maize microsatellite primers was then tested. This showed that 57 out of 76 (75%) give highly reproducible amplification with *Miscanthus* DNA. Amplification products differed in size from those in maize but there was no bias toward higher or lower molecular weights. Microsatellite polymorphism produced by 17 primer pairs was studied in detail in a panel of 11 *Miscanthus* clones belonging to the species *Miscanthus sinensis*, *Miscanthus sacchariflorus*, *Miscanthus ×giganteus* and *Miscanthus condensatus*. Intra- and inter-specific length polymorphisms were frequent between the tested *Miscanthus* clones with length polymorphisms being found for all primer pairs, detecting 3–22 alleles. Polymorphism information content (PIC) values for microsatellites ranged from 0.48 to 0.94 with an average of 0.83. Species-specific amplicons were produced by two microsatellites. Genetic similarity coefficients of the *Miscanthus* clones ranged from 0.35 to 0.92, with an average of 0.57. Five polymorphisms were studied in a segregating population, where they showed Mendelian inheritance. In addition, two microsatellite markers mapping 1.3-cM apart on maize chromosome 7 were linked in *Miscanthus* at an estimated distance of 8 cM, suggesting collinearity. The high transferability of microsatellite markers from maize will enhance the power and resolution of genome analysis in *Miscanthus*.

Keywords *Miscanthus* · Microsatellite · RFLP · Maize · Biomass crop · Diversity

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Introduction

Several plant species have been investigated for their potential as biomass crops. As biomass-conversion techniques have advanced, interest has focussed on the cultivation of *Miscanthus* species, which have a potential for high biomass yields under European conditions (25 to 35 t ha^{-1} $year^{-1}$ once the crop has been established: Schwarz 1993; van der Werf et al. 1993; Holtz 1996; Himken 1997; Venendaal et al. 1997). The genus *Miscanthus* Anderss. (Andropogoninae: Poaceae) contains C_4 perennial grasses of tropical/subtropical origin with a wide area of distribution, which is a result of their high adaptability to different environmental conditions. *Miscanthus ×giganteus* was first introduced into Europe as an ornamental and has subsequently attracted interest for biomass production. This accession is sterile but is easily propagated vegetatively. Therefore, the available genetic diversity is limited to somaclonal variation. According to Greef and Deuter (1993) and Linde-Laursen (1993), *M. ×giganteus* is a triploid (2n=3*x*=57) originating from the cross between diploid *Miscanthus sinensis* (2n=2*x*=38) and allotetraploid *Miscanthus sacchariflorus* (2n=4*x*=76). The two putative parents have one genome in common and therefore *M. ×giganteus* has two genomes with high homology to *M. sinensis* and a third genome with low homology. *M. ×giganteus* is not appropriate material for genetic analysis because of its genomic constitution and sterility. *M. sinensis* is preferred because it is diploid and the donor of two of the genomes of *M. ×giganteus*.

There is a need to study the factors affecting the combustion quality in *Miscanthus* in greater depth. Targets for genetic improvement are the reduction of emission pollutants and reduction of the fouling, sludging and corrosion that can occur in biomass power plants. Avoidance of these problems requires the minimisation of inorganic elements such as potassium (K), chlorine (Cl), silica (Si), phosphorous (P) and calcium (Ca), while maintaining the present low contents of sodium (Na) and sulphur (S). The complexity of these quality traits and the high costs of chemical analysis require the generation of molecular markers suitable for marker-assisted selection and breeding.

To-date, molecular studies of *Miscanthus* have been limited to isozyme (Von Wuhlisch et al. 1994) and AFLP analyses (Greef et al. 1997). The latter enable genetic maps to be constructed rapidly, but such maps cannot be aligned with those of other species. Therefore, it is useful to identify anchor markers that allow linkage groups to be defined with respect to other members of the Gramineae. RFLP markers are well suited to this but it would be valuable if simple sequence repeat markers (SSRs or microsatellites) could be used as these are PCR-based, require only small amounts of DNA, generally show codominant inheritance and are amenable to high-throughput analysis.

Microsatellites offering good coverage of the genome are available in only a few plant species. Due to their high development costs it is unlikely that they will be developed in *Miscanthus*, at least in the near future. Microsatellites can be identified using DNA database searches (Akkaya et al. 1992; Devos et al. 1995) but this is not feasible for *Miscanthus* where relatively few sequences are currently available. An alternative is to use microsatellites developed in related species which have been studied more extensively, utilising conserved flanking regions around the microsatellite sequences (see Peakall et al. 1998 for a review).

The main objective of the present work was the development of cost-effective molecular markers in *Miscanthus* spp. for mapping and for variability studies. Cross-species hybridization and microsatellite amplification approaches were explored and the latter optimized for the generation of suitable markers for combustion quality breeding of *Miscanthus*.

Materials and methods

Plant material

M. sinensis lines F1.1 and F1.7 were used for the initial RFLP and microsatellite transferability study. These are two different hybrids between *M. sinensis* lines MS-90–2 and MS-88–110 which were selected as parents because of their contrasting combustion quality traits. Marker segregation was studied in 45 individuals from the F1.1×F1.7 progeny. To test the usefulness of microsatellite markers in diversity studies, germplasm belonging to *M. sacchariflorus*, *M. sinensis*, *M. ×giganteus* and *Miscanthus condensatus* was screened (Table 1). The maize line Tx303 was used as a control.

DNA extraction

DNA was extracted from young frozen leaf or stem tissue using the CTAB method of Murray and Thompson (1980) with some modifications. Oxidation adversely affected DNA quality in initial experiments and, to overcome this, plastic tubes for tissue collection were frozen before adding 0.7–1 g of cut leaves or stems. Tissue was frozen quickly, ground in liquid nitrogen and, before the tissue thawed, 3 ml of CTAB buffer containing 0.5% sodium bisulfite and 25 mM of dithiothreitol (DTT) were added. The solution was incubated for 40 min at 65°C with occasional mixing. After the tissue was extracted once with an equal volume of 24:1 chloroform:octanol, DNA was precipitated with isopropanol, spooled out using a glass hook, washed in 70% ethanol and air-dried. The DNA was dissolved in 500 µl of TE buffer. RNase was added (0.03 mg) and the DNA was dissolved overnight at 4°C. The DNA stock solutions were diluted 1:20 in water for microsatellite amplification. For RFLP analysis, the DNA solution was extracted once with phenol:chloroform:isoamylalcohol (24:24:1) and alcohol-precipitated.

RFLP analysis

Genomic DNA from the parents and the mapping population was digested with *Eco*RI, *Eco*RV, *Hind*III, *Bam*HI or *Dra*I (Hoffmann La Roche). Electrophoresis, Southern blotting and hybridization after labelling of probes with [^{32}P] were carried out using standard procedures. Estimation of linkage used Joinmap version 2.0 (Stam and Van Ooijen 1995) with the Kosambi mapping function.

PCR amplification and microsatellite analysis

PCR primer sequences were obtained from the http://burr.bio.bnl.gov/acemaz.html web site. Amplification reactions were carried out in 10-µl volumes containing 25 ng of DNA, 0.5 units of *Taq* DNA Polymerase (Hoffmann La Roche), 200 µM of each

Table 1 *Miscanthus* genotypes used for diversity assessment

Acc. no.	Species/cultivar	Code	Chrom no. (2n)	Ploidy	Source
3	M. *sacchariflorus*	93M0005049	76	4*x*	Dr. M. Deuter
1	*M. sacchariflorus*	93M0005053	76	4*x*	Dr. M. Deuter
2	*M. sacchariflorus*	93M0185002	76	4*x*	Dr. M. Deuter
6	*M. sacchariflorus*	92M0185011	76	4*x*	Dr. M. Deuter
4	*M. sacchariflorus*	92M0185042	76	4*x*	Dr. M. Deuter
5	*M. sacchariflorus*	92M0185010	76	4*x*	Dr. M. Deuter
7	*M. sinensis* var 'Große Fontane'	91M0015	38	2*x*	Dr. M. Deuter
8	*M. sinensis* var 'Zebrinus'		38	2*x*	Kew Gardens
10	*M. sinensis* F1.7		38	2*x*	
9	*M. ×giganteus*	INSAC151	57	3*x*	Kew Gardens
18	*M. condensatus*		57	3*x*	Kew Gardens

dNTP (Hoffmann La Roche), 0.2 µM of each primer, 2.5 mM of $MgCl_2$, 50 mM of KCl and 10 mM of Tris-HCl, pH 8.3. Cycling conditions were as follows: an initial denaturation step of 4 min at 94°C for 10 min was followed by 30 amplification cycles (30-sec denaturation at 95°C, 1-min annealing at 55°C, 1-min extension at 72°C) and a final extension of 5 min at 72°C. Then, 2.5-µl aliquots of each PCR reaction were run on sequencing gels (6% polyacrylamide, 8 M urea) under standard conditions, and the products were visualized by silver staining using the method described by Bassam et al. (1991) with minor modifications.

Calculation of genetic parameters and statistical analyses

The microsatellite profiles generated by the amplification of the *Miscanthus* material described in Table 1, using the primer pairs listed in Table 3, were scored visually. The presence or absence of a band in a genotype was coded as 1 or 0, respectively. Estimates of genetic similarity were calculated for all accessions according to Nei and Li (1979) as:

$$F = \left(\frac{2n_{xy}}{(n_x + n_y)} \right),$$

where F is the ratio of shared bands between *x* and *y*, $2n_{xy}$ is the number of shared bands, and n_x and n_y are the number of bands observed in individual *x* and in individual *y*, respectively. The accessions were grouped by cluster analysis using the unweighted pairgroup (UPGMA) method. The phenogram was produced using the NTSYS-PC package for numerical taxonomy and multivariate analysis systems (Rohlf 1989). The polymorphism information content (PIC) was estimated according to Röder et al. (1995) as $1\text{-}\Sigma[p_i^2]$, where p_i is the frequency of the *i*th allele.

Results

RFLP transferability

Twenty four probes including 20 from "anchor" sets used for the comparative mapping of grass genera (Van Deynze et al. 1998) were tested for cross-hybridization with *M. sinensis* lines F1.1 and F1.7 (see Fig. 1 for an example). Twelve, including ten from the anchor set, hybridized successfully and generated polymorphisms while the remaining 12 did not hybridize. Additionally, all 15 maize probes tested gave good hybridization signals and polymorphism (Table 2).

Microsatellite transferability

M. sinensis DNA from accessions F1.1 and F1.7 was tested with 76 maize microsatellite primer pairs. Fifty seven (75%) of the primers gave reproducible amplification products from *Miscanthus* DNA with patterns similar to maize and within the same size range. Nineteen of the fifty seven (33%) were polymorphic. Five polymor-

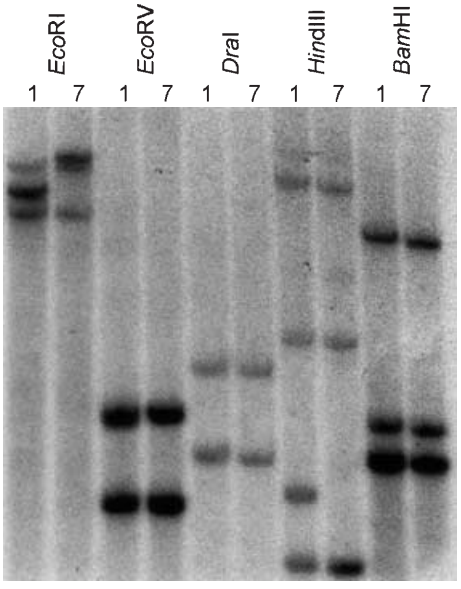

Fig. 1 RFLP hybridization pattern from *M. sinensis* lines F1.1 and F1.7 using anchor probe BCD450

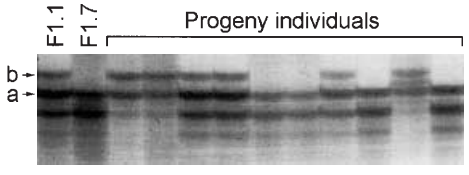

Fig. 2 Polymorphism for microsatellite bgln339 between *M. sinensis* F1.1 and F1.7 and segregation (ab×aa type) in ten progeny

Table 2 Hybridization of RFLP probes to *Miscanthus*

Hybridization to *Miscanthus*	Probes	Type
Yes	BCD98, BCD135, BCD450	Barley cDNA[a]
	CDO204, CDO393, CDO497, CDO1387	Oat cDNA[a]
	PSR571	Wheat *Pst*I genomic clone[b]
	R411	Rice cDNA[c]
	RZ69, RZ543, RZ508	Rice cDNA[a]
	CSU3, CSU39, CSU48, CSU81, CSU129, CSU207, CSU242, CSU292, CSU308, CSU481, CSU829, CSU974, CSU1138, tub4, appr109	Maize cDNA[d]
No	BCD348, BCD880, BCD926, BCD1421	Barley cDNA[a]
	CDO36, CDO344, CDO347, CDO407, CDO460, CDO795	Oat cDNA[a]
	PSB44	Barley *Pst*I genomic clone[b]
	C112	Rice cDNA[c]

[a] Cornell University, USA, anchor set
[b] John Innes Centre, UK
[c] Rice Genome Program, Japan
[d] Genome Systems Inc., USA

Table 3 Microsatellite characterization in *Miscanthus* germplasm and polymorphism information content (PIC) values

Microsatellite	Product size range (maize)	Product size range (*Miscanthus*)	No. of putative alleles	PIC
Bgln176	123–148	126–158	12	0.868
Bgln389	80–105	82–103	7	0.773
Bgln420	83–87	200–220	15	0.928
Bgln128	153–172	150–176	6	0.793
Bgln107	96–103	105–220	20	0.927
Bgln439	190–222	180–224	8	0.808
MAG.T02B10	105–127[a]	105–120	3	0.537
Bgln339	120–135	110–160	15	0.911
MAC.E01C01	71–109[a]	73–90	3	0.477
Bgln240	130–144	117–128	10	0.840
Bgln434	82–86	75–110	22	0.939
Bgln182	90–97	127–135	8	0.873
MAC.E01F06	112–146[a]	120–137	11	0.837
Bgln155	103–220	95–130	22	0.944
MAG.T02B08	102–116[a]	95–112	13	0.862
Bgln603	85–220	120–210	16	0.918
Bgln653	130–157	124–155	13	0.878
Mean			12	0.830

[a] Taramino and Tingey (1996). The other values are from this study

Table 4 Genetic similarity coefficients between *Miscanthus* clones and maize (*Z. mays*)

Species	3	1	2	6	4	5	7	8	10	9	18
M. sacchariflorus 93M0005049											
M. sacchariflorus 93M0005053	0.92										
M. sacchariflorus 93M0185002	0.81	0.76									
M. sacchariflorus 92M0185011	0.85	0.82	0.87								
M. sacchariflorus 92M0185042	0.78	0.82	0.70	0.74							
M. sacchariflorus 92M0185010	0.84	0.83	0.84	0.92	0.73						
M. sinensis var 'Große Fontane'	0.85	0.49	0.37	0.41	0.45	0.43					
M. sinensis var 'Zebrinus'	0.42	0.45	0.36	0.38	0.43	0.40	0.85				
M. sinensis F1.7	0.39	0.39	0.35	0.36	0.39	0.37	0.69	0.65			
M. ×giganteus INSAC151	0.46	0.48	0.39	0.42	0.46	0.46	0.68	0.61	0.60		
M. condensatus	0.38	0.41	0.35	0.35	0.40	0.36	0.60	0.58	0.59	0.62	
Zea mays Tx303	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.01	0.04	0.04	0.03

phisms were studied in the F1.1×F1.7 population, where they showed Mendelian inheritance. Amplification obtained with primer pair bgln339 is shown in Fig. 2. Microsatellite markers bgln339 and bgln434, which mapped 1.3-cM apart on maize chromosome 7, were linked in *Miscanthus* at an estimated distance of 8 cM, suggesting collinearity of these regions.

There is evidence that maize is an ancient tetraploid and its ten chromosomes can be divided into two subsets by reference to rice linkage segments (Moore et al. 1995). Transferable microsatellite markers were derived from all ten maize chromosomes and there was no preference for a subset ($\chi^2_{1\ d.f.}$ 0.04 ns assuming that 75% of all mapped markers were transferable). Thus, transferability showed no obvious chromosome bias, suggesting that maize microsatellites will provide good coverage of the *Miscanthus* genome.

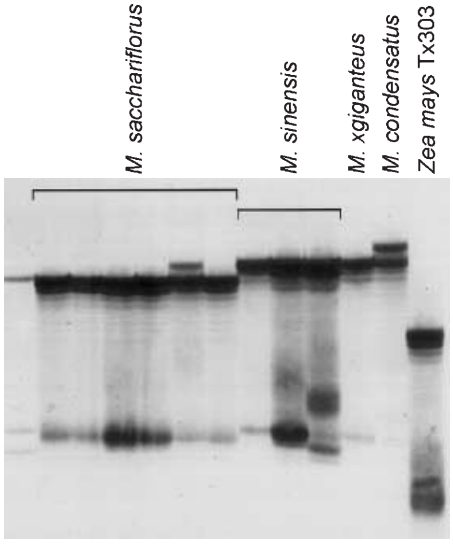

Fig. 3 Amplification products from microsatellite bgln176 in 11 *Miscanthus* genotypes (Table 1) and a maize control (Tx303)

Cluster analysis

Microsatellite polymorphisms produced by 17 primer pairs were studied in detail in a panel of 11 *Miscanthus* clones belonging to the species *M. sinensis*, *M. sac-*

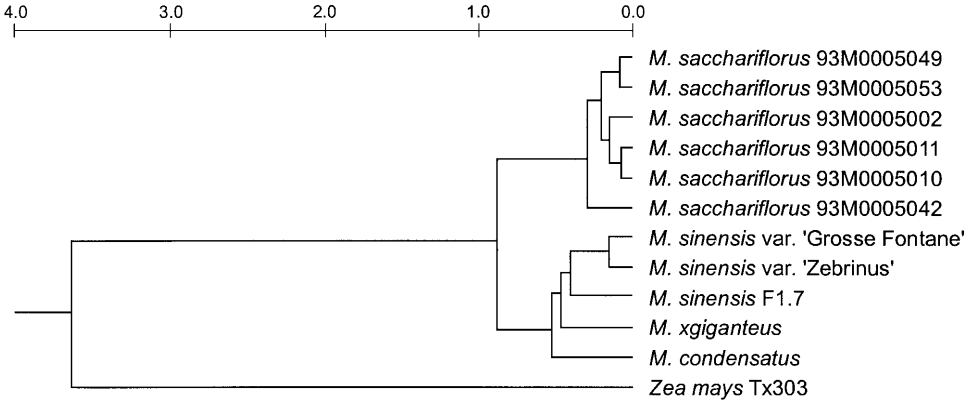

Fig. 4 Cluster analysis derived from analysis of 11 *Miscanthus* germplasm accessions (Table 1) and one maize line using 17 microsatellite primers

chariflorus, *M.* ×*giganteus* and *M. condensatus*. Length polymorphisms were detected by all primer pairs, revealing 3–22 alleles with an average of 12 (Table 3). As an example, amplification products from primer pair blgn176 are shown in Fig. 3. PIC values ranged from 0.48 to 0.94 with an average of 0.83 (Table 3). Genetic similarity coefficients ranged from 0.35 to 0.92 with an average of 0.57 (Table 4).

Species-specific amplification products were obtained with two primer pairs. MAC.E01.F06 only gave amplification from *M. sacchariflorus* clones, while bgln107 amplified from *M. sinensis*, *M.* ×*giganteus* and *M. condensatus* but not from *M. sacchariflorus*. The rest of the primers either failed to amplify from *M. sinensis* or amplified from all accessions, though with products of a different size range and banding pattern. All of the *Miscanthus* products differed in size from their equivalents in maize but there was no trend towards higher or lower molecular weights.

Results from the cluster analysis showed a clear association of *M. sinensis*, *M. condensatus* and *M.* ×*giganteus* (Fig. 4). There was variation within *M. sinensis* with F1.7 being closer to *M. condensatus* and *M.* ×*giganteus* than the other two *M. sinensis* genotypes tested. The tetraploid *M. sacchariflorus* accessions are linked to the diploid (*M. sinensis*) and triploid pools (*M. condensatus* and *M.* ×*giganteus*) but with a relatively large distance. *Zea mays* was clearly separated from the *Miscanthus* spp. cluster.

Discussion

Transferability of markers

The 50% transferability from grass anchor probes to *Miscanthus* was expected given the extensive cross-hybridization of these and other RFLP probes in previous studies (Moore et al. 1995; Van Deynze et al. 1998). Nevertheless, the 100% transferability of maize probes was surprising, and thus maize probes are likely to provide a particularly useful resource for *Miscanthus* mapping and linkage-group alignment with other grasses. The similarity of hybridization patterns between maize and *Miscanthus* encouraged us to explore the possibility that maize microsatellite primers could also be used.

Several investigators have shown that microsatellite primer pairs developed for one species can be used in close relatives (see Peakall et al. 1998 for a review), but in the Gramineae there has generally been a low level of transferability where this has been tested. Zhao and Kochert (1993) reported amplification of a microsatellite-containing product in maize and bamboo (*Bambosa vulgaris* Schreber). However, Brown et al. (1996) obtained discouraging results when using maize primers to assay polymorphism in sorghum as only 2–3% of the 67 primers tested were useful. Similarly, results from hexaploid wheat show that microsatellite markers are usually genome-specific and few amplify from related species such as barley (Bryan et al. 1997; Röder et al. 1998; Stephenson et al. 1998). There are, however, some exceptions, such as the *Xgwm165* marker from wheat (Röder et al. 1998) which has proved useful for aligning the group-4 maps of wheat and barley (Ivandic et al. 1999), and of *Hordeum chilense* (P. Hernández, unpublished results). In other cases polysomic inheritance has been reported, as in hexaploid and tetraploid sweetpotato (Buteler et al. 1999).

Clearly, the transferability of microsatellite primers is likely to be related to the taxonomic separation of species. Transferability is not easily predictable for any given sequence, but the rapidity of the microsatellite assay means that the usefulness of primers can be determined empirically. The extent of the transferability of maize microsatellite markers to *Miscanthus* (74.5%) is surprising given that they belong to different genera and that the microsatellites were derived from genomic libraries rather than from known coding regions of genes, which might be expected to be conserved. The availability of more than 1000 public maize microsatellite primer sequences amplifying 975 distinct loci (Jan 2000, <http://www.agron.missouri.edu/microsatellite.html>) constitutes an efficient and cost effective source of molecular markers for *Miscanthus* breeding and genetics.

The banding patterns of transferable microsatellites were similar for maize and *Miscanthus* species, although fragment sizes were different. We observed no bias toward higher or lower molecular-weight fragments in *Miscanthus* than in maize, and polymorphisms were equally common in both types. These results contrast

with the general trend in mammalian microsatellites, which have been reported to amplify shorter fragments in related species (Ellegren et al. 1997). Nevertheless, the loss of polymorphism is a shared feature.

Microsatellite markers for genetic mapping and germplasm assessment in *Miscanthus*

We have selected diploid *M. sinensis* for genetic mapping as it provides the simplest situation and because it is the donor of two of the three genomes of *M* ×*giganteus*. The Mendelian segregation of the five microsatellite loci tested illustrates the usefulness of these markers for *Miscanthus* mapping, and the high levels of variability and reproducibility associated with microsatellite markers will allow them to be used as anchor markers between genetic maps of *Miscanthus* and maize. In this way they will facilitate marker-assisted selection in *Miscanthus* crosses and serve to integrate trait analysis with other Gramineae species.

The estimated similarity between the *M. sacchariflorus* accessions was 70–92%; 65–85% between the *M. sinensis* accessions and 62% between the autotriploid *M. condensatus* and the allotriploid *M.* ×*giganteus* (Table 4). Our results indicate a closer clustering of the *M.* ×*giganteus* clone used in this study to the *M. sinensis* cluster than to the *M. sacchariflorus* cluster. This is a logical outcome, because *M.* ×*giganteus* shares two genomes with *M. sinensis* and one with *M. sacchariflorus*. Nevertheless, this preliminary interpretation should be treated with caution because of the limited number of lines used in this study. Moreover, the relationships could change when using a different set of reference lines or additional markers. For example, Greef et al. (1997) obtained closer clustering of two *M.* ×*giganteus* accessions to the *M. sacchariflorus* pool using AFLP markers.

We did not observe increased numbers of DNA fragments with increased ploidy levels, nor did the amplification patterns indicate hybrid or alloploid origins. This probably reflects genome-specificity of the microsatellite markers as has been observed in wheat (Bryan et al. 1997; Röder et al. 1998; Stephenson et al. 1998). If this explanation is correct the large number of alleles (up to 22) observed with some primer pairs would reflect a high level of heterozygosity in the accessions studied. Alternatively, these primer pairs may amplify sequences from additional sites in the *Miscanthus* genome. These alternatives can be resolved by future mapping studies and sequence analysis of PCR products.

In conclusion, maize RFLP and microsatellite markers offer fast and reproducible methods for analysing the relatively unknown *Miscanthus* gene pool, for genetic mapping and for alignment of genetic maps with other Gramineae species. They therefore provide valuable data that complement existing cytological, morphological (Adati and Shiotani 1962; Greef and Deuter 1993; Linde-Laursen 1993; Lafferti and Lelley 1994) and molecular (Von Wuhlisch et al. 1994; Greef et al. 1997) studies.

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