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Characterization of a Genetic Resource Collection for *Miscanthus* (Saccharinae, Andropogoneae, Poaceae) using AFLP and ISSR PCR

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Amplified fragment length polymorphism (AFLP) and inter-simple sequence repeat markers were employed to characterize a genetic resource collection of *Miscanthus*, a grass under trial in Europe as a biomass crop. The 26 polymorphic markers produced by two ISSR fingerprinting primers were able to discriminate taxa and identify putative clones. AFLP fingerprints were fully reproducible and produced a larger number of markers for the three primer pairs tested, of which 998 were polymorphic (representing 79·3 % of all bands). AFLP markers distinguished species, infra-specific taxa (varieties and cultivars) and putatively clonal material. They were also used to assess the inter-relationships of the taxa, to investigate the origin of important hybrid plants and to estimate the overall level of genetic variation in the collection. They were useful for assessing the species status of certain taxa such as *M. transmorrisonensis*, an endemic from Taiwan that was clearly distinct from *M. sinensis*; whereas other taxa of disputed species status, such as *M. condensatus* and *M. yakushimanum* were not genetically distinct from *M. sinensis*. The AFLP markers detected a high degree of infra-specific variation and allowed subdivisions of the genetic resource collection to be made, particularly within *M. sinensis*.

Key words: *Miscanthus*, AFLP, ISSR, inter-microsatellites, biomass, genetic resources, taxonomy, hybrids, molecular phylogenetics.

INTRODUCTION

Reform of the European Common Agriculture Policy led to the introduction of the set-aside scheme, in which land must be taken out of food production or used for growing non-food crops (Kilpatrick *et al*., 1994). A strong candidate for a new crop suited to this style of agriculture is the grass genus *Miscanthus* Anderss., which has received considerable attention in northern Europe as a biomass source for renewable energy production and as a raw material for the cellulose and paper industries. Currently, biomass accounts for approx. 14 % of the world's non-solar energy (Kilpatrick *et al*., 1994). Evaluation trials assessing biomass production of these perennial species have produced annual yields of 20–44 t d. wt ha^{-1} and crop heights in excess of 3 m (Bullard *et al*., 1995, 1997). In the United Kingdom the prospects for *Miscanthus* have been improved by the introduction of the non-fossil fuel obligation scheme (NFFO) and the development of short-rotation coppicing of willow and poplar trees, which is now near the point of commercial exploitation (Kilpatrick *et al*., 1994). The first commercial plantings of *Miscanthus* in the UK have been made recently (Bullard, pers. comm.) since there is a market for a herbaceous alternative to short-rotation coppice of willow and poplar. *Miscanthus* is also used for thatching, forage, wind breaks, erosion protection, silk dyeing and in breeding programmes with sugar cane (Hodkinson, data gathered from labels on herbarium specimens at Kew; Wikberg, 1990), and it is a popular hardy garden plant.

Most research investigating the productivity and economic potential of *Miscanthus* has centred on two taxa: *M. sacchariflorus* (Maxim.) Benth. & Hook., a species from northern China and Japan, and *M.* × *giganteus* Greef & Deuter ex Hodkinson & Renvoize (Hodkinson and Renvoize, 2001), a putative hybrid between *M. sacchariflorus* and *M. sinensis* Anderss. (Adati and Shiotini, 1962; Linde-Laursen, 1993). *Miscanthus* × *giganteus* Greef & Deuter ex Hodkinson & Renvoize is also known as *M.* × *giganteus* Greef & Deuter (Greef and Deuter, 1993) but the latter name is invalid under the rules of the International Code of Botanical Nomenclature because the diagnosis and description were in English and no type specimen was designated (Greuter *et al*., 2000). *Miscanthus* × *giganteus* is also incorrectly known as *M. giganteus* or *M. sinensis* 'Giganteus' and is often mistaken for *M. sacchariflorus.* See Hodkinson and Renvoize (2001) and Hodkinson *et al*. (2002) for further discussion of the nomenclature of *M.* × *giganteus*. The agricultural community is fully aware of the hazards and limitations of relying on single clones for high yields because these are at high risk from pests and diseases and are not likely to be adapted to local climatic or edaphic conditions. However, few attempts have been made to broaden the genetic base of the crop, and little effort has been given to evaluating and characterizing the genetic diversity available within the genus (Renvoize *et al*., 1997).

Studies using DNA sequences from the internal transcribed spacer of nuclear ribosomal DNA (nrITS) have

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elucidated species inter-relationships in the genus but were unable to resolve the differences between cultivars or varieties of *Miscanthus* (Hodkinson *et al*., 1997). Little variation was detected for the variants of *M. sinensis*, and it is at this taxonomic level that further information is required. This is in contrast to some other studies that have successfully used DNA sequence data to assess infra-specific variation in plants (Jorgensen and Cluster, 1988; Cox *et al*., 1992; Ramakrisha *et al*., 1995). Two further DNA regions, 5S nuclear ribosomal DNA spacers (Baum and Appels, 1992; Cox *et al*., 1992; Sastri *et al*., 1992) and the *trnL-F* intron and intergenic spacer (Taberlet *et al*., 1991) were also sequenced by Hodkinson *et al*. (1997) but did not discriminate between the infra-specific taxa.

Isozyme analysis has been used to assess genetic diversity within *Miscanthus* spp. (Chou *et al*., 1987; Chou and Chang, 1992; Von Wuhlish *et al*., 1994), and DNA markers were first used by Greef *et al*. (1997), who applied amplified fragment length polymorphism (AFLP™) fingerprinting (Vos *et al*., 1995) to assess variation in three *Miscanthus* species. The study was limited in terms of species, infra-specific taxa and cultivars sampled, but did prove that the markers were suitable for assessing genetic infra-specific variation in *Miscanthus*.

Two DNA fingerprinting methods, amplified fragment length polymorphisms (AFLPs) and inter-simple sequence repeat (ISSR, also known as inter-microsatellites; Weising *et al*., 1995) were used to characterize genetic diversity in the collections held at the Royal Botanic Gardens, Kew, UK, and ADAS Arthur Rickwood Research Station, Cambs, UK. The collections include a number of different species but are composed mainly of *M. sinensis* cultivars which represent an important resource for both biomass production and horticulture. We provided a preliminary overview of our AFLP work on *Miscanthus* in Hodkinson *et al*. (1997), but present the empirical data here for the first time.

ISSR PCR (Zietkiewicz *et al*., 1994; Weising *et al*., 1995) uses a single primer and is experimentally similar to random amplified polymorphic DNA (RAPD; Williams *et al*., 1990), but differs in a number of important details. It uses primers based on simple sequence repeats (microsatellites) such as $(GACA)_4$ or $(CA)_8$. Such sequences are common in the genome and are therefore good targets for such a PCR-based fingerprinting technique. The selectivity of the primers can also be modified by adding an anchor such as RG to one end (anchored ISSR PCR; Zietkiewicz *et al*., 1994). Regions between these microsatellites are amplified, separated typically by agarose gel electrophoresis and then detected by ethidium bromide/UV light. Primers are usually longer (16–20 bp) than those employed in RAPD (10 bp), and this allows increased stringency. Such an approach should theoretically improve reliability and reproducibility in comparison with RAPD (Weising *et al*., 1995).

The AFLP technique has several advantages over other marker systems currently in use (Vos *et al*., 1995; Reeves *et al*., 1998; Ridout and Donini, 1999). It produces a high number of polymorphic informative markers per primer pair, is highly sensitive, requires small amounts of DNA, and has proved to be robust, reliable and reproducible (Mueller and Wolfenbarger, 1999; Ridout and Donini, 1999; Hodkinson *et al*., 2000), unlike some other PCR-based techniques, such as RAPD (Karp *et al*., 1996). AFLP fingerprinting is based on the selective amplification of restriction fragments from a digest of total genomic DNA and has been adapted for use on an automated DNA sequencer.

The complex multi-locus fingerprints produced by AFLP provide a large number of informative markers derived from loci widely dispersed throughout the nuclear genome (Ridout and Donini, 1999). For example, in barley (*Hordeum vulgare* L.), AFLP markers are located on the long and short arms of all seven chromosomes, with a strong correlation between the number of markers per chromosome and the length of the chromosome (Waugh *et al*., 1997). In rice, *Oryza sativa* L., Mackill *et al*. (1996) used AFLP to map an F_2 population and found that the 50 AFLP markers were spread across nearly all chromosomes. However, clustering of AFLP markers within certain regions has been reported in other taxa (Qi *et al*., 1998).

MATERIALS AND METHODS

Plant material

Only fully vouchered material from collections at RBG Kew and the ADAS Arthur Rickwood Research Station (Mepal, Ely, Cambridgeshire, UK; a site for *Miscanthus* biomass trials) were included in the molecular analysis so that results could be compared with morphology of the plants in question. In total, 75 accessions were included in the study and are listed in Table 1.

DNA extraction

DNA was extracted from 0·5–1·0 g of fresh leaf material using the modified 2 × CTAB procedure of Doyle and Doyle (1987) and precipitated using 100 % ethanol for at least 48 h at –20 °C. The DNA was then pelleted, washed with 70 % ethanol and purified via caesium chloride/ethidium bromide (1·55 g ml^{-1}) gradient centrifugation with subsequent dialysis. DNA was then stored in TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8·0) at –20 °C until use. Total genomic DNA was quantified by measuring light absorption using a Philips PV 8820 UV/VIS spectrophotometer.

ISSR PCR

The ISSR primers screened for PCR fingerprinting of *Miscanthus* were $(GACA)_4$, $(CA)_8RY$, $(CA)_8RG$, $(GATA)_4$ and $(CA)_7NNN$. PCR was performed using 100 ng template DNA, 100 μM of each dNTP, 2 mM $MgCl_2$ in a 100 μl reaction using 0·5 units of *Taq* polymerase (Promega Ltd, Southampton, UK). The thermal cycling for all PCR reactions comprised 40 cycles, each with 20 s denaturation at 93 °C, 1 min annealing at 50 °C and an extension of 20 s at 72 °C. A final extension of 6 min at 72 °C was also included. Amplification products (10 μl) were separated using standard 2 % agarose or 2 % NuSieve 3 : 1 agarose (FMC Bioproducts; Flowgen, Lichfield, UK), a gel suited

for the separation of DNA fragments of less than 1 kb in length.

AFLP

Reactions were performed using the AFLP Plant Mapping Kit of PE Applied Biosystems Inc. (ABI, Warrington, UK), and DNA fragments were detected on an ABI 377 automated DNA sequencer with ABI GeneScan 2·02 and Genotyper version 1·1 software. Thirty-two primer pairs with various three-base anchors were used initially to screen six accessions and to establish the most informative combinations for *Miscanthus* fingerprinting (Table 2). Suitable primer pairs were defined as those that produced a large number of well defined, polymorphic bands. DNA fragments ranging from 50 to 500 bp in size from the AFLP analysis were scored. Three primer pairs (indicated in Table 2) were used to fingerprint 75 accessions of *Miscanthus,* including a large number of *M. sinensis* clones, *M. floridulus* (Labill.) Warb., *M. sacchariflorus*, *M.* × *giganteus*, *M. oligostachyus* Stapf., *M. nepalensis* (Trin.) Hack. and *M. transmorrisonensis* Hayata. *Saccharum officinarum* L., being the closest known relative of *Miscanthus* (Clayton and Renvoize, 1986), was also included for comparison. Replicate samples and samples of known clonal origin were used to evaluate reproducibility of the fingerprints and to estimate scoring error in the analysis.

Data analysis

Neighbour-joining (NJ) analysis was applied to a distance matrix based on mean character difference (PAUP 4·0; Swofford, 1998) for both the ISSR and AFLP data. An NJ analysis was also applied using Nei and Li distances (Nei and Li, 1985), and the results (not presented) were not significantly different from the NJ tree based on mean character difference. Internal support for groupings was assessed using 1000 bootstrap replicates (Felsenstein, 1985). Principal coordinates analysis (PCO) was performed with Le Progiciel R v4·0d (Casgrain, 1999) using Dice distances (Dice, 1945).

RESULTS

ISSR PCR

Two of the five ISSR primers, $(GACA)_4$ and $(CA)_8RG$, gave clear reproducible banding patterns with some levels of polymorphism. Three of the primers, $(CA)_8RY$, $(GATA)_4$ and $(CA)_7NNN$, did not produce suitable molecular markers. The fingerprints produced with the two good primers were difficult to score when a large number of accessions were included, and therefore only a few individuals could be handled reliably using this method. A total of 26 markers (all polymorphic) was produced from the two ISSR primers, of which 14 were shared by more than one individual. In the NJ analysis (Fig. 1), strong support was found for the grouping of *M. sinensis* with 100 % support (bootstrap percentage, BP) and *M.* × *giganteus* clones (99 BP). *Miscanthus* × *giganteus* was approximately equidistant from *M. sinensis* and *M. sacchariflorus*, its putative parental species. Considerable variation was detected between the *M. sinensis* accessions (varieties and cultivars), but no genetic variation was detected between *M.* × *giganteus* accessions. Similar groupings were evident in the PCO analysis (Fig. 2). The accessions can be separated using the first two axes of the PCO, and these cumulatively account for 87·2 % (73·7 % and 13·5 %, respectively) of the data variance, the third axis (not shown) representing 7·4 %.

AFLP

Three primer pairs produced a total of 1259 DNA markers, of which 998 were polymorphic (representing 79·3 % of all bands). The AFLP fingerprints distinguished *Miscanthus* species and infra-specific taxa. Representative fingerprints of *Miscanthus* accessions are given in Fig. 3, which also illustrates the power of the technique to detect clonal material (i.e. the traces from two plants are remarkably consistent). Two plants initially labelled as different taxa, *M. sacchariflorus* and *M.* × *giganteus*, were morphologically indistinguishable and gave identical fingerprints in all AFLP primer combinations tested (and were therefore invariant at otherwise highly polymorphic loci). Other cultivars of *Miscanthus* were also accurately assessed, and they separated out as distinct groups in the NJ tree (Fig. 4). Many of the taxa in our collection had not been allocated species names, and the AFLP data, combined with morphological examination, allowed an accurate identification to be made. The taxa presented in Table 3 were identified in this way.

Miscanthus transmorrisonensis is the closest relative of *M. sinensis* (89 BP). A group consisting of *M. sacchariflorus, M.* × *giganteus* and *M. oligostachyus* represents the next most genetically similar species to this major group. Accessions of *M. sinensis* and a number of subgroups are genetically distinct. For example, a group containing a number of taxa with name Yakushima or similar (such as *M. sinensis* 'Yakushimanum' and *M. sinensis* 'Yakushima') has strong bootstrap support.

DISCUSSION

Measuring genetic diversity using ISSR PCR

The ISSR PCR and anchored ISSR methods provided evidence for the clonal nature of *M.* × *giganteus* accessions and indicated that incorrectly labelled material, such as one *M. sacchariflorus* accession, was actually *M.* × *giganteus*. From the NJ tree (Fig. 1) and the PCO (Fig. 2), it can be seen that considerable infra-specific variation is detected in the *M. sinensis* accessions, but no variation was detected at these otherwise polymorphic loci in the *M.* × *giganteus* accessions.

We considered ISSR methods less efficient than AFLPs for screening large numbers of *Miscanthus* accessions. One problem is the manual scoring of bands on agarose gels in which the sizing of fragments is not as accurate as automated genotyping methods. It is also difficult to compare results from samples run on different agarose

TABLE 1. *Grasses and associated voucher specimens held at the Royal Botanic Gardens Kew, UK and ADAS Arthur Rickwood Research Station used in the study*

Taxon	ID	Voucher and/or Kew accession number
M. floridulus (Labill.) Warb. ex K. Schum. & Lauterb.	72	*Hodkinson 30. 1978–1387*
M. × *giganteus* Greef & Deuter ex Hodkinson & Renvoize	2	*Renvoize s.n. 1990 381*
M. × *giganteus*	8	*Gilbert s.n. 13/11/90. 1969–19097*
M. × *giganteus*	22	*Hodkinson s.n. 1993–1779*
M. × *giganteus*	23	*Kew living 1993–1780*
M. × *giganteus*	26	*Living ADAS MB93/01*
M. × *giganteus*	60	*ADAS MB95/30*
M. × *giganteus*	187	*ADAS PN96/19*
M. × *giganteus*	188	*ADAS PN96/20*
M. × *giganteus*	189	*ADAS PN96/21*
M. × *giganteus*	190	*ADAS PN96/22*
M. × *giganteus*	180	*ADAS PN96/05*
M. nepalensis (Trin.) Hack.	25	*Hodkinson 1*
M. nepalensis	66	*Hodkinson 22*
M. oligostachyus Stapf.	16	*Hodkinson 13*
M. oligostachyus 'Nanus Variegatus'	161	*Hodkinson s.n. 1996–1065*
M. sacchariflorus (Maxim.) Benth. & Hook. 'Purpurascens'	61	*Hodkinson s.n. 1987–2727*
M. sinensis Anderss. var. *variegatus* Beal	1	*Hodkinson 33*
M. sinensis 'Silver Feather'	3	*Hodkinson 24. 1975–930*
M. sinensis 'Silberspinne'	4	*Hodkinson s.n. 1988–2519*
M. sinensis	5	*Hodkinson 40. 1978–1389*
M. sinensis ssp. *condensatus* (Hackel) T. Koyama	7	*Renvoize s.n. 1969 19091*
M. sinensis var. *zebrinus* Beal	10	*Hodkinson 35*
M. sinensis 'Poseidon'	11	*Hodkinson s.n. 1995–1866*
M. sinensis 'Undine'	12	*Hodkinson 26*
M. sinensis 'Silver Feather'	13	*Hodkinson s.n. 1975–915*
M. sinensis var. *zebrinus*	14	*Hodkinson 34*
M. sinensis var. *variegatus*	17	*Hodkinson s.n. 1973–10370*
M. sinensis 'Graziella'	18	*Hodkinson 29*
M. sinensis 'Kleine Silberspinne'	19	*Hodkinson s.n. 1995–1865*
M. sinensis sp.	20	*Hodkinson 36*
M. sinensis	21	*Hodkinson 3*
M. sinensis	24	*Hodkinson 28*
M. sinensis 'Goliath'	27	*ADAS MB93/02*
M. sinensis 'Gracillimus'	28	*Hodkinson s.n. MB94/05*
M. sinensis 'Roland'	29	*Hodkinson s.n. ADAS MB94/06*
M. sinensis	30	*ADAS MB94/07*
M. sinensis 'Grosse Fontäne'	31	*Renvoize s.n. PN95/01*
M. sinensis 'Malepartus'	33	*ADAS PN95/03*
M. sinensis 'Ferner Osten'	36	*ADAS PN95/06*
M. sinensis 'Kleine Fontäne'	38	*ADAS PN95/08*
M. sinensis 'Kleine Silberspinne'	40	*ADAS PN95/10*
M. sinensis 'Vorläufer'	42	*ADAS PN95/12*
M. sinensis 'Kaskade'	43	*ADAS PN95/13*
M. sinensis 'Roland'	46	*ADAS PN95/16*
M. sinensis 'Poseidon'	47	*ADAS PN95/17*
M. sinensis 'Wetterfahne'	48	*ADAS PN95/18*
M. sinensis 'Gewitterwaike'	49	*ADAS PN95/19*
M. sinensis 'Sirene'	50	*ADAS PN95/19*
M. sinensis 'Nippon'	51	*ADAS PN95/21*
M. sinensis 'Afrika'	56	*ADAS PN95/26*
M. sinensis 'Zwergelephant'	57	*ADAS PN95/27*
M. sinensis var. *variegatus* Beal	62	*Hodkinson s.n. 1973–2834*
M. sinensis 'Yakushimanum'	63	*Hodkinson 21. 1987–1148*
M. sinensis	64	*Hodkinson 23*
M. sinensis 'Variegatus'	67	*Hodkinson s.n. 1969–34750*
M. sinensis	68	*Hodkinson 19*
M. sinensis 'Silver Feather'	69	*Hodkinson 18*
M. sinensis var. *gracillimus*	71	*Hodkinson s.n. 1969–19098*
M. sinensis 'Sirene'	113	*Hodkinson s.n.*
M. sinensis 'Nippon'	143	*Hodkinson s.n. 1996–823*
M. sinensis 'Grosse Fontäne'	144	*Hodkinson s.n. 1996–1294*
M. sinensis 'Pünktchen'	145	*Hodkinson s.n.*
M. sinensis 'Ferne Osten'	147	*Hodkinson s.n. 1996–1303*
M. sinensis	48	*Hodkinson s.n.*
M. sinensis 'Malepartus'	149	*Hodkinson s.n. 1996–1301*
M. sinensis 'Sarabande'	150	*Hodkinson s.n.*

TABLE 1. *Continued*

Taxon	ID	Voucher and/or Kew accession number
M. sinensis 'Yakushima Dwarf'	151	*Hodkinson s.n. 1996–822*
M. sinensis 'Kleine Silberspinne'	152	*Hodkinson s.n. 1996–820*
M. sinensis 'Strictus'	153	*Hodkinson s.n. 1996–1297*
M. sinensis 'Kaskade'	154	*Hodkinson s.n. 1996–1064*
M. sinensis 'Yakushima'	178	*Hodkinson s.n. ADAS 96/03*
M. sinensis	184	*ADAS PN96/14*
M. sinensis 'Goliath'	194	*ADAS PN96/30*
M. transmorrisonensis Hayata	65	*Hodkinson 20. 1990–2748*
Saccharum officinarum L.	104	*RBG Kew 1973–12242*

TABLE 2. *Selective amplification primers tested for AFLP analysis*

*Eco*RI primer anchor	Fluorescent label	*Mse*I primer anchor	EcoRI primer anchor	Fluorescent label	*Mse*I primer anchor
01 AAC	TAMRA	CAG	17 AAC	TAMRA	CTA
02 AAC	TAMRA	CAC	**18 AAC***	**TAMRA**	**CAT**
03 AAG	JOE	CAG	19 AAG	JOE	CTC
04 AAG	JOE	CAC	20 AAG	JOE	CAT
05 ACA	FAM	CAG	21 ACA	FAM	CTA
06 ACA*	**FAM**	**CAA**	22 ACA	FAM	CAT
07 ACC	TAMRA	CAG	23 ACC	TAMRA	CTA
08 ACC	TAMRA	CAC	**24 ACC***	**TAMRA**	**CAT**
09 ACG	JOE	CAG	25 ACG	JOE	CTA
10 ACG	JOE	CAC	26 ACG	JOE	CAT
11 ACT	FAM	CAG	27 ACT	FAM	CTA
12 ACT†	**FAM**	**CAA**	28 ACT	FAM	CTC
13 AGC	TAMRA	CAG	29 AGC	TAMRA	CTA
14 AGC	TAMRA	CAC	30 AGC	TAMRA	CAT
15 AGG	JOE	CAG	31 AGG	JOE	CTA
16 AGG	JOE	CAC	32 AGG	JOE	CAT

See ABI kit for further details on primer composition.
* The most successful primers that were used to screen a further 75 *Miscanthus* accessions.
† A successful primer combination used in Hodkinson *et al*. (2002) but not in this study.

gels, which increases scoring error. Standard 2 % agarose gels cannot accurately differentiate between fragments of similar length, whereas 2 % NuSieve 3 : 1 gel, used in this study, improved the resolution but was still limited in its ability to separate fragments differing by less than 10 bp in length. Detection of ISSRs could be automated by fluorescently labelling the primers, but many of the markers would be lost because they are too long for detection using standard acrylamide gels. This method would not provide any added benefit over that of AFLP analysis, which is generally more reliable and informative (Karp *et al*., 1996). Far fewer polymorphic markers were produced per primer using ISSR PCR (mean of 13) than a corresponding primer pair in an AFLP analysis (mean of 332).

ISSR PCR and anchored ISSR PCR have proved useful for assessing genetic diversity within various plant groups (Weising *et al*., 1995; Wilkinson *et al*., 2000), including grasses (Li and Ge, 2001), but we believe that they are better suited to screening of small numbers of plants and testing specific hypotheses regarding inter-relationships rather than large-scale screening. For example, Wilkinson *et al*. (2000) used them effectively to discover the parental species of a *Brassica* hybrid found in sympatric populations of *B. napus* (oilseed rape) and *B. rapa* (wild turnip). ISSRs are,

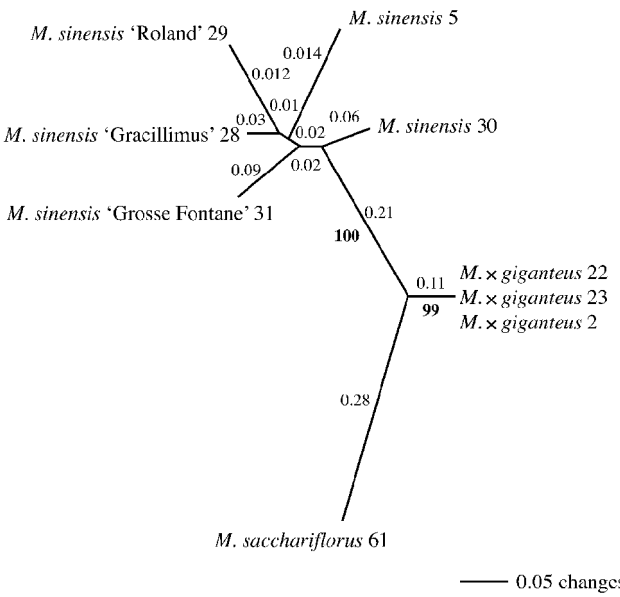

FIG. 1. Unrooted neighbour joining tree for ISSR data. The NJ tree shows the results of two primers, $(GACA)_4$ and $(CA)_8RG$. Values above the branches are genetic distances. Values below the branches are bootstrap percentages.

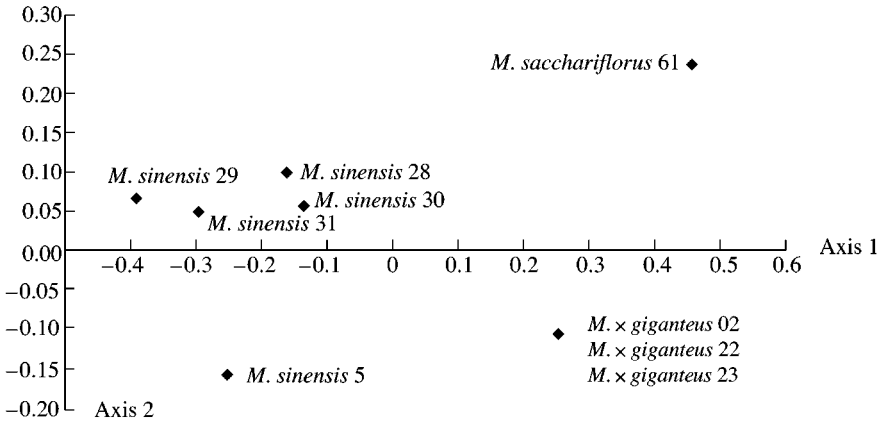

FIG. 2. Principal coordinates analysis (PCO) for ISSR data using Dice distances. The accessions can be separated using the first two axes of the PCO and these cumulatively account for 87·2 % (73·7 % and 13·5 %, respectively) of the data variance.

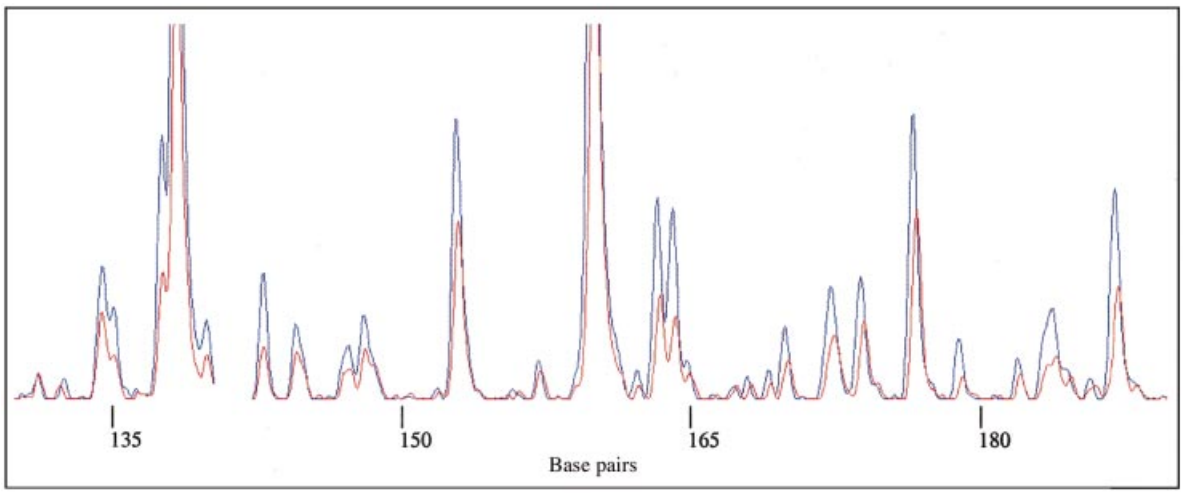

FIG. 3. AFLP traces of two *Miscanthus* accessions: *M.* × *giganteus*, originally called *M. sacchariflorus* 23 (red) and *M.* × *giganteus* 22 (blue). Note the two are identical in all fragments in the analysis.

however, cheaper than AFLP and require less technology to collect and analyse.

Measuring genetic diversity and infra-specific variation using AFLP

The AFLP technique, in contrast to ISSR analysis, proved to be a suitable method for characterizing large numbers of *Miscanthus* species and infra-specific taxa. One of the main strengths of the multi-locus AFLP technique is its ability to produce a larger number of reliable molecular markers, in this case 998 polymorphic markers from just three primer pairs.

AFLPs helped to detect clonal or nearly clonal material. For example, two morphologically indistinguishable plants growing under different species names, *M. sacchariflorus* and *M.* × *giganteus*, had identical fingerprints (Fig. 3) in all AFLP primer combinations tested and have since been shown to have the triploid chromosome complement characteristic of the latter species ($2n = 3x = 57$; Hodkinson, unpubl. res.). In the NJ analysis (based on the initial scoring of bands without subsequent detailed examination), some variation was evident among *M.* × *giganteus* accessions but this was shown to be due to scoring error (see below). Greef *et al*. (1997) assessed genetic variation in three *Miscanthus* species (*M. sinensis*, *M. sacchariflorus* and *M.* × *giganteus*) using radioactively detected AFLPs and, in agreement with our findings, also discovered little genetic variation in their *M.* ×*giganteus* accessions. There may be only two or three closely related clones of *M.* × *giganteus* in cultivation (outside of research institutes, where new hybrids are being produced; J. Clifton-Brown, pers. comm.).

The fluorescently labelled automated AFLP method utilized in this study was efficient at separating large numbers of cultivars and varieties of *M. sinensis* (Figs 3 and 4). The different cultivars are labelled with different colours, and it can be seen that these group together in most cases. For example, four accessions with the name *M. sinensis* var. *variegatus* (or similar; at the top of the tree)

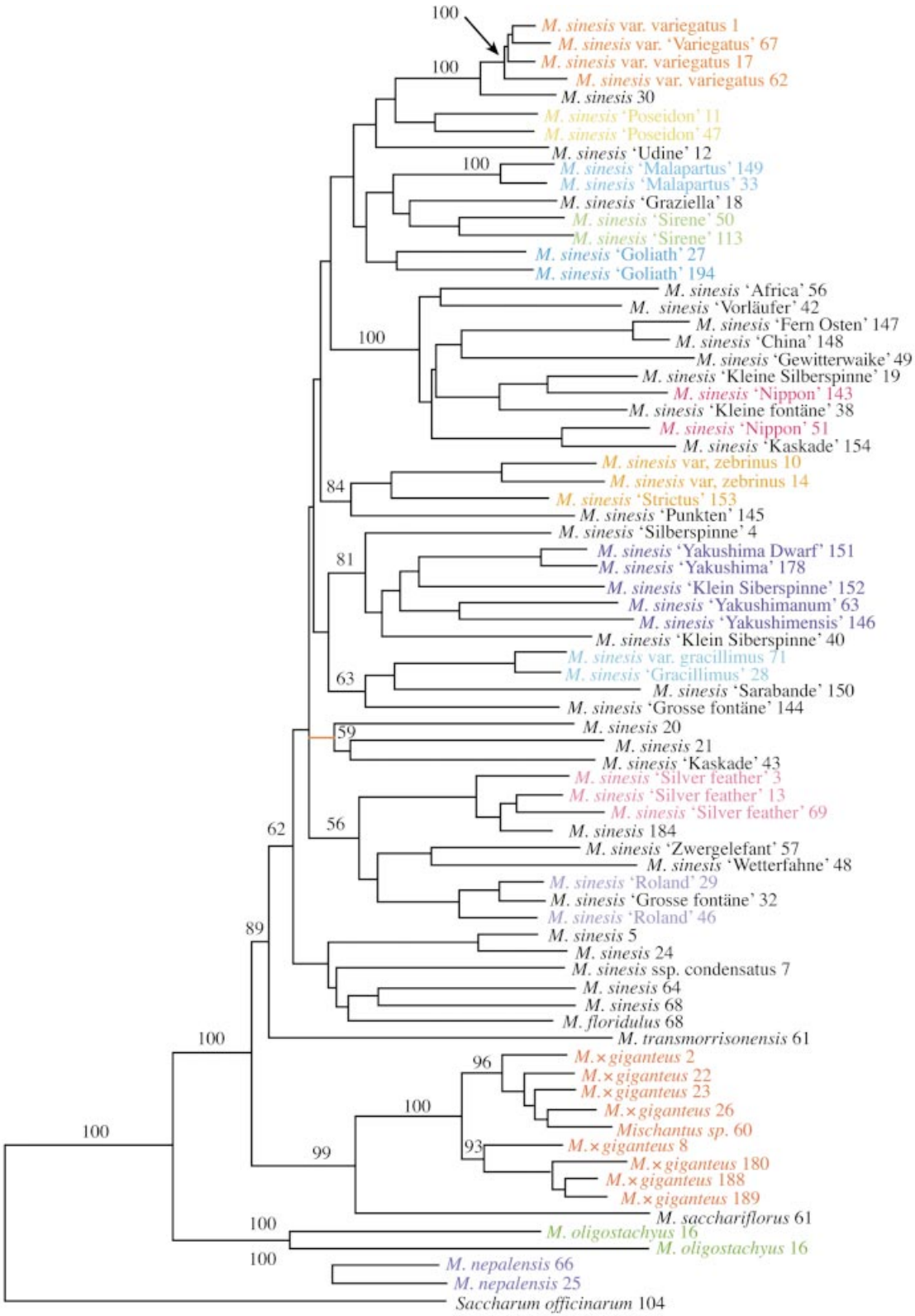

FIG. 4. Neighbour joining (NJ) tree of AFLP data. Branch length is proportional to genetic distance. Values above branches are bootstrap percentages. Species are unambiguously separated using the AFLP markers. Furthermore, cultivars are also accurately grouped on the basis of genetic distance and have been highlighted with different colours.

TABLE 3. Miscanthus *taxa growing in the living collection at ADAS Arthur Rickwood or RBG Kew identified using AFLP and morphological data*

ID	Previous identity	New identity based on AFLP data
8	*M. sacchariflorus*	*M.* × *giganteus*
20	*Miscanthus* sp.	*M. sinensis*
23	*M. sacchariflorus*	*M.* × *giganteus*
26	*M. sinensis* 'Giganteus'	*M.* × *giganteus*
60	*Miscanthus* sp.	*M.* × *giganteus*
61	*M. purpurascens*	*M. sacchariflorus*
64	*M. chinensis*	*M. sinensis*
148	*Miscanthus* sp. 'China'	*M. sinensis* 'China'
150	*Miscanthus* sp. 'Sarabande'	*M. sinensis* 'Sarabande'
161	*M. tinctorius* 'Nanus Variegatus'	*M. oligostachyus* 'Nanus Variegatus'
180	*M. sinensis* 'Giganteus'	*M.* × *giganteus*
194	*M.* × *giganteus* 'Goliath'	*M. sinensis* 'Goliath'

Note: some taxa were previously unnamed or misnamed.

group closely together with 100 % bootstrap support. Many *Miscanthus* cultivars are now available, and accurate cultivar identification is becoming important. Cultivar names are not governed by the International Code of Botanical Nomenclature (Greuter *et al*., 2000) and can often proliferate out of control. In our collection there are numerous cultivars with similar but not identical cultivar names, which when examined with AFLP can be accurately clustered. Deviation from expected groupings of taxa is rare and can be easily explained by misnaming or duplicate naming for the same taxon or group. Alternatively, slight deviations from the expected groupings may be expected because of scoring error in the AFLP analysis (see below). Known clonal material or duplicate material should be identical in AFLP profile, unless somatic variation has occurred, and no variation should be found in experimental replicates. By comparing AFLP profiles of these identical clones and replicate samples it has been estimated that the scoring error lies approximately in the range of 1–2 %, which is consistent with that estimated in other studies (Mueller and Wolfenbarger, 1999).

There is clearly a high level of variation within *M. sinensis*, and subgroups can be identified. A group containing a number of taxa with name Yakushima or similar (such as *M. sinensis* 'Yakushimanum' and *M. sinensis* 'Yakushima'; 81 BP) has been considered as a separate species but is clearly a member of the *M. sinensis* group.

Establishing the inter-relationships of the taxa

In the NJ tree (Fig. 4), the various species are well separated and this system closely matches identification based on gross morphology. Most taxa in the living genetic resource collections are identified as *M. sinensis*, some as *M.* × *giganteus*, one as *M. sacchariflorus*, two as *M. nepalensis*, two as *M. oligostachyus* and one as *M. transmorrisonensis*. *Miscanthus transmorrisonensis* is morphologically similar to *M. sinensis*, and we considered it as an infra-specific taxon of *M. sinensis* until both DNA sequence data (Hodkinson *et al*., 1997) and AFLP highlighted its uniqueness. It is endemic to Taiwan and may have diverged sufficiently on a molecular level to merit species recognition. A group consisting of *M. sacchariflorus* and *M.* × *giganteus* accessions is closely related to the major *M. sinensis* group and *M. transmorrisonensis*. The results also support the hypothesis that *M.* × *giganteus* is a hybrid between *M. sinensis* and *M. sacchariflorus*. A hybrid would be expected to inherit approximately equal numbers of AFLP markers from its two parent species. *Miscanthus* × *giganteus* has an intermediate position between *M. sinensis* and *M. sacchariflorus* on the first axis of the PCO analysis (Fig. 2) but is displaced on the second axis, which may indicate that *Miscanthus* × *giganteus* has some unique variation not present in the sampled representatives of its parental species. We do not, therefore, have the exact two parental genotypes of our *M.* × *giganteus* accessions.

Miscanthus oligostachyus (Japan) and *M. nepalensis* (Himalayas) are separated from the rest of the *Miscanthus* species, a result consistent with DNA sequence data (Hodkinson *et al*., 1997). Hodkinson *et al*. (1997) found that *M. oligostachyus* is sister to a core *Miscanthus* group including *M. sinensis*, *M. floridulus* and *M. sacchariflorus*, and should therefore be included in a strictly defined *Miscanthus* group. *Miscanthus nepalensis*, in contrast, should not be classified as *Miscanthus sensu stricto* since it groups more closely with other genera of Saccharinae. The results of the AFLP analysis are broadly congruent with the taxonomic treatment of Lee (1964*b*, *c*, *d*). However, *Miscanthus sinensis* ssp. *condensatus* and *M. floridulus* are embedded within *M. sinensis* (Fig. 4), and their species status is questionable. Nevertheless, on morphological examination, they were found to be correctly identified. *Miscanthus floridulus* is primarily Pacific in its distribution and overlaps with *M. sinensis* in a limited area; *M. sinensis* is distributed mainly within continental southern and eastern Asia as well as Japan, Taiwan and Malaysia. It is morphologically distinguished from *M. sinensis* by its smaller spikelets and longer inflorescence axis in relation to its racemes. Intermediates are evident, and the two species are difficult to separate using quantitative morphometric data (Renvoize *et al*., 1997). The data presented here do not support any obvious division. More *M. floridulus*

individuals need to be added to this analysis to reach a firm conclusion about its species status. *Miscanthus sinensis* ssp. *condensatus*, a taxon endemic to Japan, was separated from *M. sinensis* on the basis of leaf anatomy by Lee (1964*a*) but was considered a subspecies by Koyama (1987). The AFLP analysis presented here also finds no evidence in support of its distinct species status.

Providing a reliable marker system for future plant breeding and utilization

The living collections at ADAS Arthur Rickwood Research Station and RBG Kew represent important resources for future exploitation. The AFLP marker system used in this study has proved to be efficient for screening a large genetic resource collection. If *Miscanthus* is to become a viable biomass crop in northwestern Europe, it must be bred for tolerance of cold weather, particularly in the early part of the growing season. A number of cold-tolerant varieties exist, such as *M. sinensis* ssp. *condensatus*, which appear to be less influenced than other accessions of *Miscanthus* by late spring or early autumn cold weather. The AFLP fingerprints are held in a database and can be assessed further. Using breeding experiments, the markers can be utilized for marker-aided selection and quantitative trait loci analysis (Godwin, 1997; Kearsey, 1997). The AFLP method also proved efficient at identifying horticultural varieties and cultivars of *M. sinensis* and would therefore be useful as a method to demonstrate the uniqueness of newly developed horticultural varieties for patenting purposes. AFLP will undoubtedly have potential in the future to help distinguish cultivars for commercial purposes.

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