

# GENETIC DIVERSITY AND RELATIONSHIPS REVEALED BY AFLP MARKERS AMONG *SACCHARUM SPONTANEUM* AND RELATED SPECIES AND GENERA

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## ABSTRACT

Modern sugarcane cultivars are genetically vulnerable because the narrow gene pool from which they are derived originated from a few inter-specific hybrids between *Saccharum officinarum* and its wild relatives, including *S. spontaneum*. Efforts are being made to broaden the genetic base of cultivated sugarcane through wide crosses with *S. spontaneum* and other related species and genera. The objective of this study was to evaluate genetic diversity and interrelationships among accessions in a collection of *Saccharum* germplasm maintained at the USDA-ARS-SRRC Sugarcane Research Laboratory at Houma, Louisiana, using AFLP markers. The accessions included 21 *S. spontaneum*, three *S. officinarum*, two *S. robustum*, one *S. sinense*, three hybrids (*S. officinarum* x *S. spontaneum* F<sub>1</sub>), 13 cultivars, two cultivar-derived mutants, two *Erianthus* spp., one *Miscanthus sinense*, and one sorghum (*Sorghum bicolor*). Six AFLP primer combinations generated a total of 607 bands. Of these, 595 were polymorphic, with an average polymorphic rate of 98 %. Two multivariate techniques, cluster analysis and multi-dimensional scaling, were used to analyze the data. Cluster analysis portrayed *Sorghum* and *Miscanthus* as distinct from the rest of the entries, which together belong to the so-called *Saccharum* complex. Similar results were revealed by the multi-dimensional scaling biplot. Among the *Saccharum* complex accessions, *S. robustum* was placed in the same cluster as *Erianthus* spp. and shared a closer relationship with *S. officinarum* relative to *S. spontaneum*. *S. sinense* was found in a cluster with some *S. spontaneum* entries, and its proximity to Badila (*S. officinarum*) in the biplot tends to support its hybrid origin. Contrary to previous reports suggesting closer ties with *S. officinarum*, cultivars and hybrids were mostly found in clusters with *S. spontaneum*. This discrepancy is likely attributable to a combination of factors, including the disproportionate number of *S. spontaneum* entries in the study, the complex aneuploid nature of cultivars and hybrids, and the lack of locus specificity of AFLP markers. Similar to previous reports, high levels of genetic diversity (similarity ranged from 0.43 to 0.57) were revealed among the *S. spontaneum* entries with country of origin having no influence on the pattern of diversity.

## INTRODUCTION

Modern sugarcane cultivars are derived mainly from interspecific hybridization between two major *Saccharum* species, namely *S. officinarum* ( $2n = 80$ ) and *S. spontaneum* ( $2n = 40-128$ ) (Irvine, 1999). *S. officinarum* (also known as “noble cane”) is a high sucrose content species, while *S. spontaneum*, is lower in sucrose content but resistant to various biotic and abiotic stresses. The  $F_1$  hybrids of crosses between *S. officinarum*  $\times$  *S. spontaneum* were repeatedly backcrossed to *S. officinarum* to minimize the negative effects of the wild germplasm. This process, termed “nobilization” by sugarcane breeders, is an excellent example of the important contribution of wild germplasm to the genetic improvement and survival of an economically important crop. “Nobilization” stabilized productivity as a result of increased disease resistance, ratooning ability, and adaptability (Roach, 1972). While sugarcane breeding has thrived all over the world largely by intercrossing the original interspecific hybrid clones and their derived progeny, only a few clones were involved in the original crosses. Thus, the genetic diversity in cultivated sugarcane is alarmingly narrow (Berding and Roach, 1987). In the U.S. for example, only two ancestors were found to have contributed germplasm to 90 % or more of the cultivars surveyed from Louisiana (Deren, 1995).

To guard against genetic vulnerability in the Louisiana sugar industry, a basic breeding program was established by the USDA-ARS-SRRC at the Sugarcane Research Laboratory (SRL) with two main objectives: 1) broaden the genetic base of cultivated sugarcane and 2) identify and introgress agronomically useful genes into the cultivated background. The basic breeding component of the SRL sugarcane varietal development program maintains a working collection of about 100 germplasm accessions belonging to the *Saccharum* complex (Mukherjee, 1957), including species, such as *S. officinarum*, *S. spontaneum*, *S. robustum*, *S. giganteum*, *Erianthus* spp., and *Miscanthus* (Mukherjee, 1957; Daniels et al., 1975). A much larger collection of *Saccharum* complex germplasm is held at one of two world collections located in Miami, Florida.

*Saccharum spontaneum* remains the most extensively and widely used source of germplasm in the SRL basic breeding program. *S. spontaneum* clones are better adapted to the temperate climates of Louisiana than *S. officinarum*, which is of tropical origin (Artschwager and Brandes, 1958). Additionally, *S. spontaneum* represents an important source of genes for vigour, ratooning ability, cold tolerance, and host plant resistance to some common diseases of cultivated sugarcane in Louisiana (Dunckelman and Breaux, 1969). However, despite the fairly large number of *S. spontaneum* accessions in the SRL germplasm collection, only a few have actually been used to develop new cultivars. For instance, the *S. spontaneum* accession US56-15-8, collected from the banks of a stream in the Fang District of Northern Thailand, was originally evaluated and hybridized in a quest for a superior source of mosaic resistance (Dunckelman and Breaux, 1972). Today, many commercially recommended varieties in Louisiana, LCP 85-384 (leading commercial variety), HoCP 85-845, L97-128, and HoCP 96-540 have US56-15-8 in their pedigree.

Thus, although the issue of nuclear genetic diversity in sugarcane is being addressed, research into factors that could encourage use of more diverse sources of *S. spontaneum* germplasm in the collection is warranted. Knowledge of the genetic diversity and relationships

among clones in the collection would greatly enhance this effort. The AFLP marker technique (Vos et al. 1995) has been used to assess genetic diversity in sugarcane cultivars and germplasm (Besse et al., 1998; Lima et al., 2002). Recently AFLP markers were used to distinguish tropical and subtropical sugarcane cultivars from India based upon their *S. spontaneum* content (Selvi et al., 2005). The main objective of this study was to characterize the extent and pattern of genetic diversity among a collection of *S. spontaneum* germplasm using AFLP markers. The relationship among members of the *Saccharum* complex is not well understood (Irvin 1999). Therefore, clones of other *Saccharum* species and related genera in the collection were included in the study, the secondary objective of which was to evaluate their interrelationships.

## MATERIALS AND METHODS

### *Plant materials*

A total of 49 accessions representing four genera were sampled from the germplasm collection held at the SRL (Table 1). They comprised the following: 21 *S. spontaneum*, three *S. officinarum*, two *S. robustum*, one *S. sinense*, three hybrids (*S. officinarum* x *S. spontaneum* F<sub>1</sub>), 11 cultivars and two experimental clones from Australia, Bangladesh, China and U.S., two cultivar-derived mutants, one *Miscanthus sinense*, two *Erianthus* spp., and one sorghum (*Sorghum bicolor*). Except for the sorghum that was derived from seed, the accessions were maintained clonally through vegetative propagation. Genomic DNA was isolated from young leaves using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. DNA concentration was checked using a 1 % agarose gel and was standardized to 50 ng/ $\mu$ l.

### *AFLP protocol*

The AFLP protocol was performed as described by Vos et al. (1995) using the LI-COR IR Dye AFLP<sup>®</sup> kit following the manufacturer's instructions with slight modification of the amplification stage. Pre-selective amplification (Step 1) was carried out with a single nucleotide (i.e. *EcoR* I + A and *Mse* I + C) followed by selective amplification (Step 2) using three instead of two selective extension nucleotides. A total of six primer combinations were used (Table 2). The fluorescently-labelled fragments were separated and visualized using LI-COR's Global IR<sup>2</sup> DNA analysis system. Image data was automatically collected and simultaneously recorded during electrophoresis. AFLP<sup>®</sup> fragment scoring was facilitated using the SAGA-MX software and verified manually. Each polymorphic AFLP marker was identified by the primer combination consisting of six letters plus a base pair position. Bands were scored as present (1) or absent (0).

### *Data analysis*

Genetic similarity (GS) was estimated among all pairs of accessions using the Jaccard-similarity coefficient (Jaccard, 1908) as follows:  $GS_{ij} = a/(a+b+c)$  where  $GS_{ij}$  is the genetic similarity measurement between individuals  $i$  and  $j$ , the number of polymorphic bands common to both individuals is represented by  $a$ , whereas  $b$  and  $c$  are the number of bands exclusive to individual  $i$  and  $j$ , respectively. Cluster analysis (CA) was performed on the GS matrix using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm following the Sequential Agglomerative Hierarchical Nested Cluster Analysis (SAHN)

**Table 1.** Characteristics of 49 accessions from a sugarcane germplasm collection held at the USDA-ARS-SRRC sugarcane research laboratory at Houma, Louisiana.

Accession †	Classification	Origin ‡	Accession †	Classification	Origin ‡
HoCP96-540	Cultivar	US	IND81-165	<i>S. spontaneum</i>	India
TucCP77-42	Cultivar	US	SES323A	<i>S. spontaneum</i>	India
US01-40	Experimental	US	SES66-084B	<i>S. spontaneum</i>	India
L98-207	Experimental	US	SES006	<i>S. spontaneum</i>	India
HoCP91-555	Cultivar	US	SES084B	<i>S. spontaneum</i>	India
Q153	Cultivar	Australia	SES147B	<i>S. spontaneum</i>	India
Q158	Cultivar	Australia	SES231	<i>S. spontaneum</i>	India
Q160	Cultivar	Australia	HOLÉS	<i>S. spontaneum</i>	India
Q172	Cultivar	Australia	SPONT17	<i>S. spontaneum</i>	Iran
GX1	Cultivar	China	IMP9068	<i>S. spontaneum</i>	Indonesia
GX11	Cultivar	China	MOL1032B	<i>S. spontaneum</i>	India
GX17	Cultivar	China	MPTH97-213	<i>S. spontaneum</i>	Thailand
ISD25	Cultivar	Bangladesh	MPTH98-326	<i>S. spontaneum</i>	Thailand
NG77-214	<i>Erianthus</i>	New Guinea	PCAV84-12B	<i>S. spontaneum</i>	Philippines
SES372	<i>E. ravennae</i>	India	PQ84-3	<i>S. spontaneum</i>	Philippines
IMP3057	<i>Mischanthus</i>	China	S66-084A	<i>S. spontaneum</i>	Taiwan
BADILA GREEN	<i>S. officinarum</i>	New Guinea	SES234-B	<i>S. spontaneum</i>	India
GERMAN	<i>S. officinarum</i>	New Guinea	US56-15-8	<i>S. spontaneum</i>	Thailand
MUNTOKJAVA	<i>S. officinarum</i>	New Guinea	CUBA	Species hybrid	US
NG51-088	<i>S. robustum</i>	New Guinea	DWARF1 <sup>§</sup>	Cultivar-derived mutant	US
IS76-184	<i>S. robustum</i>	Indonesia	DWARF2 <sup>§</sup>	Cultivar-derived mutant	US
KATHA	<i>S. sinense</i>	China	LAROSE	Species hybrid	US
GUANGXI87-21	<i>S. spontaneum</i>	China	CLOVERLY	Species hybrid	US
GUANGXI87-22	<i>S. spontaneum</i>	China	ATX30-42	Sorghum	US
IND81-144	<i>S. spontaneum</i>	India			

† GREEN GERMAN and MUNTOK JAVA could possibly be species hybrids with *S. officinarum* as one of the parents.

‡ Record from the Germplasm Resources Information Network (GRIN) indicating country of origin.

§ Dwarf 1 and Dwarf 2 are genetic mutants derived from the experimental clone LCP81-137 (Burner, 1999).

module of NTSYS (Sneath and Sokal, 1973). Multidimensional scaling (MDS) analysis was also performed on the GS values to further explore possible underlying dimensions of relationship among the accessions in the study. A minimum-length spanning tree (MST) was superimposed on the MDS plot to help detect local distortion because pairs of points which look close together in a plot may actually be far apart if other dimensions are taken into account. Analyses were performed using NTSYSpc software, version 2.11L (Rohlf, 2000). For the purpose of comparison between clusters and also to determine the robustness of the cluster, bootstrap analysis was done with 10,000 replications using the Nei and Li (1979) method to estimate GS. The PAUP version 4.0b10 software was used for this analysis (Swafford, 2002).

## RESULTS AND DISCUSSION

### *AFLP polymorphism*

In an AFLP analysis of genetic similarity among Brazilian cultivars that used two selective nucleotides in Step 2, the polymorphism rate was on average 50 % per primer combination (Lima et al., 2002). An AFLP marker study with 28 Indian cultivars had an average polymorphism rate of 63 % per primer combination (Selvi et al., 2005). The Indian cultivars have more *S. spontaneum* clones in their genetic background relative to the Brazilian cultivars. In our study, the high stringency imposed (by using three instead of two selective nucleotides) reduced the total number of amplified fragments compared with a trial run where two selective nucleotides were used. Furthermore, it was generally easier and more straightforward to score and validate gels produced using three instead of two selective nucleotides.

Notwithstanding the stringency imposed in Step 2, sufficient polymorphism was revealed using six primer combinations (Table 2). The six primer combinations amplified a total of 607 fragments, of which 595 (98 %) were polymorphic. The most polymorphic primer pair amplified a total of 182 fragments, all of which were polymorphic. The least polymorphic primer combination amplified 45 fragments, of which 40 (89 %) were polymorphic.

**Table 2.** AFLP primer combinations, number of bands amplified, and the number and percentage of polymorphic bands revealed among 49 *Saccharum* germplasm accessions.

Primer pair	No. of bands amplified	No. of polymorphic bands	Polymorphism (%)
E-AACC/M-CCAC	182	182	100
E-AAAG/M-CCAA	75	74	99
E-AACG/M-CCAA	104	103	99
E-AACA/M-CCTA	105	104	99
E-AACC/M-CCTG	45	40	89
E-AAGG/M-CCTG	96	92	96
Total/average	607	595	98

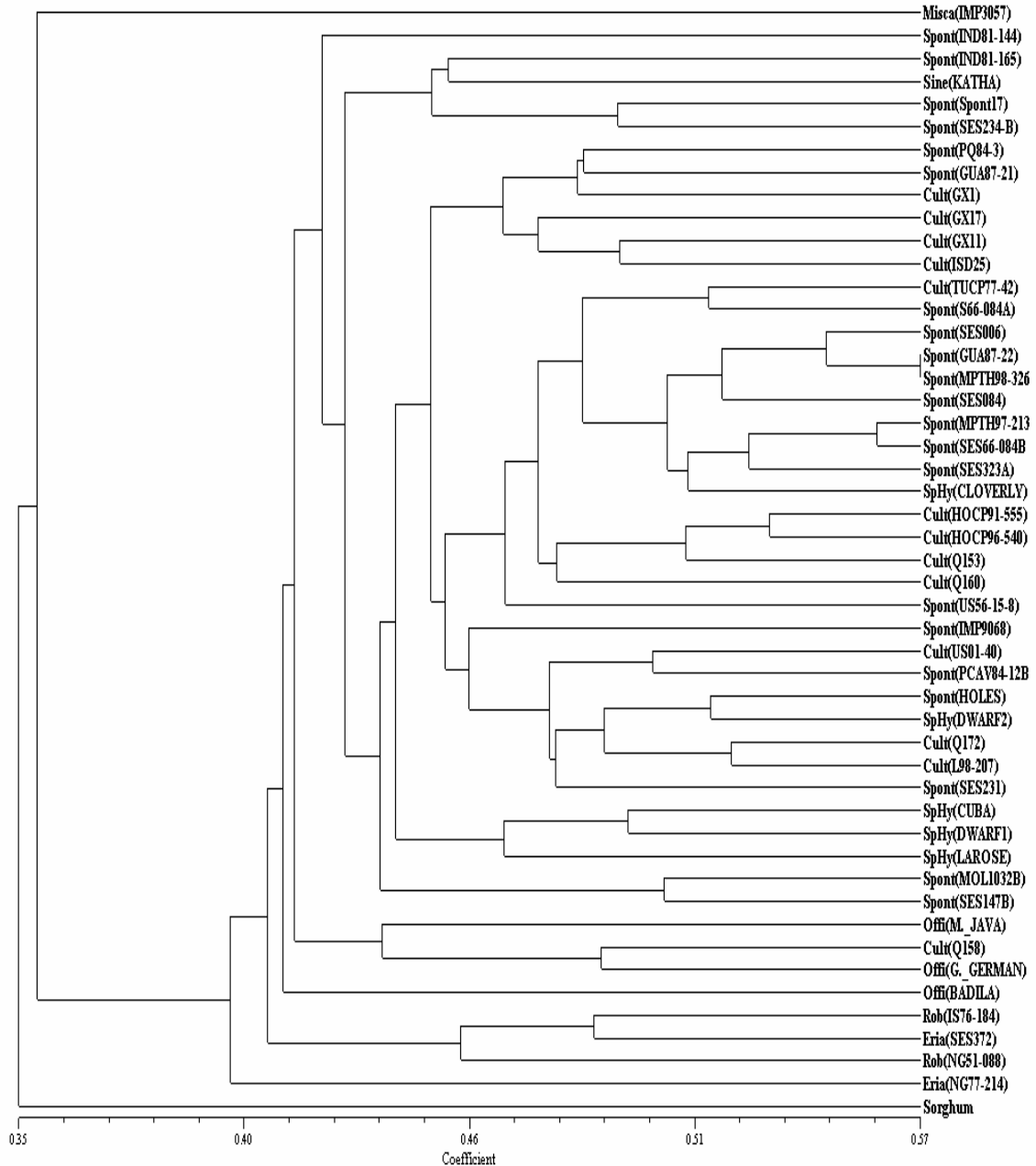
### *Genetic diversity and relationships among species and genera*

*Sorghum* and, to a lesser extent, *Miscanthus* were identified as ‘out-groups’ relative to members of the so-called *Saccharum* complex (Mukherjee, 1957) (Fig. 1). Bootstrap analysis, based on the 50 % majority-rule, revealed three distinct groups comprising *Sorghum*,

*Miscanthus*, and members of the *Saccharum* complex with no clear distinction among the members of the *Saccharum* complex (Fig. 1). However, the genetic distance between *Sorghum* and *Miscanthus* vs. the *Saccharum* complex (0.35 – 0.40) relative to distances among some clones within the *Saccharum* complex (0.40 – 0.57) indicates that *Sorghum* and *Miscanthus* share some level of synteny with members of the *Saccharum* complex. Paterson et al. (1995), Ming et al. (1998, 2002), and Jordan et al. (2004), using comparative mapping and co-linearity analysis found sorghum to be the closest relative of sugarcane relative to other cultivated grass species, while Daniels et al. (1975) have argued for the inclusion of *Miscanthus* sect. *Diandra* Keng into the *Saccharum* complex.

As mentioned earlier, no clearly discernable clusters or groups were apparent among species within the *Saccharum* complex (Fig. 1) although the portrayal of this in the cluster and MDS plot (Figs. 1 and 2) appeared to be more pronounced when *Sorghum* and *Miscanthus* were included in the analysis. Dropping *Sorghum* and *Miscanthus* from the analysis made the results portrayed in Figs. 1 and 2 easier to visualize (Data not shown). Ignoring the 50 % majority rule, the first group within the *Saccharum* complex comprised a loose association among the two *S. robustum* and two *Erianthus* clones (Fig 1). *Erianthus* clone SES 372 grouped closer to *S. robustum* clones IS 76-184 and NG51-088 than to *Erianthus* NG77-214 (Fig. 1). In the MDS plot, the *Erianthus* NG77-214 and *S. robustum* NG51-088 clones were not at close proximity and appeared at the fringes of the *Saccharum* complex group. The old world *Erianthus* clones (NG77-214 and SES 372) used in this study, are generally accepted as a separate but related genus and form part of the *Saccharum* complex. New world *Erianthus* species (e.g. *E. giganteus*; not included in this study), on the other hand, are not recognized as a separate genus by some authors and have been classified as *S. giganteum* (Webster and Shaw, 1995). Using AFLP markers, Besse et al. (1998) placed the Old world *Erianthus* with *Saccharum* species in the same group and the New World *Erianthus* in a distinct group. Using SSR markers, Cordeiro et al. (2003) placed *Erianthus* species in a group separate from *Saccharum* species, but there was a clear distinction between the Old and New world *Erianthus* species.

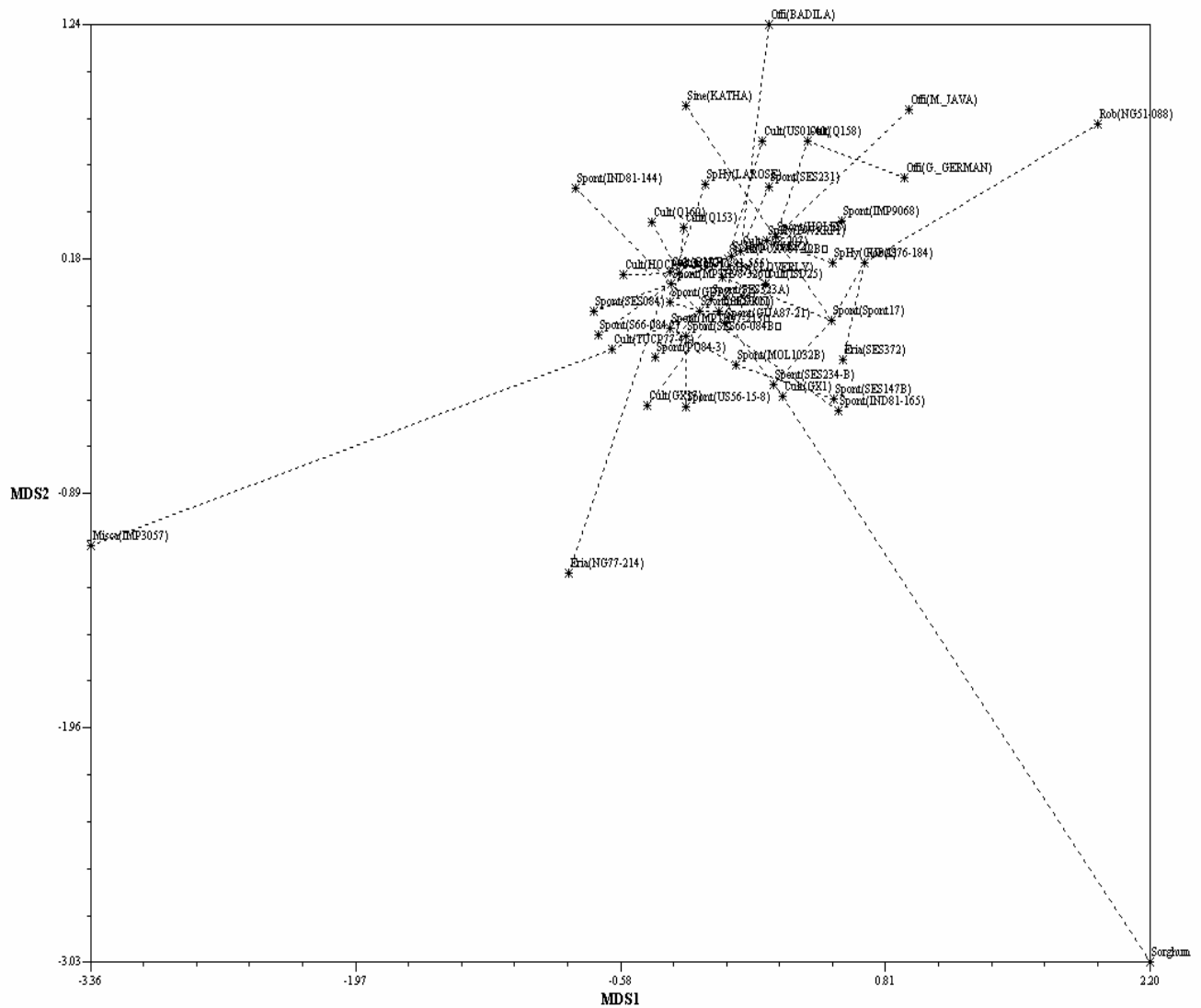
It was surprising that the *S. robustum* clones were closely aligned with *Erianthus* compared with *S. officinarum*, cultivars, and species hybrids (Fig. 2). However, they shared a closer relationship with *S. officinarum* compared with *S. spontaneum*. *S. robustum* has been cited as a likely progenitor of *S. officinarum* (Brandes, 1958; Sreenivasan et al., 1987). Target region amplification polymorphism (TRAP) (Hu and Vick, 2003) marker analysis showed *S. robustum* to be closely aligned with *S. officinarum* compared with *Erianthus* (Alwala et al., 2006). However, Daniels and Roach (1987) described *S. robustum* as a group of plants in flux crossing among themselves and other genera, including *Erianthus*. *S. robustum*, as a group, has the widest distribution and the greatest variation in New Guinea with some morphological characteristics of *Erianthus* (Krishnamurthi and Koike, 1982).



**Figure 1.** Genetic relationships among a collection of sugarcane germplasm accessions as revealed using AFLP markers and cluster analysis.

The *S. officinarum* clones along with a cultivar, Q158, from Australia formed the next group of *Saccharum* complex species. Badila was found on a single branch in the dendrogram (Fig. 1), and in the MDS plot (Fig. 2), the other two *S. officinarum* clones, Green German and Muntok Java, appeared closer to each other than to Badila. Green German and Muntok Java were found in the same branch as the cultivar, Q158 (Fig. 2). Although listed as *S. officinarum* at the USDA, ARS, Sugarcane Field Laboratory, Canal Point, FL, chromosome numbers of  $2n > 80$  have been recorded for these Green German and Muntok Java clones, casting doubt as to their

classification as true *S. officinarum* (Ming et al., 2002). They could possibly be species hybrids of unknown ancestry but with *S. officinarum* as one of the parents, which may explain their clustering behavior relative to Badila.



**Figure 2.** Genetic relationships among a collection of sugarcane germplasm accessions as revealed using AFLP markers and multidimensional scaling analysis (nearest neighbour module).

The single *S. sinense* clone, Katha, was found in the next group along with some *S. spontaneum* clones (Fig. 1). In the MDS plot (Fig. 2) it was found in close proximity to Badila (*S. officinarum*), and the minimum spanning tree linked it with a *S. spontaneum* clone, Spont 17. *S. sinense* are believed to be natural hybrids between *S. officinarum* and *S. spontaneum* (Daniels and Roach, 1987; D’Hont, 2002), and sugarcane and maize-derived microsatellite markers placed *S. sinense* clones in clusters intermediate between *S. officinarum* and *S. spontaneum* clusters (Cordeiro et al., 2003; Selvi et al., 2003).

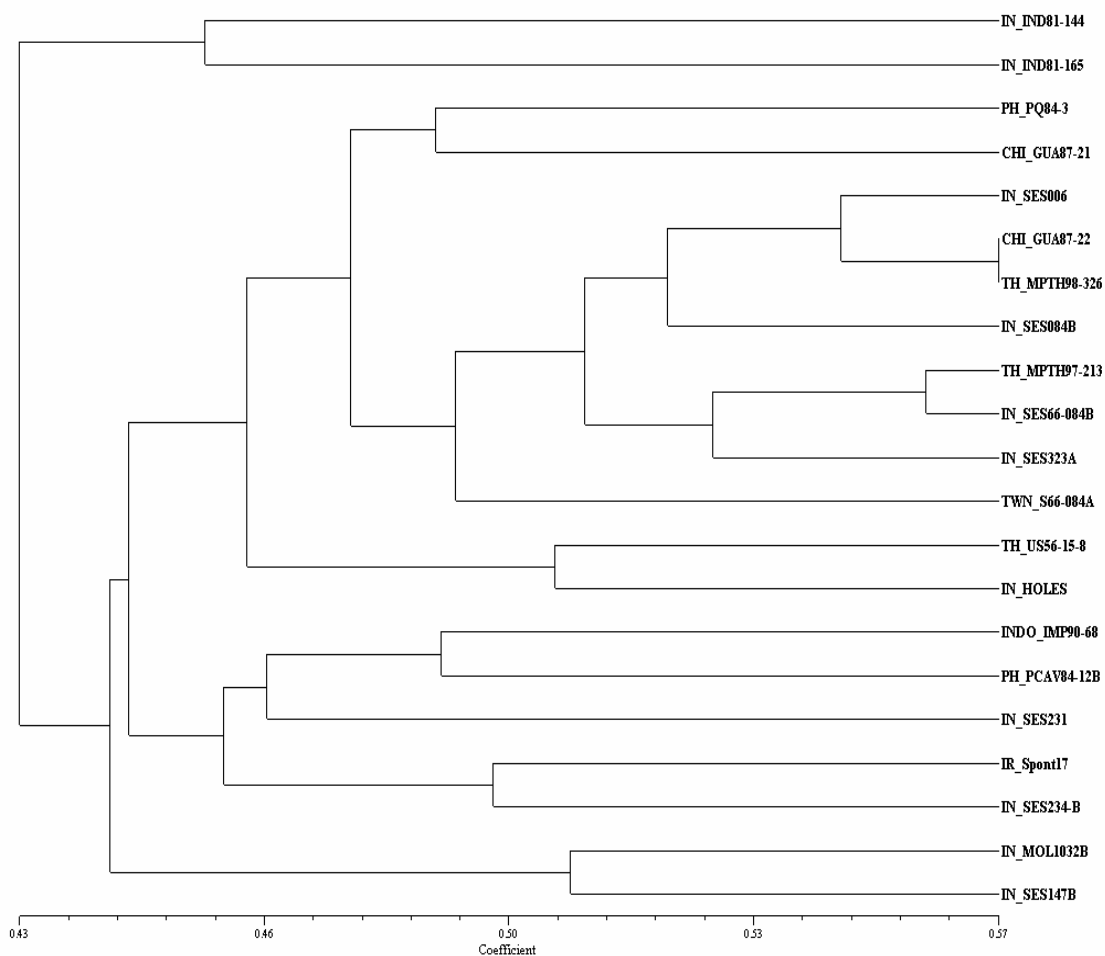
All cultivars and species hybrids, except Q158, were grouped with *S. spontaneum* clones (Fig. 2). These results were not intuitive because cultivars are known to have inherited 80 % of their genome from *S. officinarum* and only 20% from *S. spontaneum* (Bhat and Gill, 1985; Bremer, 1961; D'Hont et al., 1994; 1996). These disparities could likely be attributed to the disproportionate number of *S. spontaneum* clones included in the study. When studying interspecies relationships, methods such as that used in this study would probably function more accurately when equal numbers of entries are used for each group (species). A similar remedy was recommended to correct for the so-called 'long branch attraction' where lineages are inferred to be closely related, regardless of their true evolutionary relationships (Felsenstein, 2004).

The *S. spontaneum* clone, US56-15-8, was used in the development of many recent Louisiana cultivars and as expected was found in the same sub-cluster with two of the three Louisiana cultivars. Two cultivars from Australia also were found in this sub-cluster. On the other hand, the *S. spontaneum* clone SES 147b is listed in the ancestry of the cultivar TucCP 77-42, but they were placed in separate and distant sub-clusters (Fig. 1). Mislabelling or the effects of selection during breeding may be responsible for these results (Skinner et al., 1987). However, selection alone cannot adequately explain why the two mutants, Dwarf 1 and Dwarf 2, both believed to be derived from the same cultivar (Burner, 1999), were placed in different sub-clusters. Dwarf 1 and Dwarf 2 were the most closely related entries in our previous study in which TRAP markers were used (Alwala et al., 2006), and selection was one reason proposed to explain why they clustered together in the TRAP study. The aneuploid nature of sugarcane cultivars, in which different sets of chromosomes may be missing in different cultivars, could also explain the general lack of conformity in the way the cultivars clustered. This is further complicated by the fact that AFLP markers are not locus specific, and co-migration is possible between similar size bands of different sequences.

#### *Pattern of genetic diversity among S. spontaneum clones*

Our results revealed a substantial amount of genetic diversity among the *S. spontaneum* clones surveyed. *S. spontaneum* is generally considered the most diverse species in the genus *Saccharum* with its natural ecosystem extending from Japan and New Guinea through the Indian subcontinent to the Mediterranean and Africa (Daniels and Roach, 1987). This diversity also is exhibited by its wide polyploid series with chromosome numbers ranging from  $2n = 40$  to 128, with the most frequent counts being  $2n = 48, 64, 80,$  and 96 (Irvine, 1999). Genetic similarity coefficient among the *S. spontaneum* clones in this study ranged from 0.43 to 0.57 (Fig. 3) which is low compared to the 0.64 to 0.92 range reported among elite Louisiana varieties using similar AFLP markers (Kimbeng et al., 2003). Similar to reports by Pan (2000, 2004) and Tai (1995), the pattern of clustering among the *S. spontaneum* clones were irrespective of country of origin (Table 1; Fig. 3). A few *S. spontaneum* clones from India clustered together, but others from the same regions grouped elsewhere. We consider this (the apparent clustering of clones from India) an artefact stemming from the disproportionate number of clones from India in this study. The MDS biplot (Fig. 4) similarly reflected the substantial variability among the *S. spontaneum* clones and the failure of country of origin to influence the grouping pattern. The results from this study with regards to the pattern of diversity observed among the *S. spontaneum* clones will help inform strategies to maintain and utilize genetically diverse clones in the collection for basic

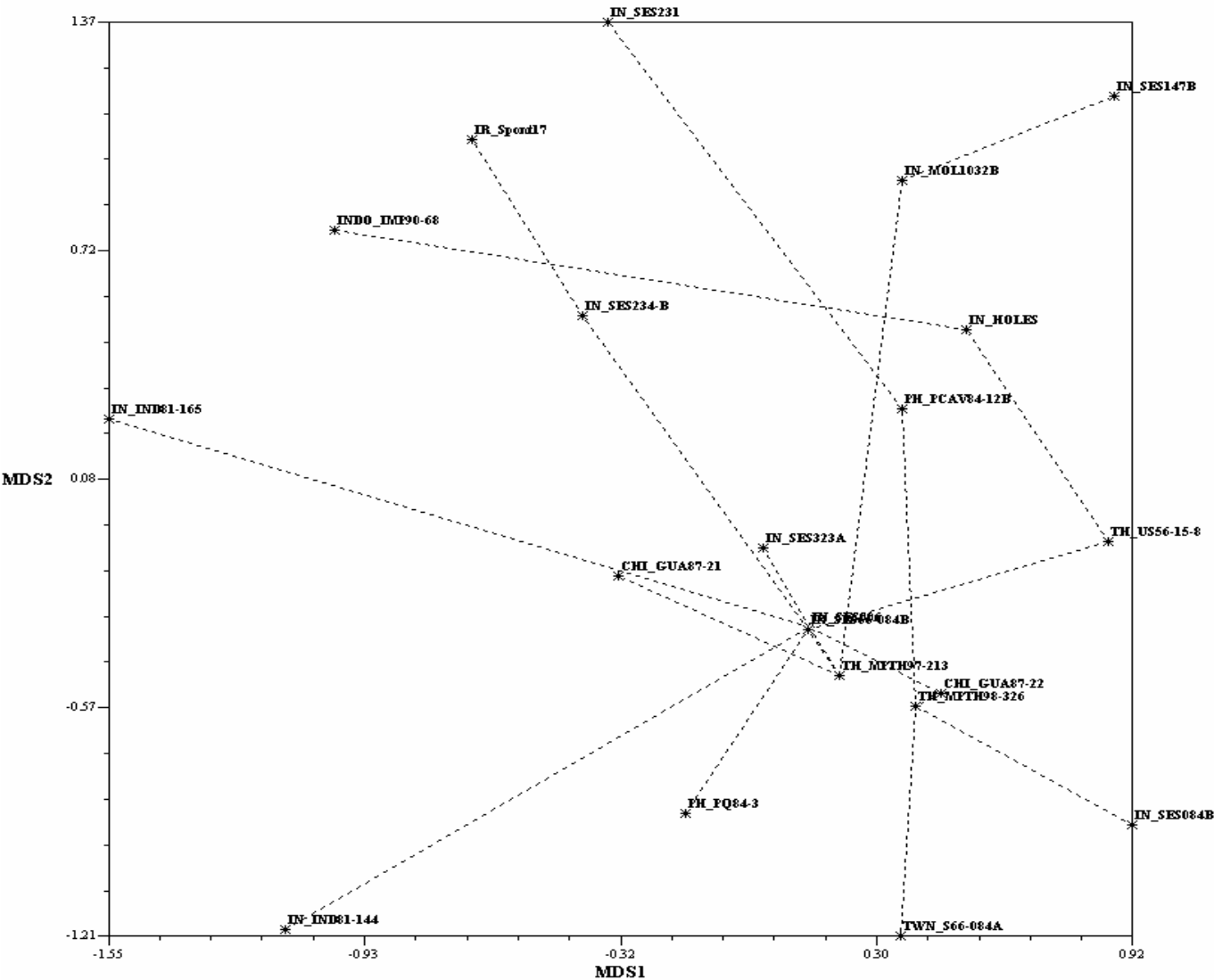
breeding. For example, when the objective is to increase genetic diversity, less emphasis will be placed on country of origin during sampling of *S. spontaneum* clones.



**Figure 3.** Genetic relationships among 21 *S. spontaneum* accessions as revealed using AFLP markers and cluster analysis.

### CONCLUSIONS

These results confirm the utility of AFLP markers to study genetic diversity and interrelationships among clones in a *Saccharum* germplasm collection. Analysis of AFLP marker polymorphism classified *Sorghum* and *Miscanthus* as ‘outgroups’ relative to species within the *Saccharum* complex. However, the analysis revealed some relationships among *Saccharum* complex species that were not consistent with previous results and expectations. The differing relationships found in this study are inconclusive, since species were represented by vastly disproportionate numbers of clones which possibly may have contributed to the inconsistencies. Finally, the results were consistent with previous reports in revealing high levels of genetic diversity among *S. spontaneum* clones in the collection and the failure of country of origin to influence the pattern of this diversity.



**Figure 4.** Genetic relationships among 21 *S. spontaneum* accessions as revealed using AFLP markers and multidimensional scaling analysis (nearest neighbor module).

**ACKNOWLEDGMENTS**

This work was funded through grants from the USDA-NRI Program and the American Sugar Cane League of the U.S.A, Inc. Jie Arro received vital training on molecular marker techniques at the laboratory of Dr. M. Timko (Dept. of Biology, University of Virginia, Charlottesville, VA). Dr. S. Edme (USDA, ARS, Sugarcane Field Laboratory, Canal Point, FL), and anonymous reviewers provided critical comments on the manuscript. All are gratefully acknowledged.

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