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Molecular Dissection of the 5S Ribosomal RNA-Intergenic Transcribed Spacers in *Saccharum* spp. and *Tripidium* spp.

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Abstract: Due to complex polyploid, sugarcane whole genome sequencing and characterization lag far behind other crops. PCR-based DNA markers are a viable low-cost option to evaluate genetic diversity and verify genotypes. In this study, the 5S ribosomal RNA-intergenic spacer (ITS) of 171 accessions of *Saccharum* spp. and *Tripidium* spp. was dissected, including 30 accessions of *S. officinarum*, 71 of *S. spontaneum*, 17 of *S. robustum*, 25 of *S. barberi*, 13 of *S. sinense*, 2 of *S. edule*, 5 sugarcane cultivars (*Saccharum* spp. hybrids), 6 of *Tripidium* spp. (formally *Erianthus* spp.), and 2 of unknown species. The ITS spacers were amplified from 10 ng of the leaf DNA of each accession with the universal PCR primers PI and PII. The PCR-amplified spacers (amplicons) were analyzed by both agarose gel and capillary electrophoresis (CE). While agarose gel electrophoresis revealed five banding patterns, a total of 42 polymorphic amplicons, ranging from 60 to 506 bp, were detected by CE. Three amplicons, 234-, 235-, and 236-bp in size, were amplified from all accessions of six *Saccharum* species, except for three *S. robustum* accessions (Molokai 5573, NG 57-054, and NG 77-235) that lacked the 236-bp amplicon. The 234-, 235-, 236-bp banding pattern found in *S. spontaneum* was less consistent than other *Saccharum* species, sometimes missing a few but not all the bands in this region. An amplicon of 61-bp was amplified only from the sugarcane hybrid varieties. The PI/PII patterns indicated diversity and subpopulations within *Saccharum*, which could potentially be used in Breeding. Moreover, all *Saccharum*-specific amplicons were mostly absent in *Tripidium* spp. accessions, which produced 405-bp and 406-bp amplicons, and any pattern of the exceptions indicated misidentification. The *T. bengalense* accession Kalimpong had a unique CE-banding pattern that was different from all other accessions. Although the clustering pattern of the 42 amplicons only discriminated at the genus level, these amplicons helped identify nine misclassified accessions. This study further demonstrates that these PI/PII amplicons could be particularly useful markers for breeders at sugarcane field stations to quickly confirm and discriminate among the accessions of germplasm collections.

Keywords: 5S rRNA-ITS; capillary electrophoresis; germplasm; sugarcane; *Saccharum* spp.; *Tripidium* spp.

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1. Introduction

Sugarcane is thought to have originated in New Guinea where a rich source of *Saccharum* germplasm exists in East Indonesia/New Guinea [1]. Modern sugarcane cultivars are complex interspecific hybrids of *Saccharum officinarum* and *S. spontaneum*, although *S. robustum*, *S. barberi*, and *S. sinense* may also have contributed genes to the modern sugarcane cultivars [1]. *Saccharum*, *Erianthus*, *Miscanthus*, and *Sclerostachya* are so closely related that Mukherjee [2] referred to them as the '*Saccharum* Complex'; however, *Erianthus* was

renamed as *Tripidium* [3] and taken out from the '*Saccharum* Complex' by Lloyd Evans et al. [4] based on the chloroplast genome sequence. Berding and Roach [5] remarked that all sugarcane cultivars originated from a few parental clones. Later, Arro et al. [6] further emphasized that modern sugarcane cultivars (*Saccharum* spp. hybrids) were genetically vulnerable because of their origination from a few interspecific hybrids between *S. officinarum* and *S. spontaneum*. A local breeding germplasm collection at the USDA-ARS, Sugarcane Research Unit (SRU) in Houma, Louisiana, USA, and the World Collection of Sugarcane and Related Grasses (WCSRG) at the USDA-ARS, Sub-tropical Horticulture Station in Miami, Florida, USA, have a large number of *Saccharum* and related species accessions for use as germplasm in sugarcane introgression breeding programs. The USA sugarcane breeding programs are continuously introgressing wild relatives to broaden the genetic base of sugarcane [7]. Additional accessions are still being added, so it is necessary to identify the material being imported. Since phenotypic identification can be difficult for species where the plants are required to be in flower [8], and introgression efforts are needed to accurately identify species because some germplasm accessions are more difficult to introgress [4], DNA markers that can quickly and inexpensively identify taxonomy are needed, such as those developed from the rapidly evolving non-transcribed intergenic spacers of 5S rRNA.

The 5S rRNA of higher eukaryotes is primarily organized in tandem arrays that have a transcription unit and non-transcribed intergenic spacer sequence. The 5S rRNA is highly conserved in length and sequence, whereas its non-transcribed spacer changes more rapidly [9]. The variability of the non-transcribed spacers of the 5S rDNA has been found to be useful in phylogenetic studies in several species, including *Arabidopsis* [9], *Capsicum* [10], flax [11], pea [12], rye [13], sea barley [14], sugar beet [15], sugarcane [16–18], wheat [19], and even animal species such as mussels [20]. Cox et al. [19] developed PI/PII primers from the conserved 5S-rRNA locus to amplify the more variable non-transcribed intergenic spacers to rapidly find inter- and/or intra-specific variations within species. These primers were used by Pan et al. [17] to study the phylogenetic relationship among *Saccharum* and related taxa and evaluate the markers' ability to discriminate between species. The 5S rRNA has been frequently used to identify the intergeneric hybrids between *Tripidium* and *Saccharum* [16,21–24]. The 5S rRNA has also been used to identify chromosomes in genetic cytology experiments. In 2021, Fu et al. [25] conducted sequential fluorescence in situ hybridization (FISH) experiments with maize chromosome painting probes to establish the karyotype of *T. arundinaceum*. They also localized the 5S rDNA and 35S rDNA on chromosomes 5 and 6, respectively, in a putative hexaploid *T. arundinaceum* accession Hainan92-77 ($2n$ = 60, x = 10). In 2022, Wu et al. [26] developed three *E. arundinaceus*-specific DNA markers, Ea086-128, Ea009-257, and EaITS-278, covering all *E. arundinaceus* chromosomes based on suppression subtractive hybridization (SSH) and fluorescence in situ hybridization (FISH) experiments. The Ea086-128 and Ea009-257 markers were used to identify authentic F_1, BC_1, BC_2, BC_3, and BC_4 progenies from crosses between sugarcane and *E. arundinaceus*.

This study was launched to expand on the earlier work of Pan et al. [17] by surveying many more accessions of the six *Saccharum* species (*S. officinarum*, *S. robustum*, *S. spontaneum*, *S. barberi*, *S. sinense*, and *S. edule*), sugarcane cultivars (*Saccharum* spp. hybrids), and three *Tripidium* spp. from a local germplasm collection at the USDA-ARS, SRU, Houma, Louisiana, and the WCSRG, Miami, Florida. The goals were to identify the genetic diversity among this germplasm based on the length polymorphism of 5S rRNA-ITS and to identify DNA markers for taxonomic identity from a broader spectrum of germplasm.

2. Materials and Methods

2.1. Plant Materials

A total of 171 accessions were included in this study (Table 1), including 30 accessions of *S. officinarum*, 17 of *S. robustum*, 71 of *S. spontaneum*, 25 of *S. barberi*, 13 of *S. sinense*, 2 of *S. edule*, 5 of *Saccharum* spp. hybrids (sugarcane cultivars), 8 of *Tripidium* spp. (formally

Erianthus spp.), and 2 of unknown species. Leaf tissues were collected from plants from the germplasm collection in Houma, Louisiana, USA (85 accessions), or the WCSRG in Miami, Florida, USA (86 accessions). During the collection, the leaf samples were placed inside sealing plastic bags and kept on ice in a cooler. The bags were transferred to a −80 °C freezer until DNA extraction upon returning to the lab.

Table 1. A list of 171 accessions from a local sugarcane germplasm collection (Houma, Louisiana) and the World Collection of Sugarcane and Related Grasses (Miami, Florida).

Genus	Species	Accession	Total	Gel Banding Type
Tripidium	*arundinaceum*	IS76-218, MPTH97-194, MPTH98-283, MPTH98-326	**4**	**IV**
	bengalense	IMP9751	**1**	**IV**
	procerum	Kalimpong	**1**	**V**
Saccharum	*officinarum*	Akoki24 *, Badila Fiji *, Badila Java *, Badila, Bandjermasin Hitam *, Big Ribbon *, EK02 *, Fiji1, Fiji24 *, Fiji47, Green German*, Haw Orig 36 *, IJ76-418B *, IJ76-521 *, IM76-245 *, IN84-024 *, IN84-68A, Louisiana Purple*, Mentor4745, Muntok Java, NG21-003 *, NG21-017 *, NG57-223 *, NG77-066 *, NG77-142 *, NG77-241 *, NG96-024 *, NH70-069 *, Oi Deng, Saipan *	**30**	**I, II**
	spontaneum	Coimbatore, Dacca *, Djatiroto, Glagah *, GuangXi86-5, GuangXi87-21, GuangXi87-22, IMP9068, IMP9089, IN76-086 *, IN84-010 *, IN84-033 *, IN84-089 *, IN84-21, IND81-043 *, IND81-101 *, IND81-144, IND81-155 *, IND81-161, IND81-80, IND82-257A, IND82-311, IS76-121 *, Isiolo *, JW570, JW599, M. Moentai *, MOL1032A, MOL1032B, MPTH97-003, MPTH97-113, MPTH97-200, MPTH97-204, MPTH97-209, MPTH97-213, MPTH97-216, MPTH97-218, MPTH97-233, MPTH97-461, MPTH98-388, MPTH99-476, NG77-169 *, Okinawa #01 *, PCANOR84-2A, PCAV84-12A, PCAV84-12B, PCAV84-12C, PIN84-1B, PO84-3, PPGN84-08 *, S001 *, S66-121A, S66-84A, S66-84B, SES006, SES114, SES147B, SES189, SES205A, SES231, SES297B *, SES323A, SES84-58, S spont Pakistan *, Saudi Arabia *, SM7916 *, Tainan, US56-013-07 *, US56-15-1, US56-15-8, US57-141-05 *	**71**	**I, II**
	robustum	IJ76-339 *, IJ76-534 *, IM76-232 *, IN84-045 *, IN84-076 *, M3035/66 *, Molokai 5573, NG28-289 *, NG57-054, NG57-055 *, NG77-021 *, NG77-084 *, NG77-147 *, NG77-159 *, NG77-235 *, NH70-015 *, Teboe SalakToewa *	**17**	**I, II, III**
	barberi	Agoule *, Chin, Chunnee *, Dark Pindaria *, Dhaula, Ganapathy, Hatuni, Hemja *, Hullu Kabbu *, Kalari, Katha, Khagzi, Manga sic *, Nargori *, Panura, Pathri *, Paunra *, Rena, Rhea Sport, Rounda *, Ruckri *, Semari *, Sunnabile *, Tereru *, White Pararia *	**25**	**I, II**
	sinense	Chuk Che *, Khakai *, Lu Cane *, Mcikum, Merthi Zell, Mia Lan *, Nepal 3 *, Tanzhon bamboo *, Tekcha Okinawa *, Tukuyu 1, Uba Del Natal *, Uba India *, Uba Naquin *	**13**	**I, II**
	edule	IJ76-375 *, NG77-010 *	**2**	**I**
	spp. hybrids	Ho01-564, HoCP00-950, HoCP04-838, HoCP96-540, LCP85-384	**5**	**III**
	Unknown	US4515-9200, SPS-269	**2**	**II**

* Indicates accession was from World Collection of Sugarcane and Related Grasses.

2.2. DNA Extraction and Quantification

Leaf tissues were homogenized in cetyltrimethylammonium bromide (CTAB) buffer, either by a Bead-Beater device [www.biospec.com (accessed on 25 April 2022)] for the samples collected in Houma, LA, or by manual grinding in liquid nitrogen with mortar/pestle for the samples collected in Miami, FL. DNA was extracted following a modified protocol of Doyle and Doyle [17,27]. All DNA samples were aliquoted and stored in −20 °C freezers until use. Prior to the study, DNA concentrations were determined on NanoDrop™ [www.thermofisher.com (accessed on 25 April 2023)], re-checked by 2.0% agarose gel elec-

trophoresis in 0.5X TBE buffer, and manually adjusted. The concentrations of all working samples were approximately 10 ng/μL upon dilution with sterile distilled water.

2.3. PCR Amplification, Agarose Gel, and CE Analyses

The oligonucleotide sequence of primer PI was 5′ TGGGAAGTCCT(C/T)GTGTTGCA and of PII was 5′ (T/G)T(A/C)G(T/C)GCTGGTATGATCGCA [19]. Primer PI was labeled with 6-carboxy-fluorescein (FAM) to enable the detection of PCR amplicons by capillary electrophoresis (CE). PCR reactions were conducted in 96-well PCR plates on a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a 25-μL volume. Each PCR reaction mix contained 12.5 μL of GoTaq-Green buffer (Promega, Madison, WI, USA), 2.1 μL each of PI and PII, 1 μL of DNA (or H_2O as the negative control), and 7.3 μL of H_2O. The thermal cycling program was set at 95 °C for 5 min, with 40 cycles of (93 °C for 55 s, 60 °C for 10 s, and 72 °C for 40 s) and a final extension at 72 °C for 10 min, and then held at 4 °C. PCR reactions were repeated three times. Upon completion of PCR, 6 μL of reaction mix was separated in 100 mL of 2% agarose gels stained with 10 μL of SYBR Safe DNA Gel Stain (ThermoFisher Scientific, Carlsbad, CA, USA) for 60 min at 80 volts. Gels were visualized and images were taken on a UVP UVsolo touch Imaging System [Analytik Jena US, Upland, CA, USA) following the manufacturer's instructions. The GeneRuler™ 100 bp DNA ladder (ThermoFisher Scientific, Waltham, MA, USA) was used as size markers.

To remove the green dye, the PCR amplicons of the remaining 19 μL reaction mix were precipitated by mixing with 60 μL of cold 95% ethanol, spinning at 4000 rpm for at least 15 min in a precooled centrifuge, gently inverting the plates to drain the supernatant, and briefly spinning the plate to collect the PCR amplicons. The steps were repeated one more time. After air drying, the PCR amplicons were rehydrated with 20 μL of dH_2O. A spot check on the NanoDrop™ showed an average concentration of 90 ng/μL and a 260/280 ratio of 1.8. A Hamilton's Microlab Star Liquid Handling Station (Hamilton Company, Reno, NV, USA) was used to dilute the purified PCR amplicons 75-fold with H_2O. One μL of the diluted amplicon solution was mixed with 9 μL of Hi-Dye formamide/GeneScan™ 1200 LIZ™ dye Size Standard (Applied Biosystems, Inc., Foster City, CA, USA) to make up each CE analysis solution. The CE solutions in 96-well plates were subjected to CE on an ABI3730xl DNA Analyzer following the manufacturer's instructions (Applied Biosystems, Inc., Foster City, CA, USA). During the CE process, the separation of the amplicons was recorded automatically into GeneScan files, which were processed with GeneMarker™ Software V1.8 (SoftGenetics, LLC, State College, PA, USA) to visualize the amplicons. The sizes of the amplicons were calibrated automatically by the GeneScan™ 1200 LIZ™ size standards. The presence of an amplicon was given a score of "1" while its absence was given a score of "0", which were recorded in a binary Excel data sheet.

2.4. Data Analysis

The software Orange v 3.35 [28], a machine learning and data mining suite for data analysis through Python scripting and visual programming, was used for data analysis. The binary CE data for amplicon's presence (1) or absence (0) were used to create a distance matrix using Manhattan distance function and the distance matrix was used to construct a classical hierarchical clustering tree using a hierarchical clustering function based on complete linkage algorithm.

3. Results and Discussion

When the PI/PII-primed PCR reaction mixtures were analyzed by 2% agarose gel electrophoresis, there were five banding types across all the accessions. Eighty-four accessions shared Type I with only an intensely stained band of about 235 bp in size (Figure 1, lane 2), 89 accessions shared Type II with two bands, the 235 bp band and a faint band of about 540 bp (lane 3), 9 accessions had Type III with two bands, the 235 bp band and a faint band of about 60 bp (lane 4), 6 accessions shared Type IV with an intensely stained band of about 410 bp (lane 5), and Type V was presented only by the *T. procerum* accession

Kalimpong with two bands, the 410 bp band and a faint band of 510 bp (lane 6). It is noted that the Types I and II banding patterns were not species-specific, but by accessions from all *Saccharum* species (Table 1). The Type III banding pattern was only observed for *Saccharum* spp. hybrids. These three banding types of PI/PII amplicons were also observed in the agarose gel electropherograms reported from earlier studies in sugarcane (see Figure 2 in [16] and Figure 3 in [29]).

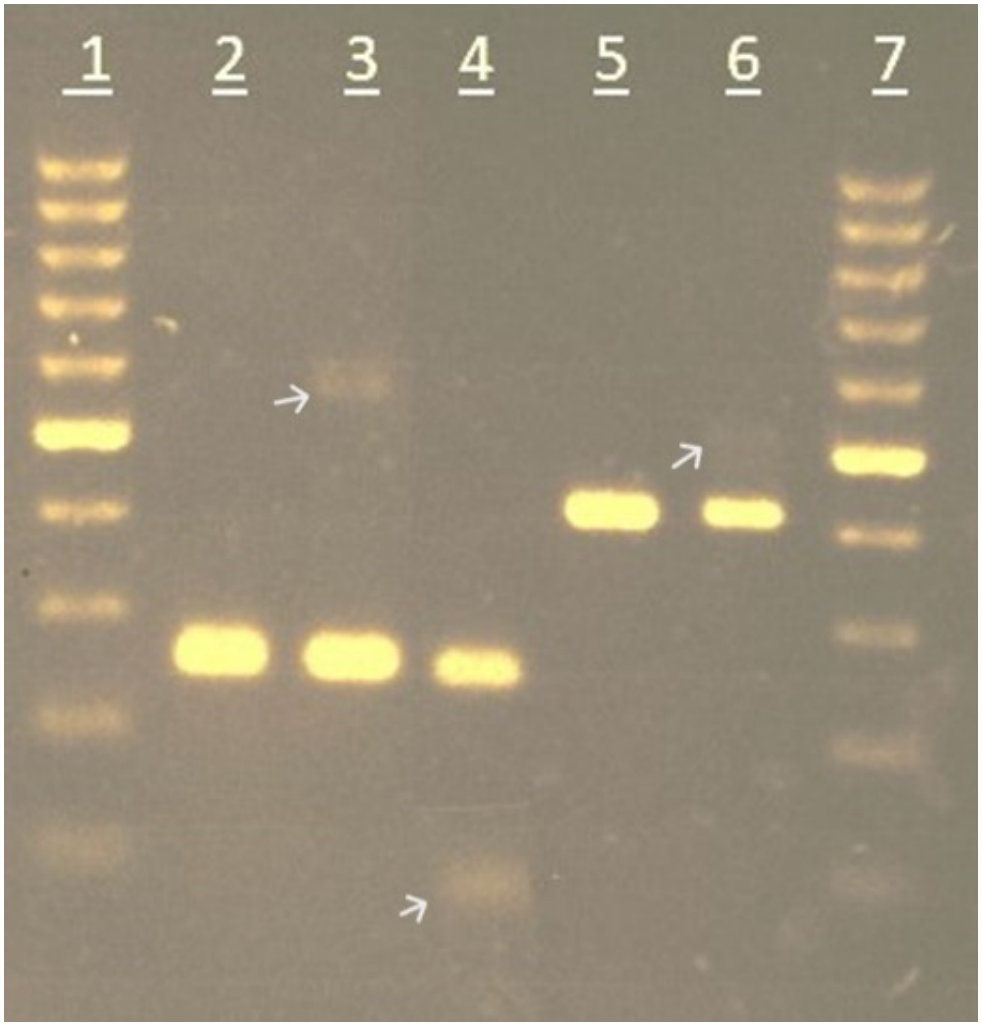

Figure 1. Five types of Apex gel dye-stained agarose gel electropherograms of FAM-PI/PII-primed PCR amplificons of sugarcane leaf tissue DNA. Lane designations: 1 and 7, GeneRuler 100 bp DNA Ladder (from top down, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp, respectively); 2, IJ 76-521 (*S. officinarum*) (Type I); 3, SES 84-58 (*S. spontaneum*) (Type II); 4, M 3035-66 (*S. robustum*) (Type III); 5, MPTH 97-194 (*T. arundinaceum*) (Type IV); and 6, Kalimpong (*T. procerum*) (Type V).

Five types of CE electropherograms were also revealed by the GeneMarker^{TM} V1.8 software among the 171 <.fsa> run files, which were in agreement with the five banding types from agarose gel electrophoresis (Figure 2). However, in contrast to agarose gel electrophoresis, the same PI/PII amplicons were detected by CE in continuous arrays of peaks (Figure 2). There were 42 different amplicons of sizes varying from 60 bp to 506 bp (Table 2). All accessions of the *S. officinarum*, *S. robustum*, *S. barberi*, *S. sinense*, *S. edule*, and *Saccharum* spp. hybrids produced three amplicons of 234-, 235-, and 236-bp in size, except for the three *S. robustum* accessions, Molokai 5573, NG 57-054, and NG 77-235, which lacked the 236-bp amplicon. The 237-bp amplicon was occasionally produced in the *S. barberi*, *S. edule*, *S. officinarum*, *S. robustum*, *S. sinense*, and *Saccharum* spp. hybrids.

However, the 238-bp amplicon was rarely observed and only in *S. officinarum*, *S. robustum*, and *S. spontaneum*. Some *S. spontaneum* accessions from China and Thailand also produced additional *S. spontaneum*-specific 224- and 225-bp amplicons. The 234-, 235-, and 236-bp banding pattern found in S. *spontaneum* was less consistent than other *Saccharum* species, sometimes missing a few but not all the bands in this region.

Figure 2. (**A**) Five panels of capillary electropherograms of FAM-PI/PII-primed PCR amplificons (blue color) of sugarcane leaf tissue DNA using GeneMarker™ Software (SoftGenetics, LLC, State College, PA). Panel designations (from top down): Type I (IJ 76-521, *S. officinarum*); Type II (SES 84-58, *S. spontaneum*); Type III (M 3035-66, *S. robustum*); Type IV (MPTH 97-194, *T. arundinaceum*); and Type V (Kalimpong, *T. procerum*). In each panel, values on the Y-axis represent relative fluorescence intensity (*rfu*) and values on the X-axis are the sizes of PCR amplicons (base pair) (blue color) or GeneScan™ 1200 LIZ™ dye Size Standard (orange color). Eight fingerprints were detected, which were labeled with letters a, b, c, d, e, f, g, and h, respectively (red fonts). (**B**) Cropped images of the eight fingerprints as continuous arrays of amplicon peaks.

In this study, there were nine duplicate accessions sampled from both the SRU and WCSRG germplasm collections, namely, Chuk Che, Green German, IND 81–80, Khagzi, Ruckri, Tekcha Okinawa, Uba Del Natal, Uba India, and Uba Naquin. The PI/PII-amplicons from these duplicated accessions produced the exact same fingerprints by both agarose and capillary electrophoreses.

Accessions US 4515-9200 and SPS-269 are unknown species that were donated to the world collection by the Shakarganj Sugar Research Institute in Pakistan. In the US GRIN system, US 4515-9200 is listed as a donation from Pakistan without an identification. It could be that the accession listed as US 4515-9200 is US 4515, which is labeled as *S. spontaneum*. Less is known about the SPS-269 accession. However, since these two unknown accessions only produced the 234-, 235-, and 236-bp amplicons, displaying a banding Type II during agarose gel electrophoresis, they are most likely members of one of the *Saccharum* species (Table 1).

Table 2. PI/PII-primed PCR amplicons from 171 accessions of sugarcane, six species of *Saccharum*, three species of *Tripidium* genera, and two unknown species.

Genus	***Saccharum***							***Tripidium***			
Species	***barberi***	***edule***	***hybrid***	***officinarum***	***robustum***	***sinense***	***spontaneum***	***arundinaceum***	***bengalense***	***procerum***	***unknown***
No. Accessions	25	2	5	30	17	13	71	4	1	1	2
Amplicons (bp)	Percent of each amplicon per species										
61	0	0	100	0	0	0	0	0	0	0	0
224–225	0	0	0	0	0	0	11.3	0	0	0	0
227	0	0	0	0	0	0	4.2	0	0	0	0
228–229	0	0	0	0	5.9	0	7	0	0	0	0
230–231	0	0	0	0	0	0	4.2	0	0	0	0
233	0	0	0	0	2.8	0	5.6	0	0	0	0
234	100	100	100	100	100	100	73.2	25 †	100	0	100
235	100	100	100	100	100	100	94.4	25.0 †	100	0	100
236	100	100	100	100	82.4	100	91.5	25.0 †	100	0	100
237	36	100	60	36.7	17.6	84.6	42.3	0	0	0	0
238	0	0	0	3.3	8	0	2.8	0	0	0	0
247–248	0	0	0	0	0	0	2.8	0	0	0	0
382–385	0	0	0	0	0	0	2.8 †	50	0	0	0
387, 388, 402	0	0	0	0	0	0	1.4 †	0	0	0	0
403–405	0	0	0	0	0	0	1.4 †	0	0	100	0
406	0	0	0	0	0	0	0	0	0	100	0
407	0	0	0	0	0	0	2.8 †	50	0	0	0
408–409	0	0	0	0	0	0	2.8 †	0	0	0	0
463–466	0	0	0	0	0	0	0	0	0	100	0

† Includes accessions IS 78–218 and IMP 9751 that have marker patterns like *Saccharum* and may have been misidentified.

Most *Tripidium* accessions produced unique amplicons in the 382- to 466-bp range, but two *Tripidium* accessions, IS 78-218 (*T. arundinaceum*) and IMP 9751 (*T. bengalense*), only produced 234-236 bp amplicons as the *Saccharum* accessions did. It could be that the two accessions were misidentified. The other two accessions of *T. arundinaceum* showed peaks in the 407–409 bp region and both produced amplicons of 408- and 409-bp. The only *T. procerum* accession, Kalimpong, had peaks in the 403–406 bp and 463–466 bp region.

Unexpectedly, the *S. spontaneum* accessions Isiolo, MPTH 97-113, and MPTH 99-476 did not produce amplicons in the 234–235-236 bp region. Instead, larger sized amplicons were observed in the 382–405 bp regions for Isiolo, 407–409 bp region for MPTH 97-113, and 382–385 bp and 407–409 bp regions for MPTH 99-476. These amplicons were not produced by most of the *Saccharum* accessions, and it probably means that these accessions were also misidentified.

On the other hand, five *S. spontaneum* accessions, namely, SES288, MPTH97-194, MPTH97-221, MPTH98-283, and MPTH98-326, did not amplify the 234–236 bp amplicons, but amplified larger sized amplicons distinctive of *Tripidium*. Therefore, these accessions are also mislabeled *T. arundinaceum* accessions and have since been listed as *Tripidium* [30]. It seems that misidentification is a common problem between *S. spontaneum* and *T. arundinaceum*. If the PI/PII markers could be confirmed as genus-specific for *Tripidium*, then they could be a great tool to quickly identify unknown accessions such as *Saccharum* or *Tripidium*. Since the larger sizes of *Tripidium*-associated PI/PII amplicons are distinct, they could be particularly useful to identify true hybrids from the intergeneric crosses between *Saccharum* and *Tripidium* [16,24], and may perhaps be useful in confirming *Tripidium* inheritance in backcrossing and introgression. These PI/PII amplicons could be combined with other *Tripidium* genus-specific markers, such as Ea086-128, Ea009-257, and EaITS-278 [26] to accurately identify *Tripidium* accessions.

According to the cluster tree, the accessions can be divided into two clusters at the Manhattan distance value of 18 (Figure 3). The bottom cluster included the *Tripidium* and alike accessions and the top cluster included the *Saccharum* accessions. The next split into two large clusters among the *Saccharum* accessions was at the Manhattan distance value of 9. Most *Saccharum* species were found in both clusters, but the *S. edule* and *Saccharum* spp. hybrids were only found in the top cluster. The top cluster was further split into two at the Manhattan distance value of 6, with the *S. spontaneum* accessions Dacca and Guang Xi-series accessions in one cluster and the remaining accessions in the other. The bottom cluster split at the Manhattan distance value of 8, with Japanese and Indian *spontaneum* clones Okinawa-01 and S66-84A and B in one cluster and the remaining accessions in the other. After that, the bottom cluster split at the Manhattan distance value of 6, and the *S. spontaneum* MPTH-series accessions were in one cluster and the remaining in another. Both the top and bottom *saccharum* clusters further split at the Manhattan distance value of 4, with the top cluster having all the hybrids and the *S. spontaneum* accession IS 76-121 in the minority cluster and the bottom having the *S. robustum* accession IN 84-076 and two *S. spontaneum* JW accessions in the smaller cluster. This level of diversity revealed by the clustering indicates potentially divergent populations within the *Saccharum* genus that should be further investigated. From these results, it appears that the PI/PII markers can discriminate only between the *Saccharum* spp. and *Tripidium* spp. accessions, although some diversity was also identified within the S. *robustum* and S. *spontaneum* species.

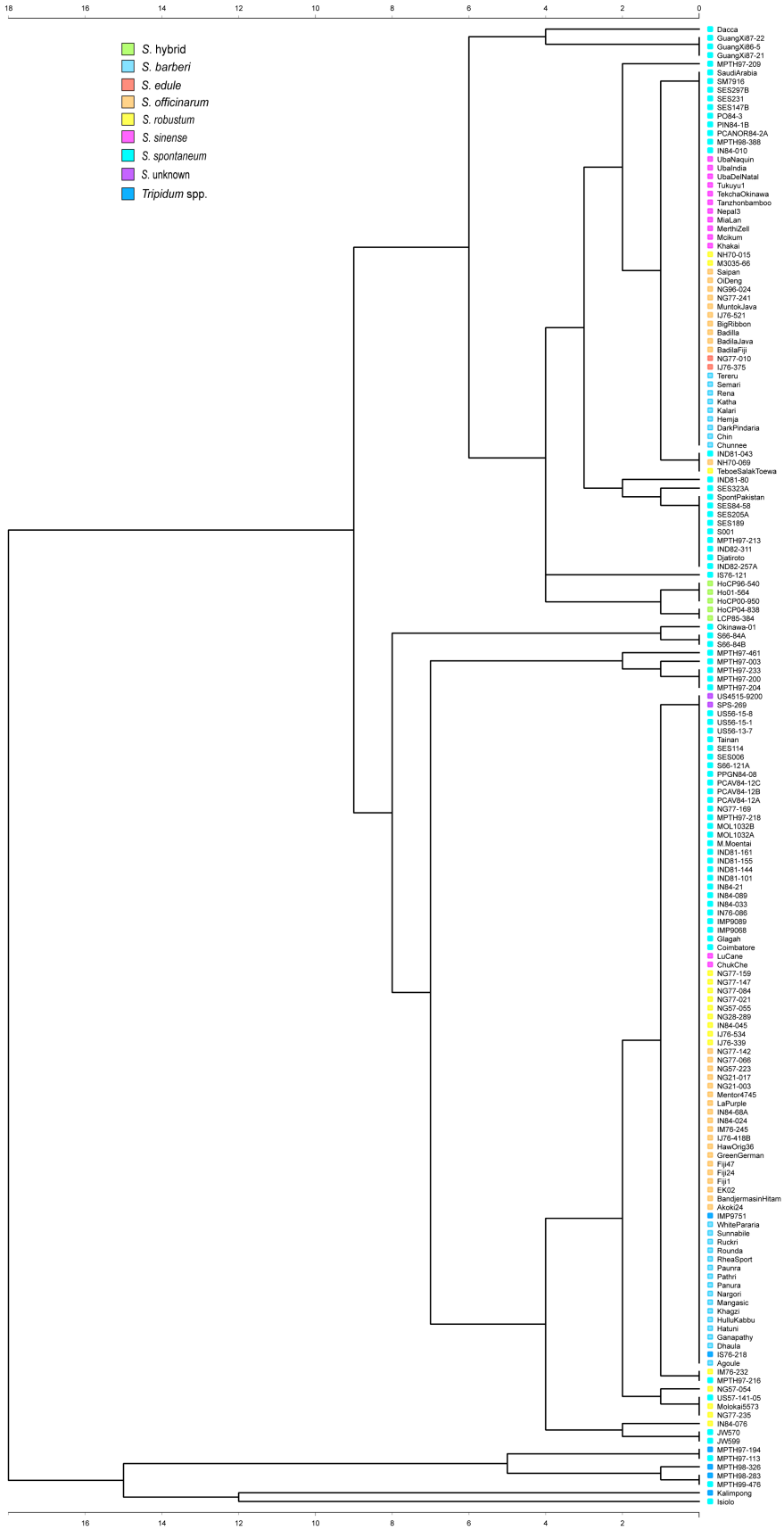

Figure 3. A phenogram of *Saccharum* spp. and *Tripidium* spp. accessions constructed by the hierarchical clustering function of Orange v 3.35 [28] based on Manhattan distance values of PI/PII-amplicon data of 5S rRNA-ITS and complete linkage algorithm.

The results from this study are comparable to Pan et al. [17], in which the size of the PI/PII-amplicons from *S. officinarum* ranged from 231 to 237 bp, which is similar to the 234–238 bp range found in this study. The size range of the PI/PII-amplicons from the *Saccharum* spp. hybrids (233–237 bp) was also similar to the size range in this study (234–237 bp). One significant difference between the two studies is that the sizes reported in Pan et al. [17] were obtained through the Sanger sequencing of cloned PI/PII-amplicons, whereas in this study, the sizes of the PI/PII-amplicons were calibrated by the Liz1200^{TM} size standards through CE. In addition, there were 75 accessions of *S. spontaneum* accessions included in this study, resulting in a larger range of sizes, 224–248 bp (excluding the data from the five *S. spontaneum* accessions that are likely mislabeled *Tripidium*), compared with six *S. spontaneum* accessions and 228–238 bp in Pan et al. [17]. The size range for *Tripidium* spp. found in Pan et al. [17] was 385–410 bp, which was consistent with the higher bp size for *Tripidium* found in this study, but we had an accession of *Tripidium procerum*, Kalimpong, which increased the range (382–466 bp). Similarly, Pachakkil et al. [23], using the same primers, found that the band size was 280 bp for *Saccharum* and 420 bp for *Tripidium arundinaceum* using agarose electrophoresis. In Pan et al. [17], the *S. officinarum*, *S. spontaneum,* and *Saccharum* spp. hybrids also clustered together, indicating an agreement that the *Saccharum* species cannot be distinguished using the PI/PII markers, but certain accessions within each species with relatively rare bands such as 61 bp and 224 bp could be used to verify crosses made with those accessions.

4. Conclusions

PI/PII-amplicons from the 5S ribosomal RNA-intergenic transcribed spacers in *Saccharum* spp. and *Tripidium* spp. were molecularly dissected through agarose gel electrophoresis, fluorescence labeling, and capillary electrophoresis. Some PI/PII-amplicons could potentially quickly identify *Tripidium* spp. accessions from *Saccharum* spp. A few PI/PII-amplicons were identified that could indicate diversity and subpopulations within *Saccharum* spp. These markers have the potential to be used by breeders at sugarcane field stations to expand their ability to manage germplasm and validate the backcrossing that existed prior, but needs to be evaluated first.

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