PHYLOGENETIC ANALYSIS OF *SACCHARUM S.L.* (POACEAE; ANDROPOGONEAE), WITH EMPHASIS ON THE CIRCUMSCRIPTION OF THE SOUTH AMERICAN SPECIES[1]

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- *Premise of the study:* Polyploidy and reticulate evolution are often a complication for discovering phylogenetic relationships between genera and species. Despite the huge economic importance of sugarcane (*Saccharum officinarum*—Poaceae, Andropogoneae), the limits of the genus *Saccharum* and its species are complex and largely unresolved, involving both polyploidy and reticulate evolution. This study aimed to assess the phylogenetic relationships of *Saccharum s.l.*, including *Erianthus* and *Tripidium*, as well as investigate the taxonomic circumscription of the South American species of the genus.
- *Methods:* Molecular cloning and sequencing of five regions of four low-copy nuclear loci were performed, including *Aberrant panicle organization*1 (*apo*1), *Dwarf*8 (*d*8), two exons of *Erect panicle*2 (*ep*2-*ex*7 and *ep*2-*ex*8), and *Retarded palea*1 (*rep*1). Concatenated trees were reconstructed using Maximum Parsimony, Maximum Likelihood, and Bayesian Inference analyses.
- *Key results:* The allopolyploid origin of *Saccharum* was demonstrated using evidence from nuclear genes. The samples of *Saccharum s.l.* grouped in two distinct clades, with *S. arundinaceum* and *S. ravennae* (= *Tripidium,* or *Erianthus* sect. *Ripidium*) apart from all other species analyzed of the genus. *Saccharum angustifolium, S. asperum,* and *S. villosum* correspond to distinct clades (different species). The plants with intermediate morphology between *S. angustifolium* and *S. villosum* presented a pattern of paralogues consistent with a hybrid origin.
- *Conclusions: Saccharum s.l.* is polyphyletic and *Tripidium* should be recognized as a distinct genus. However, no strong evidence was found to support the segregation of *Erianthus.* The taxonomic circumscription of the South American species of the genus was resolved and the occurrence of natural hybrids was documented. Better understanding of the phylogenetic relationships of *Saccharum* and relatives may be useful for sugarcane breeders to identify potential taxa for interspecific and intergeneric crosses in the genetic improvement of sugarcane.

Key words: *Erianthus*; hybridization; low-copy nuclear loci; polyploidy; *Ripidium*; species complex; species delimitation; sugarcane; *Tripidium.*

Polyploidy and reticulate evolution often complicate efforts in phylogenetic reconstruction of relationships between genera and species (McDade, 1992; Triplett et al., 2012). The two processes are common in the genus *Saccharum* L. (Poaceae; Andropogoneae), which includes one of the most important crops in the world, sugarcane (*S. officinarum* L.), whether measured by tons harvested or by dollar value (Boddey et al., 2008; Bonnett and Henry, 2011). In addition to being the major source of sugar for human consumption, sugarcane is also a source of ethanol for biofuel that powers many parts of the world, notably Brazil (Boddey et al., 2008). Despite this immense value, the limits of the genus and the species within it are complex, contentious, and largely unresolved. Species from the New World have been variously classified in *Saccharum* and *Erianthus* Michx., with different authors combining the two or keeping them separate (Mukherjee, 1958; Molina, 1981; Clayton and Renvoize, 1986; Amalraj and Balasundaram, 2006). Adding to the confusion has been difficulty in determining the limits of species (Welker and Longhi-Wagner, 2012).

Saccharum and *Erianthus* are both members of the tribe Andropogoneae, in the subfamily Panicoideae of the Poaceae. The tribe Andropogoneae, erroneously called Sacchareae by some

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authors (see Welker et al., 2014), comprises approximately 90 genera and 1060 species with a cosmopolitan distribution (Sánchez-Ken and Clark, 2010). It includes some of the world's most economically important plants, such as sugarcane, maize (*Zea mays* L.), and sorghum (*Sorghum bicolor* (L.) Moench), as well as many ecologically dominant species of tropical and temperate grasslands. Andropogoneae is strongly supported as monophyletic, and *Arundinella* Raddi (tribe Arundinelleae) is its sister group (Mathews et al., 2002; Sánchez-Ken and Clark, 2010). Recent phylogenetic analyses suggested that the very short branches along the backbone of the trees were caused by a rapid evolutionary radiation near the base of the Andropogoneae clade (Mathews et al., 2002; Teerawatananon et al., 2011; Estep et al., 2014). Phylogenetic analyses indicate the presence of a "core Andropogoneae" clade, including *Andropogon* L., *Schizachyrium* Nees, *Hyparrhenia* Andersson ex E. Fourn., and *Bothriochloa* Kuntze, among others. *Saccharum* and *Miscanthus* Andersson are closely related genera, and are placed outside the "core Andropogoneae" (Hodkinson et al., 2002; Mathews et al., 2002; Estep et al., 2014).

The genus *Saccharum*, in the broad sense including the species of *Erianthus*, comprises 35–40 species from tropics and subtropics of the world (Clayton and Renvoize, 1986). Some authors have considered *Erianthus* as a distinct genus, with approximately 28 species from North and South America, Africa, Europe, and Asia (Mukherjee, 1958; Molina, 1981; Watson and Dallwitz, 1992). The main morphological difference between these genera is the awned spikelets in *Erianthus* and the awnless spikelets in *Saccharum s.s.* (Mukherjee, 1958). The Old World species of *Erianthus* are grouped in a different section (*Erianthus* sect. *Ripidium* (Trin.) Henrard) or a distinct genus (*Ripidium* Trin.) by different authors (Grassl, 1972; Besse et al., 1997; Hodkinson et al., 2002). However, *Ripidium* Trin. is an illegitimate name, and the name *Tripidium* H. Scholz was proposed to replace it (Valdés and Scholz, 2006). *Narenga* Bor and *Miscanthidium* Stapf. were also accepted as distinct genera by a few authors (e.g., Clayton, 1972; Watson and Dallwitz, 1992) instead of including their species in *Saccharum* or *Miscanthus*.

The circumscription of *Saccharum* and related genera is controversial. Several phenetic studies indicated strong molecular differentiation between *Saccharum* and *Erianthus* (Besse et al., 1998; Nair et al., 2005; Selvi et al., 2006). On the other hand, a phylogenetic analysis based on the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (Hodkinson et al., 2002) found no support for this division, even though it suggested that *Saccharum s.l* is polyphyletic. However, only a few species of *Erianthus* were included in that analysis, and only one of them was from New World (North America). On the other hand, Hodkinson et al. (2002) suggested that *Tripidium* (under *Ripidium*) may be considered as a distinct genus, because its species are grouped together but are separate from other *Saccharum s.l.* species in all ITS equally most parsimonious trees (but without support in the trees). The taxonomic delimitation between *Saccharum* and *Miscanthus* is also not clear, with intergeneric hybrids occurring between them (Clayton and Renvoize, 1986; Hodkinson et al., 2002).

In addition to the taxonomic controversy at the generic level, the circumscription of the South American species of *Saccharum s.l.* is also convoluted. Filgueiras (2003) recognizes three native species of *Saccharum s.l.* in the region, *Saccharum angustifolium* (Nees) Trin., *S. asperum* (Nees) Steud., and *S. villosum* Steud., in addition to the introduced sugarcane, reducing six previously recognized species of *Saccharum*/*Erianthus* (Swallen, 1966; Molina, 1981; Smith et al., 1982) to synonymy. Five of the synonyms are assigned to *Saccharum villosum* (Filgueiras, 2003; Morrone et al., 2008): *Erianthus balansae* Hack., *E. clandestinus* Swallen, *E. glabrinodis* (Hack.) Swallen, *E. purpureus* Swallen, and *E. trinii* (Hack.) Hack. Specimens identified as *Saccharum villosum s.l.* are morphologically variable, especially in the dimensions and indument of the leaves and culms, and in the shape of the leaf blades, suggesting that *S. villosum* might be more than one taxon (Welker and Longhi-Wagner, 2012).

Saccharum villosum is morphologically similar to *S. angustifolium*, a sympatric species in South America. *Saccharum angustifolium* can be distinguished mainly by the linear leaf blades, narrower than in *S. villosum*, with a conspicuous midvein. *Saccharum villosum* has lanceolate leaf blades with the midvein inconspicuous in the upper portion of the blade (Welker and Longhi-Wagner, 2012) (see Table 1 and Fig. 1). However, some specimens collected in Southern Brazil, Argentina, and Uruguay present an intermediate morphology between the two species. These specimens were identified as *Saccharum* aff. *villosum* Steud. by Welker and Longhi-Wagner (2012). Based on morphological aspects, Welker and Longhi-Wagner (2012) suggested that these specimens with intermediate leaf morphology might be natural hybrids between *S. villosum* and

TABLE 1. Comparison of morphological and biogeographical characters of South American taxa of *Saccharum s.l.*

	S. angustifolium	***S. asperum***	***S. villosum***	***S. villosum*** ("wide leaf blades")	***S.* aff. *villosum***
Leaf blades: shape, indument, width	linear (without pseudopetiole), glabrous, 2–6 mm wide	lanceolate (with pseudopetiole), glabrous or pilose, 7–23 mm wide	lanceolate (with pseudopetiole), generally pilose, 7–14 mm wide	lanceolate (with pseudopetiole), generally pilose, 14–20 mm wide	lanceolate (with pseudopetiole), glabrous or pilose, 3–6 mm wide
Leaf blades: midvein	conspicuous up to the apex of the blade, wider than or as wide as the lateral portion of the blade	inconspicuous in the upper portion of the blade, narrower than the lateral portion of the blade	inconspicuous in the upper portion of the blade, narrower than the lateral portion of the blade	inconspicuous in the upper portion of the blade, narrower than the lateral portion of the blade	inconspicuous in the upper portion of the blade, narrower than the lateral portion of the blade
Glumes of the spikelets: indument	pilose	glabrous	pilose	pilose	pilose
Habitat	dry grasslands	marshlands	marshlands and wet grasslands	marshlands	marshlands and wet grasslands
Geographical distribution	Colombia and Venezuela to Argentina and Uruguay	Colombia and Venezuela to Argentina and Uruguay	Mexico to Argentina and Uruguay	Argentina and Brazil	Argentina, Brazil, and Uruguay

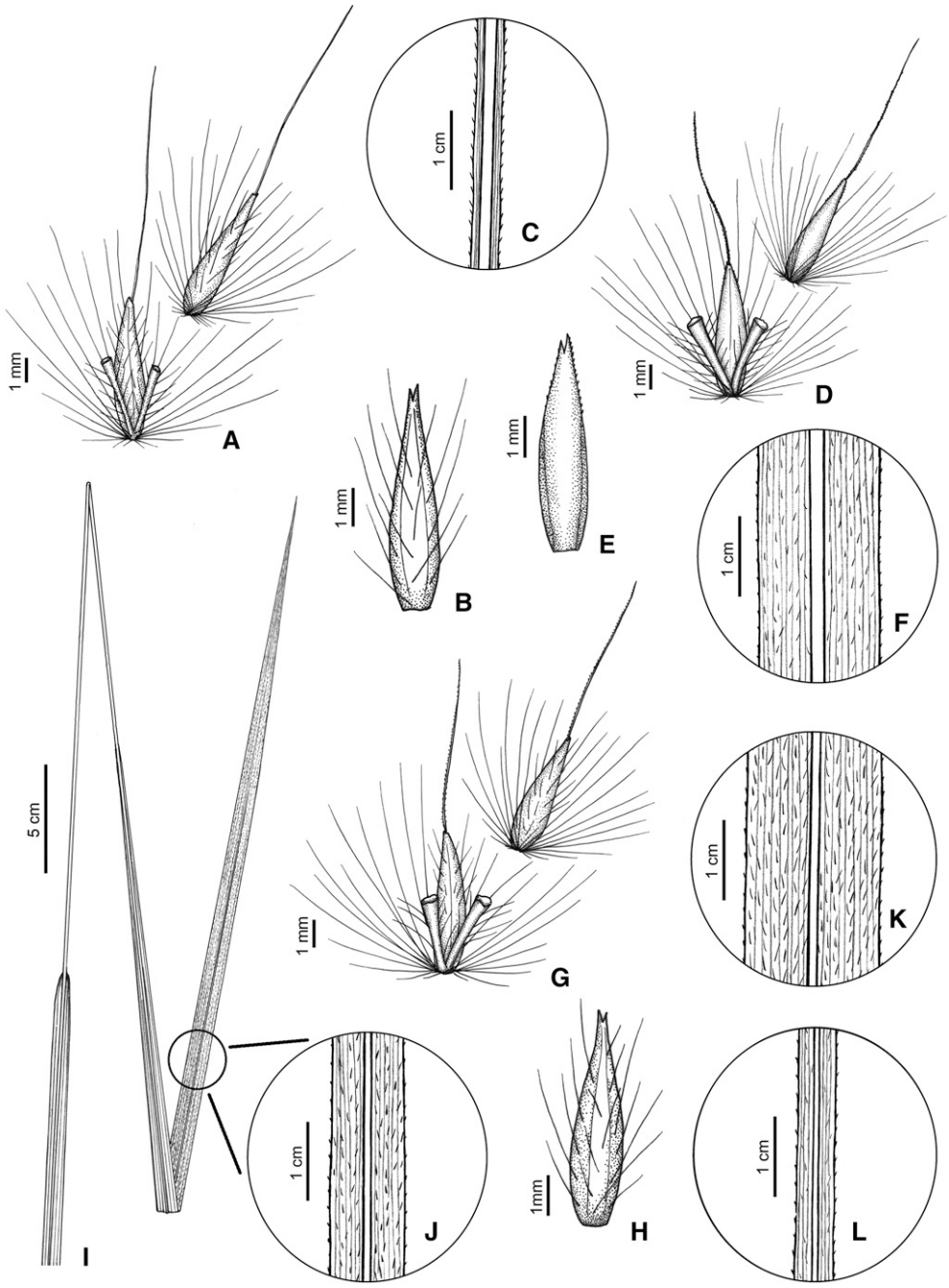

Fig. 1. Morphological details of the South American species of *Saccharum s.l.* A–C. *Saccharum angustifolium*. A. Pair of spikelets, pedicel, and rachis internode. B. Lower glume of the sessile spikelet. C. Middle portion of the leaf blade. D–F. *Saccharum asperum*. D. Pair of spikelets, pedicel, and rachis internode. E. Lower glume of the sessile spikelet. F. Middle portion of the leaf blade. G–J. *Saccharum villosum*. G. Pair of spikelets, pedicel, and rachis internode. H. Lower glume of the sessile spikelet. I. Leaf with lanceolate blade and pseudopetiole. J. Middle portion of the leaf blade. K. *Saccharum villosum* ("wide leaf blades"). Middle portion of the leaf blade. L. *Saccharum* aff. *villosum*. Middle portion of the leaf blade. Illustrations by C.A.D. Welker.

S. angustifolium. Both *S. villosum* and *S. angustifolium* have spikelets with pilose glumes, a morphological characteristic that distinguishes them from *S. asperum*, another sympatric species in South America, in which the glumes are glabrous (Table 1, Fig. 1). Specimens of *S. asperum* do not present as much morphological variability as the species mentioned above (Welker and Longhi-Wagner, 2012).

Polyploidy and reticulate evolution are common in Andropogoneae, as well as in the clade including sugarcane and relatives (Kim et al., 2014; Estep et al., 2014). A recent study documented that at least one third of Andropogoneae species resulted from allopolyploidy, with a remarkably high number of independent allopolyploidization events (Estep et al., 2014). Because of this reticulate history, data from low-copy nuclear loci are required to resolve phylogenetic relationships between genera and species (Sang, 2002; Estep et al., 2012; Triplett et al., 2012; Liu et al., 2014). Although plastid markers and ITS have been widely used, the low sequence variability in the

plastid genome, and the high number of paralogues, plus incomplete concerted evolution in ITS, make them inadequate for this purpose (Sang, 2002; Álvarez and Wendel, 2003). Phylogenetic trees inferred from nuclear genes are useful to understand the relationships of polyploid taxa and identify allopolyploidization events, because they produce characteristic double-labeled tree topologies in which the polyploid species appear twice (Sang, 2002; Triplett et al., 2012; Estep et al., 2014). In such trees, allopolyploids can be recognized even in the absence of chromosome counts (Estep et al., 2014).

The current study aimed to (1) test the monophyly of *Saccharum s.l.* and assess its phylogenetic relationships to other genera of Andropogoneae, (2) define the taxonomic circumscription of the South American species of *Saccharum s.l.,* and (3) better understand the identity of the specimens with intermediate morphology between *S. villosum* and *S. angustifolium* (*Saccharum* aff. *villosum*).

MATERIALS AND METHODS

***Plant material*—**Twenty-nine specimens of *Saccharum s.l.* were included in the analysis, as well as 43 species belonging to 34 other genera of Andropogoneae. Two species of *Arthraxon* P. Beauv. were used as outgroup, because it is well supported as the sister genus to the rest of the tribe (Estep et al., 2014). The sample included material from the type species of the genera *Saccharum*, *Erianthus*, and *Tripidium*: *S. officinarum*, *E. giganteus* (Walter) P. Beauv. (= *Saccharum giganteum* (Walter) Pers.), and *T. ravennae* (L.) H. Scholz. (= *Saccharum ravennae* (L.) L. / *Erianthus ravennae* (L.) P. Beauv.), respectively. (The genus *Erianthus* was described by Michaux (1803) based on *Erianthus saccharoides* Michx., which is a superfluous illegitimate name and a synonym of *E. giganteus* / *Saccharum giganteum* (Tropicos, 2014a)). Voucher specimens and collection localities are listed in Table 2. GenBank accession numbers for the sequences are listed in Appendix 1.

***Molecular cloning, sequencing, and data processing*—**Total genomic DNA was extracted using the CTAB procedure (Doyle and Doyle, 1987), modified for microcentrifuge tubes. Five regions of four low-copy nuclear loci were PCR amplified following Estep et al. (2012): *Aberrant panicle organization*1 (*apo*1), *Dwarf*8 (*d*8), two exons of *Erect panicle*2 (*ep*2-*ex*7 and *ep*2-*ex*8), and *Retarded palea*1 (*rep*1). Previous works show that these loci are efficient markers to infer phylogenetic relationships in the tribe Andropogoneae (Estep et al., 2012; Estep et al., 2014).

The PCR products were purified via gel extraction using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, California, USA), following the manufacturer's protocol. To capture paralogous copies, purified products were cloned using pGEM-T Easy Vector and transformed into JM109 High-Efficiency Competent Cells (Promega, Madison, Wisconsin, USA), following manufacturer's protocols. Transformed cells were plated and selected via a blue-white screen on LB agar with X-Gal, isopropyl-beta-thio-galactoside (IPTG), and ampicillin. Between 8 and 24 positive clones of each PCR product were selected. Extracted DNA from the colonies was sent to Beckman Coulter Genomics (Danvers, Massachusetts, USA) for sequencing in both directions using universal primers (T7 and M13R). Internal primers were also used for sequencing *d*8 and *ep*2-*ex*7 loci (Estep et al., 2012; Estep et al., 2014).

Chromatogram files were trimmed of vector using Geneious 6.1.8 (Biomatters, Auckland, New Zealand) and ambiguous bases from the ends of both reads were removed manually. Forward and reverse sequences (and sequences from internal primers in *d*8 and *ep*2-*ex*7 loci) were subsequently assembled for each clone. Only clones with 80% or more double-stranded sequence were used for analysis. All good quality contigs for each sample were then aligned using Geneious and primer sequences were removed. Recombinant sequences were identified by eye, comparing them with unambiguous sequences from related species, and were removed from the alignment. The redundant clones of the same gene copy were combined into a consensus sequence, to minimize the inclusion of sequencing errors and reduce the number of sequences to one per paralogue per locus. The resulting sequences were translated and aligned using MUSCLE, as implemented in Geneious.

***Phylogenetic analyses*—**Gene trees were estimated for each locus using RAxML 8.0.9 (Stamatakis, 2006; Stamatakis et al., 2008) using the Black Box setting on the CIPRES Science Gateway (Miller et al., 2010). We used the individual gene tree topologies as a guide to identify the corresponding paralogues of each genome in the five loci, for the polyploid specimens, and create concatenated sequences, according to Estep et al. (2014). The results presented here were based on the data set with a minimum of three out of five loci per genome for each taxon, except some paralogues of the samples Welker 477, Welker 502, Welker 538, Welker & Peichoto 556, and Welker & Peichoto 584, for which we had only two loci sequenced per genome. Our data set included 23.4% missing data (for more details, see Appendix 1). The alignment of the combined data set is presented in Appendix S1 (see Supplemental Data with the online version of this article).

Concatenated trees were reconstructed using Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) analyses. The Parsimony Ratchet analysis (Nixon, 1999) was performed in PAUP* 4.0b10 (Swofford, 2002) using the companion program PAUPRat (Sikes and Lewis, 2001). Twenty independent runs were performed with 200 iterations each. Support at each node was assessed through bootstrap analysis (Felsenstein, 1985), with a heuristic search based on 1000 replicates. Bootstrap values > 50% were recorded on the trees.

The ML analysis was performed using RAxML 8.0.9 (Stamatakis, 2006; Stamatakis et al., 2008). Models of DNA evolution were determined using jModelTest (Posada, 2008) and the GTR+G model was selected. ML support was assessed via 500 bootstrap replicates, and values > 50% were recorded on the trees. The BI analysis was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) in parallel (Altekar et al., 2004) under the GTR+G model of evolution and six rate categories. Two independent runs of 20 million generations were performed and sampled every 1000 generations. The consensus tree was estimated after a burn-in of 25% of sampled trees. Convergence of the independent runs was confirmed using the AWTY system for graphical exploration of Markov chain Monte Carlo (MCMC) convergence (Nylander et al., 2008). Posterior probability (PP) values > 0.85 were recorded on the trees.

RESULTS

The aligned data matrix, including the five low-copy nuclear loci (and excluding specimens of *Saccharum* aff. *villosum*), was 4468 base pairs long, of which 1978 (44%) were variable and 1103 (25%) were parsimony informative. The MP analysis resulted in 1927 equally most parsimonious trees of 3290 steps (CI = 0.48, RI = 0.70).

The trees resulted from MP, ML, and BI analyses were very similar, with short branches along the backbone of the tree, with low support, in contrast to long external branches, with higher support (Fig. 2). The samples of *Saccharum s.l.* fell in two distinct strongly supported clades (both with full support in MP, ML, and BI analyses). The first clade is formed by *Saccharum ravennae* (type species of the genus *Tripidium*) and *S. arundinaceum* Retz. The second clade includes the remaining representatives of *Saccharum s.l.*, *Miscanthus sinensis* Andersson, and *Pseudosorghum fasciculare* (Roxb.) A. Camus (Fig. 2). However, the relationship between these two clades is unclear. The type species of both *Saccharum* (*S. officinarum*) and *Erianthus* (*E. giganteus* / *Saccharum giganteum*) fell in this second clade, along with all South American species of *Saccharum s.l.* (Fig. 3, type species in bold). This clade included two paralogues per sample for all taxa except *Pseudosorghum fasciculare* (Figs. 2 and 3). Because the genetic loci that we sampled are unlinked, we infer that the two paralogous clades represent the history of two independent genomes that came together in an allopolyploidization event that preceded diversification. For convenience we call these genomes A and B.

We did not discover a paralogue from genome A in the sugarcane (*S. officinarum*) sample included in our analyses, but did find two copies from genome B (called B1 and B2). The two

copies grouped closely to paralogue B of the Asian *Saccharum narenga* (Nees ex Steud.) Wall. ex Hack., with strong support in the BI analysis (1 PP), but with weak support in MP and ML analyses (73% MP and ML bootstrap). Paralogue A of *S. narenga* grouped with paralogue A of *S. ecklonii* (Nees) Steud., with strong support (1 PP, 98% ML, 94% MP) (Fig. 3).

The phylogenetic relationships of the South American specimens inferred by both genomes (A and B) were very similar (Fig. 3). All accessions of *Saccharum angustifolium* formed a strongly supported clade in both genomes (genome A: 1 PP, 100% ML and MP; B: 1 PP, 99% ML, 100% MP), as did the specimens of *S. villosum s.l.* (A: 1 PP, 99% ML, 98% MP; B: 1 PP, 96% ML, 92% MP). The *S. villosum* clade is formed by two other well-supported clades based on the paralogues of genome A. The first clade (1 PP, 98% ML, 95% MP) contains robust plants with very wide leaf blades (specimens Welker 396, Welker 477, and Welker & Peichoto 575), and is sister to the clade with the remaining accessions of *S. villosum* (1 PP, 99% ML, 97% MP), which includes less robust plants with narrower blades. The three specimens with wide blades also grouped together in genome B, but only in the ML analysis and with moderate support (79%) (Fig. 3). On the other hand, the samples of *S. asperum* did not form a monophyletic group; two specimens formed a well-supported clade, apart from the other two specimens analyzed that formed another well-supported clade (Fig. 3).

When the specimens of *Saccharum* aff. *villosum* were included in the tree (Fig. 4), the bootstrap support for most nodes was slightly lower than in the trees without these samples. The aligned combined matrix including these specimens was 4468 base pairs long, of which 1992 (45%) were variable and 1108 (25%) were parsimony informative. The MP analysis for this data set resulted in 2885 equally most parsimonious trees of 3348 steps (CI = 0.47, RI = 0.71).

The specimens of *Saccharum* aff. *villosum* presented three different paralogues in the trees (except the sample Welker 630, with only two) and the paralogues fell in several distinct clades, both in genomes A and B (Fig. 4). For the specimen Welker 538, for example, the paralogue from genome A grouped in the *S. villosum* clade (0.99 PP, 88% ML, 79% MP), the paralogue from genome B grouped in the *S. angustifolium* clade (0.99 PP, 81% ML, 67% MP) and the third paralogue in a distinct clade (0.96 PP, 75% ML, <50% MP), not closely related with either of the former two clades. On the other hand, the paralogues of the specimen Welker & Peichoto 584 from both genomes A and B grouped in the *S. angustifolium* clades (A: 0.99 PP, 96% ML, 94% MP; B: 0.99 PP, 81% ML, 67% MP), and the third paralogue grouped with the third paralogue of Welker 538. The opposite situation was observed with specimen Welker 502, in which all three paralogues grouped into the *S. villosum* clades, both in genome A and B (A: 0.99 PP, 88% ML, 79% MP; B: 0.99 PP, 69% ML, <50% MP). The other specimens of *Saccharum* aff. *villosum* presented a pattern similar to one of the three described above. For convenience we call the third paralogues of *Saccharum* aff. *villosum* as genomes C and D, because they do not seem to be equivalent (Fig.4).

DISCUSSION

***Phylogenetic analyses of* Saccharum s.l.—**The pattern of very short branches along the backbone of the trees indicates that the early diversification in Andropogoneae was probably rapid. Similar topologies suggesting rapid radiation were found by Mathews et al. (2002), Teerawatananon et al. (2011), and Estep et al. (2014). The phylogenetic analyses demonstrate the allopolyploid origin of *Saccharum*. They also suggest that *Saccharum s.l.* is polyphyletic, in agreement with the results of Hodkinson et al. (2002) based on ITS sequence data. The species belonging to section *Ripidium* of the genus *Erianthus* (accepted as the distinct genus *Tripidium* or *Ripidium* by some authors), did not group closely with other species of *Saccharum s.l.* (Fig. 2), indicating that *Tripidium* should be recognized as a distinct genus. However, the relationship of the *Tripidium* clade with the clade containing the remaining representatives of *Saccharum s.l.* remains unclear and requires additional investigation.

There are many distinctions between *Tripidium* and *Saccharum s.l.*, which reinforce their recognition as separate genera. Several studies based on restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) indicate that the species of *Tripidium* are genetically different from other species of *Saccharum s.l.* (Besse et al., 1997, 1998; Selvi et al., 2006; under *Erianthus* sect. *Ripidium*) but the sample of taxa and the methods of data analysis do not provide a rigorous test of the nonmonophyly of the group. Biogeography and morphology also support the division. *Tripidium* includes the Old World species previously placed in *Erianthus* (*E.* sect. *Ripidium*), whereas *Erianthus*, in the strict sense, includes only the American species. According to Grassl (1972, under *Ripidium*), the Old World *Tripidium* species are distinct from New World species (*Erianthus s.s.*) in many reproductive morphological characters. The Old World species have three anthers, whereas the New World species have only two. New World species also have floral parts with strong awns and large seeds (presumably adapted for animal dispersal) whereas Old World species are apparently adapted for wind dispersal (Grassl, 1972; Hodkinson et al., 2002). All these aspects suggest that *Tripidium* should be recognized as a distinct genus, following Grassl (1972, under *Ripidium*). Considering *Tripidium* separate from *Saccharum s.l.* was also suggested by Hodkinson et al. (2002, under *Ripidium*), but with no support in their ITS tree. Note that the name *Ripidium* Trin. is illegitimate, because it is a posterior homonym of the fern genus *Ripidium* Bernh. (Tropicos, 2014b). Therefore, the correct name is *Tripidium* H. Scholz (Valdés and Scholz, 2006).

All other species of *Saccharum s.l.* (i.e., except *S. arundinaceum* and *S. ravennae*) grouped with *Miscanthus sinensis* and *Pseudosorghum fasciculare* in a well-supported clade (Fig. 3). The genus *Miscanthus* is known to be closely related to *Saccharum*, supported by both phylogenetic analysis and documented hybridization between the genera (Clayton and Renvoize, 1986; Hodkinson et al., 2002; Kim et al., 2014). According to Kim et al. (2014), *Saccharum* and *Miscanthus* shared a whole-genome duplication before diversification of genera. *Miscanthus* is polyphyletic, according to the phylogenetic analysis of Hodkinson et al. (2002). *Pseudosorghum* A. Camus is a small genus including only two Asian species. Based only on morphological aspects, the genus was considered more closely related to *Sorghum* Moench than to *Saccharum* (Clayton and Renvoize, 1986), but this was not confirmed in the present phylogenetic analysis. In our analysis, *Sorghum* grouped with *Polytrias indica* (Houtt.) Veldkamp, and this clade is the sister group of the "core Andropogoneae" clade, which includes *Andropogon*, *Schizachyrium*, *Hyparrhenia*, and *Bothriochloa* Kuntze, among others. Even though *Saccharum* and *Sorghum* are not very closely related, intergeneric hybridization between them has been documented (Nair et al., 2005).

TABLE 2. Species names, voucher specimens, and collection localities of the samples included in this study. Herbaria acronyms according to Index Herbariorum (Thiers, 2014) except THNHM (Thailand Natural History Museum), not included in that directory.

Species	Voucher	Locality
Andropogon eucomus Nees	Malcomber et al. 3089 (MO)	Tanzania, Iringa, Njombe
Andropogon virginicus L.	Kellogg 1240 (MO)	USA, Missouri, Saint Charles
Andropterum stolzii (Pilg.) C. E. Hubb.	Malcomber et al. 3091 (MO)	Tanzania, Iringa, Njombe
Apocopis siamensis A. Camus	Teerawatananon & Sungkaew 975 (THNHM)	Thailand, Sa Kaew, Watthana Nakhon
Arthraxon lanceolatus (Roxb.) Hochst.	Teerawatananon & Sungkaew 720 (THNHM)	Thailand, Tak, Mae Moei
Arthraxon prionodes (Steud.) Dandy	Kellogg PI 659331 (MO)	China, Xizang
Bothriochloa barbinodis (Lag.) Herter	Kellogg PI 204138 (MO)	Brazil, Rio Grande do Sul, Uruguaiana
Bothriochloa laguroides (DC.) Herter	Kellogg PI 283006 (MO)	Uruguay, San Jose, Sierra Mohonea
Capillipedium assimile (Steud.) A. Camus	Teerawatananon & Sungkaew 791 (THNHM)	Thailand, Chiang Mai, Mae Ngon
Chasmopodium caudatum (Hack.) Stapf	Kellogg Kew MSB 184054 (MO)	Burkina Faso, Houet
Chionachne koenigii (Spreng.) Thwaites	Kellogg Chio-6-D-93 (MO)	India
Chrysopogon gryllus (L.) Trin.	Kellogg PI 250984 (A/GH)	Republic of Macedonia, Skopje
Chrysopogon serrulatus Trin.	Kellogg PI 219580 (A/GH)	Pakistan, Bannu
Coix lacryma-jobi L.	Kellogg PI 320865 (MO)	India
Cymbopogon distans (Nees ex Steud.) Will. Watson	Kellogg PI 271552 (MO)	India, Pahlgam
Cymbopogon flexuosus (Nees ex Steud.) Will. Watson	Kellogg PI 209700 (A/GH)	India
Dichanthium annulatum (Forssk.) Stapf	Kellogg PI 240155 (A/GH)	Morocco
Diheteropogon amplectens (Nees) Clayton	Kellogg RF 1819 (MO)	South Africa, Gauteng
Diheteropogon hagerupii Hitchc.	Kellogg Kew MSB 254456 (MO)	Burkina Faso, Comoe
Dimeria fuscescens Trin.	Teerawatananon & Sungkaew 830 (BKF, THNHM)	Thailand, Loei, Phu Kradung
Dimeria ornithopoda Trin.	Teerawatananon & Sungkaew 685 (BKF, THNHM)	Thailand, Trat, Laem Ngob
Eriochrysis pallida Munro	Malcomber et al. 3086 (MO)	Tanzania, Iringa, Njombe
Germainia capitata Balansa & Poitr.	Teerawatananon & Sungkaew 834 (THNHM)	Thailand, Loei, Phu Kradung
Heteropogon triticeus (R. Br.) Stapf ex Craib	Teerawatananon & Sungkaew 733 (THNHM)	Thailand, Chiang Mai, Jom Thong
Hyparrhenia rufa (Nees) Stapf	Kellogg PI 206889 (A/GH)	Turkey, Antalya
	Teerawatananon & Sungkaew 735 (THNHM)	Thailand, Chiang Mai, Jom Thong
Imperata cylindrica (L.) P. Beauv.	Kowarat 108 (THNHM)	Thailand, Pathun Thani, Klong Luang
Ischaemum rugosum Salisb.	Kellogg Kew MSB 183574 (MO)	Burkina Faso, Gnagna
Iseilema macratherum Domin	Snow et al. 7239 (A/GH)	Australia, New South Wales, Moree
Microstegium vimineum (Trin.) A. Camus	Kellogg VA-2 (MO)	USA, Virginia, Fairfax
Miscanthus sinensis Andersson	Kellogg PI 668403 (MO)	Japan, Goto Islands, Nagasaki Prefecture, Osezaki
Pogonatherum crinitum (Thunb.) Kunth	Teerawatananon & Sungkaew 865 (THNHM)	Thailand, Nakhon Ratchasima, PakChong
Polytoca wallichiana (Nees ex Steud.) Benth.	Teerawatananon & Sungkaew 683 (THNHM)	Thailand, Kanchanaburi, Thong Pha Phum
Polytrias indica (Houtt.) Veldkamp	Kellogg 1264 (MO)	Philippines, Luzon
Pseudosorghum fasciculare (Roxb.) A. Camus	Teerawatananon & Sungkaew 698 (THNHM)	Thailand, Tak, Um Phang
Saccharum angustifolium (Nees) Trin. [= *Erianthus angustifolius* Nees]	Longhi-Wagner & Welker 10656 (CTES, ICN)	Brazil, Rio Grande do Sul, Jaquirana
	Welker 344 (ICN)	Brazil, Rio Grande do Sul, Caçapava do Sul
	Welker 498 (ICN)	Brazil, Santa Catarina, Caçador
	Welker 628 (CTES, ICN)	Uruguay, Rocha, Velázquez
	Welker 650 (CTES, ICN)	Uruguay, Tacuarembó
Saccharum arundinaceum Retz. [= *Erianthus arundinaceus* (Retz.) Jeswiet; *Ripidium arundinaceum* (Retz.) Grassl]	Teerawatananon & Sungkaew 864 (THNHM)	Thailand, Nakhon Ratchasima, PakChong
Saccharum asperum (Nees) Steud. [= *Erianthus asper* Nees]	Longhi-Wagner & Welker 10673 (CTES, ICN)	Brazil, Rio Grande do Sul, Bom Jesus
	Welker 366 (CTES, ICN)	Brazil, Santa Catarina, Guaruva
	Welker 435 (ICN)	Brazil, Rio Grande do Sul, São Francisco de Paula
	Welker & Peichoto 583 (CTES, ICN, K, SI)	Argentina, Misiones, Leandro Alem
Saccharum ecklonii (Nees) Steud. [= *Erianthus ecklonii* Nees; *Miscanthidium capense* (Nees) Stapf; *Miscanthus ecklonii* (Nees) Mabb.]	Kellogg PI 410159 (MO)	South Africa, Cape Province
Saccharum giganteum (Walter) Pers. [= *Erianthus giganteus* (Walter) P. Beauv.]	Layton & Zhong 161 (MO)	USA, Louisiana, Saint Tammany
Saccharum narenga (Nees ex Steud.) Wall. ex Hack. [= *Narenga porphyrocoma* (Hance ex Trimen) Bor]	Teerawatananon & Sungkaew 783 (THNHM)	Thailand, Chiang Rai, Phaya Meng Rai
Saccharum officinarum L.	Welker s.n. (MO)	USA, Missouri, St. Louis
Saccharum ravennae (L.) L. [= *Erianthus ravennae* (L.) P. Beauv.; *Ripidium ravennae* (L.) Trin.; *Tripidium ravennae* (L.) H. Scholz]	Vela s.n. (MO)	USA, Missouri, St. Louis
Saccharum villosum Steud. [= *Erianthus trinii* (Hack.) Hack.]	Longhi-Wagner & Welker 10570 (CTES, ICN)	Brazil, Rio Grande do Sul, Caçapava do Sul
	Longhi-Wagner & Welker 10611 (CTES, ICN)	Brazil, Rio Grande do Sul, Encruzilhada do Sul
	Welker 539 (CTES, ICN)	Brazil, Rio Grande do Sul, Santo Antônio das Missões
	Welker 547 (CTES, ICN)	Brazil, Rio Grande do Sul, São Borja
	Welker & Peichoto 560 (CTES, ICN)	Argentina, Corrientes, San Roque
	Welker 651 (CTES, ICN)	Uruguay, Tacuarembó
Saccharum villosum Steud. ("wide leaf blades")	Welker 396 (CTES, ICN)	Brazil, Paraná, Aparecida do Ivaí
	Welker 477 (CTES, ICN)	Brazil, Rio Grande do Sul, São Luiz Gonzaga
	Welker & Peichoto 575 (CEN, CTES, ICN, K)	Argentina, Misiones, Apóstoles

TABLE 2. Continued.

Species	Voucher	Locality
Saccharum aff. *villosum* Steud.	Welker 502 (CTES, ICN)	Brazil, Santa Catarina, Caçador
	Welker 538 (CTES, ICN)	Brazil, Rio Grande do Sul, Santo Antônio das Missões
	Welker & Peichoto 556 (CTES, ICN)	Argentina, Corrientes, Empedrado
	Welker & Peichoto 584 (CORD, CTES, ICN)	Argentina, Misiones, Leandro Alem
	Welker 630 (CTES, ICN)	Uruguay, Rocha, Velázquez
Schizachyrium brevifolium (Sw.) Nees ex Buse	Teerawatananon & Sungkaew 750 (THNHM)	Thailand, Chiang Mai, Muang
Schizachyrium sanguineum (Retz.) Alston	Teerawatananon & Sungkaew 751 (THNHM)	Thailand, Chiang Mai, Muang
Sorghastrum elliottii (C. Mohr) Nash	Kellogg Kew MSB 491101 (MO)	USA, Texas, Anderson County
Sorghastrum nutans (L.) Nash	Kellogg PI 315744 (A/GH)	USA, West Virginia, Hampshire
Sorghum bicolor (L.) Moench	Kellogg PI 156549 (A/GH)	Zimbabwe
	Ortiz & Gomez K-1996-1544 (K)	unknown
Thelepogon elegans Roth	Teerawatananon & Sungkaew 697 (THNHM)	Thailand, Tak, LanSang
Themeda arundinacea (Roxb.) A. Camus	Teerawatananon & Sungkaew 739 (THNHM)	Thailand, Chiang Mai, Mae Rim
Tripsacum dactyloides (L.) L.	Kellogg 1261 (A/GH)	USA, Missouri, Pettis County
Zea mays L.	Cultivar B73 (genome sequence)	unknown

Our phylogenetic analyses are consistent with two possible treatments of the *Saccharum* clade. The first treatment, which we favor, would consider *Saccharum* in the broad sense (excluding only the species of *Tripidium*), because the type species of both *Saccharum* and *Erianthus* fell in the same clade, closely related to the South American taxa and other species of *Saccharum s.l.* (Fig. 3, type species in bold). The phylogenetic analysis of Hodkinson et al. (2002) also did not support the segregation of *Erianthus*; however, that work focused on species from Old World and included only *Erianthus contortus* Elliott from North America (no species from South America). The second possibility would be to consider *Saccharum s.s.* (represented by *S. officinarum* in our analyses), *Erianthus* (represented by *Saccharum giganteum*, *S. angustifolium*, *S. asperum*, *S. villosum*, and *Saccharum* aff. *villosum*), *Narenga* (represented by *S. narenga*), and *Miscanthidium* (represented by *S. ecklonii*) as distinct genera, as accepted by some authors based on morphological aspects (Clayton, 1972; Watson and Dallwitz, 1992; Amalraj and Balasundaram, 2006).

The taxonomic uncertainty rests in part on the absence of paralogue A for sugarcane in our analyses, which may have a technical or a biological explanation. The technical explanation is simply that our PCR-based approach failed to amplify the genome A paralogues. This is plausible because sugarcane has high ploidy levels (Iwo and Agboire, 2008; Besse et al., 1997) presumably giving rise to many paralogues; however, we sequenced 16–24 clones per locus for this sample making it less likely that we simply missed the genome A paralogue for all loci. If the genome A is present and simply missed by our analyses, then it would favor a very broad *Saccharum s.l.*

The biological explanation is complex, but cannot be ruled out. In this scenario, an initial A × B hybridization event followed by allopolyploidization gave rise to *Erianthus-Miscanthidium* species, but not to *Saccharum officinarum*. Instead, the ancestor of genome B would have independently given rise to *Saccharum s.s.* In this scenario, *Narenga* might have arisen from a hybrid between *Saccharum s.s.* and *Miscanthidium*, because paralogue B of *Saccharum narenga* grouped with *S. officinarum* and paralogue A with *S. ecklonii*. This scenario would favor a more traditional circumscription of the genera (Clayton, 1972; Watson and Dallwitz, 1992) to reflect their disparate phylogenetic histories (see Fig. 5).

A larger sample is needed for a more accurate answer about the circumscription of *Saccharum* and related genera, as well as to test these hypotheses. Future studies should increase the number of species of *Saccharum s.l.* from the Old World and North America, as well as a more representative sample of the genus *Miscanthus*, which is part of the complex evolutionary history of sugarcane and relatives. Morphological character evolution and biogeography investigations may also help to better understand the evolution of this group.

***Taxonomic circumscription of South American species of* Saccharum s.l.—**The presence of two (or more) distinct paralogues per sample in the phylogenetic trees indicates that all analyzed South American taxa of *Saccharum s.l.* are polyploid, along with other species of *Saccharum s.l.* and *Miscanthus*. This is in agreement with published chromosome counts available for some of these species (Molina, 1981; Besse et al., 1998) and cytogenetic studies that are currently being performed with the specimens included in our phylogeny.

Saccharum angustifolium and *S. villosum* are clearly distinct species, as accepted by Filgueiras (2003) and Welker and Longhi-Wagner (2012). *Saccharum villosum* can be morphologically distinguished from *S. angustifolium* mainly by its lanceolate leaf blades, which are generally pilose and 7–20 mm wide, whereas in *S. angustifolium* the leaf blades are linear, glabrous, and 2–6 mm wide. Moreover, the leaf blades of *S. angustifolium* have a conspicuous whitish midvein up to the apex of the blade, which is wider than or as wide as the lateral portion of the blade. In *S. villosum*, the whitish midvein is narrower than the lateral portion of the blade and is inconspicuous in the upper portion of the blade (see Table 1 and Fig. 1). These two species also differ in their habitats: *S. angustifolium* occurs in dry grasslands, whereas *S. villosum* inhabits marshlands and wet grasslands (Welker and Longhi-Wagner, 2012). The two species have a broad geographical distribution: *S. angustifolium* is distributed from Colombia and Venezuela to Argentina and Uruguay, while *S. villosum* is distributed from Mexico to Argentina and Uruguay (Molina, 1981; Filgueiras, 2003).

Some specimens identified as *Saccharum villosum* from Southern Brazil and Argentina are robust plants with wider leaf blades than the type material and most members of the species (see Table 1 and Fig. 1). These specimens with wide blades represent a monophyletic group that is sister to the clade with the remaining specimens of *S. villosum*, based on sequences from genome A (but without good support based on genome B)

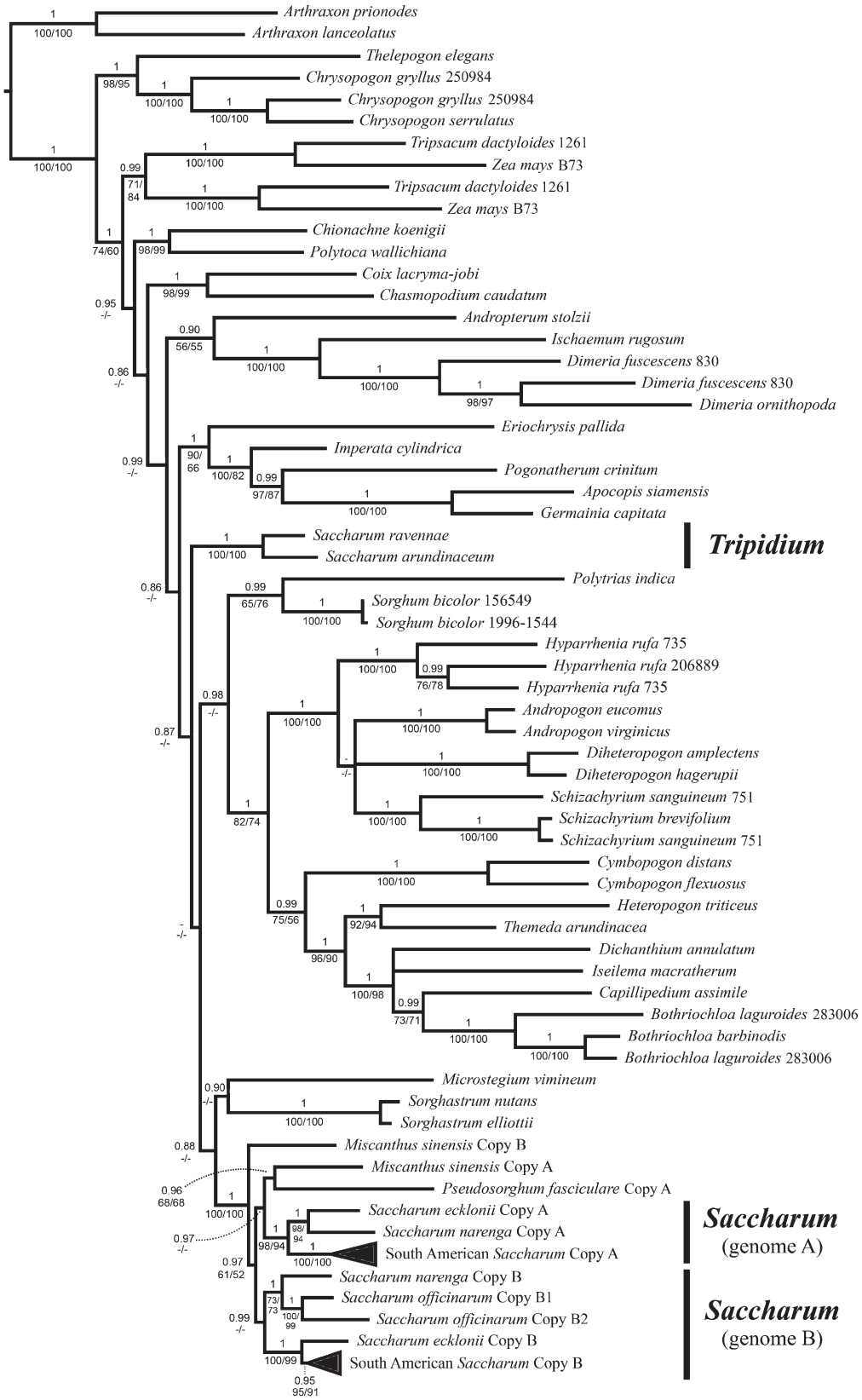

Fig. 2. Bayesian phylogeny of *Saccharum s.l.* and other genera of Andropogoneae, based on the combined data set (*apo*1, *d*8, *ep*2-*ex*7, *ep*2-*ex*8, and *rep*1), shown as a phylogram. Bayesian PP > 0.85 are shown above branches, and ML / MP bootstrap values > 50 are shown below. For species with more than one specimen or more than one paralogue in our analyses, collector number is after the binomial, according to Table 2. Clades of South American taxa of *Saccharum s.l.* were collapsed and presented in detail in Fig. 3.

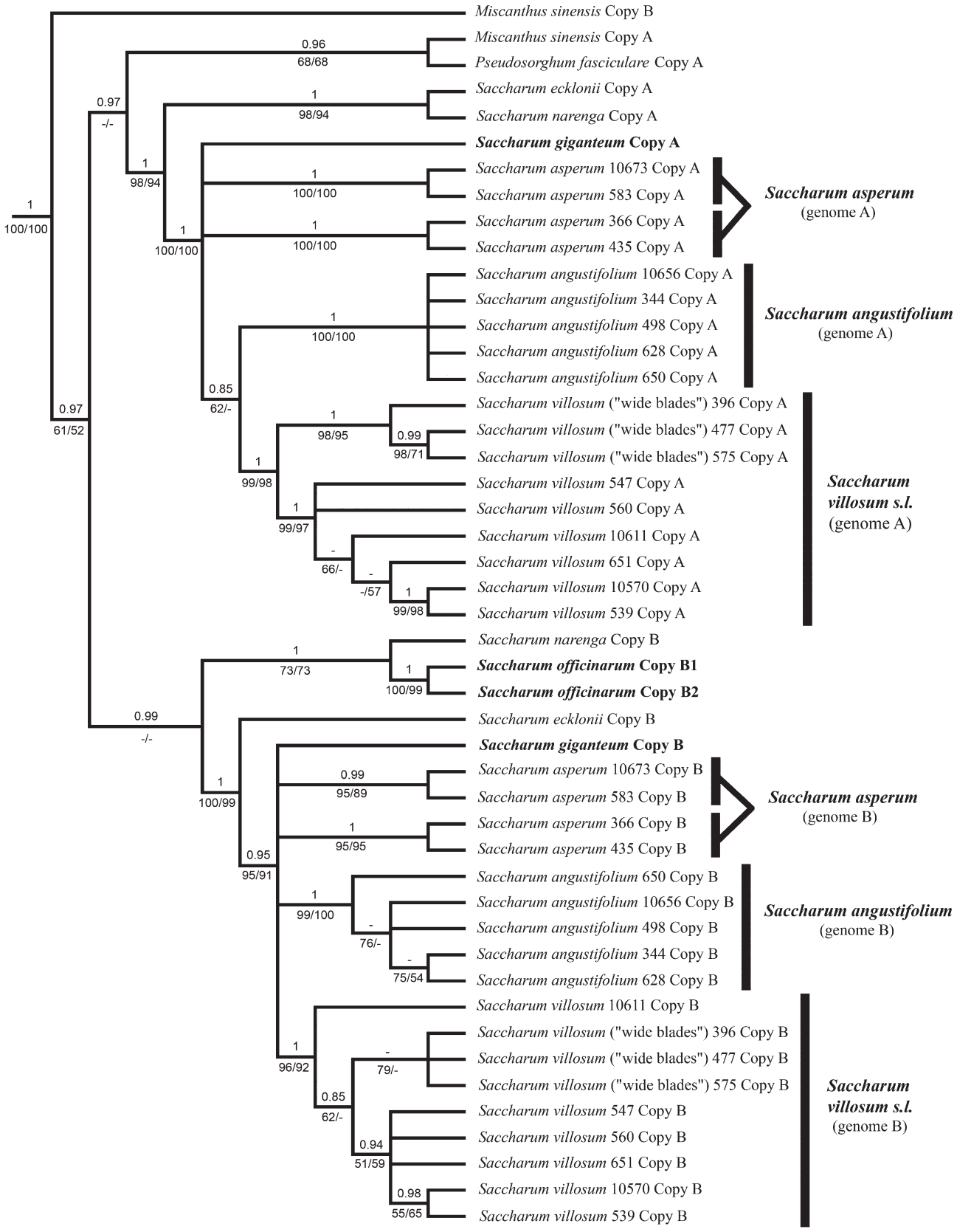

Fig. 3. Bayesian phylogeny of *Saccharum s.l.* and closely related genera, based on the combined data set (*apo*1, *d*8, *ep*2-*ex*7, *ep*2-*ex*8, and *rep*1). Bayesian PP > 0.85 are shown above branches, and ML / MP bootstrap values > 50 are shown below. For taxa with more than one specimen in our analyses, the collector number is after the binomial, according to Table 2. The type species of *Saccharum* (*S. officinarum*) and *Erianthus* (*E. giganteus* / *Saccharum giganteum*) are highlighted in bold.

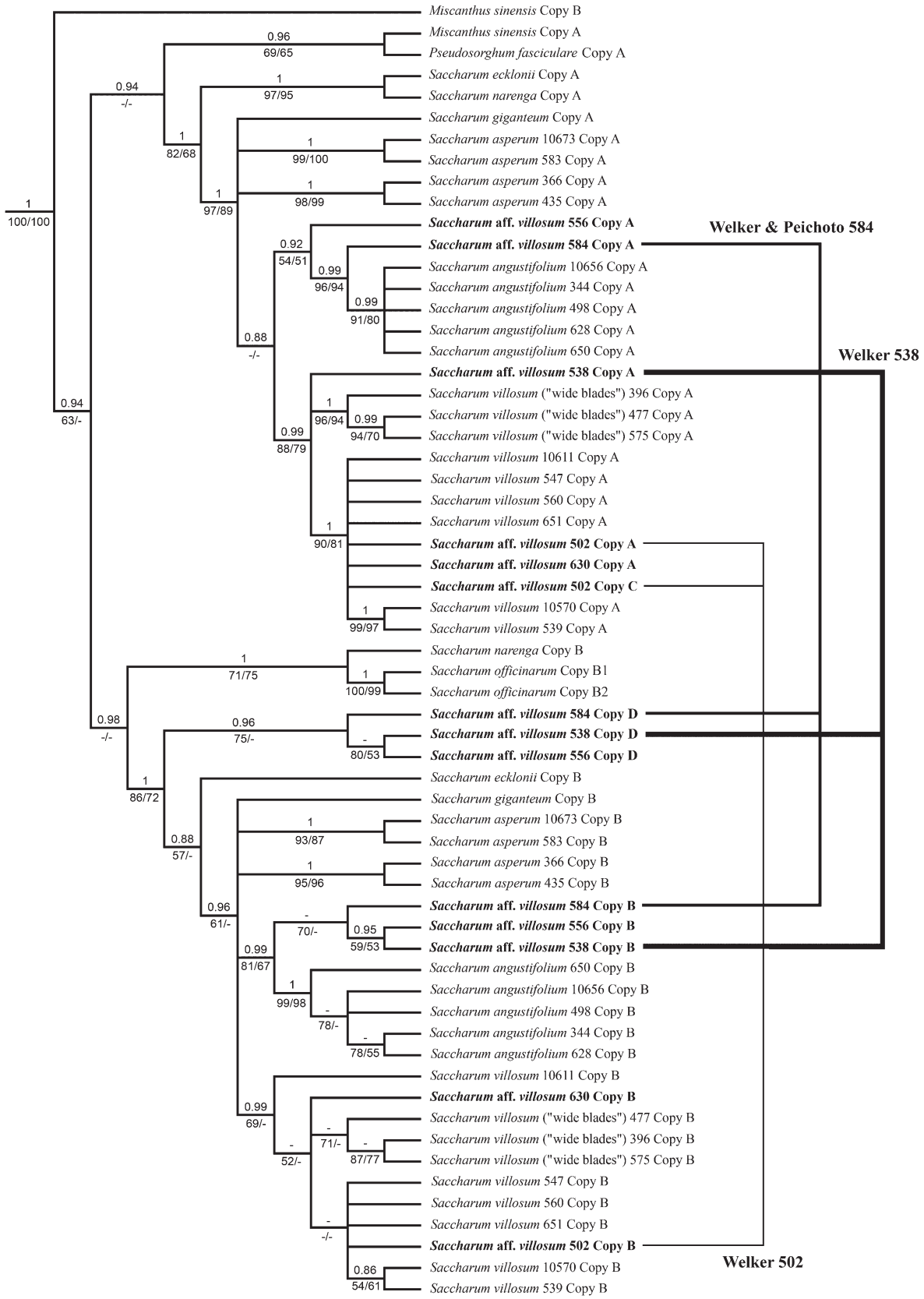

Fig. 4. Bayesian phylogeny of *Saccharum s.l.* and closely related genera, including the specimens of *Saccharum* aff. *villosum* (names in bold), based on the combined data set (*apo*1, *d*8, *ep*2-*ex*7, *ep*2-*ex*8, and *rep*1). Bayesian PP > 0.85 are shown above branches, and ML / MP bootstrap values > 50 are shown below. For taxa with more than one specimen in our analyses, the collector number is after the binomial, according to Table 2.

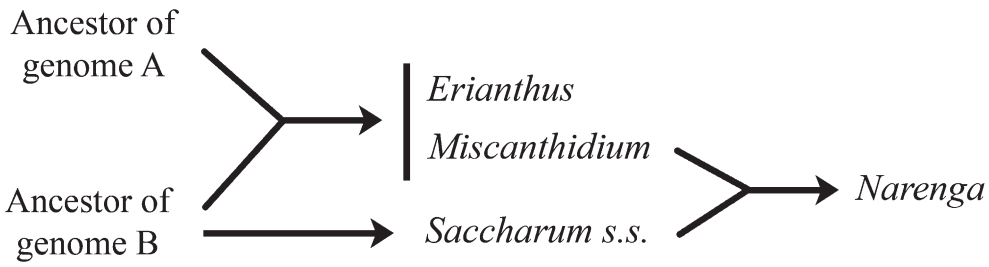

Fig. 5. Schematic representation of the possible origin of *Saccharum* and relatives.

(Fig. 3). Several binomials formerly accepted as distinct species by some authors (e.g., Swallen, 1966; Molina, 1981; Smith et al., 1982) are currently considered synonyms of *S. villosum* (Filgueiras, 2003; Morrone et al., 2008); these names include *Erianthus balansae*, *E. clandestinus*, *E. glabrinodis*, *E. purpureus*, and *E. trinii*. Although Welker and Longhi-Wagner (2012) suggested that "*S. villosum s.l.*" might include more than one taxon, investigation of the protologues and type materials of the five names cited above demonstrated that they do not correspond to the plants with very wide leaf blades, and confirmed that the five names should be considered synonyms of *S. villosum*, following Filgueiras (2003). Because the unique morphological difference between the specimens of two clades is the leaf blade width (with some overlap in some specimens), and the present phylogenetic analysis based on both genomes A and B also supports the acceptance of *S. villosum s.l.* as a single species, the specimens with wide blades are being considered here as a morphological variation of this species.

The specimens of *Saccharum asperum* did not group in the *S. angustifolium* or the *S. villosum* clades, suggesting that *S. asperum* is a distinct taxon. However, unlike the other two species, the samples of *S. asperum* did not form a monophyletic group in our analysis. The specimens formed two distinct well-supported clades in both the A and B genomes (Fig. 3). This is surprising because *S. asperum* does not present much morphological variability, and the acceptance of it as a single species was not previously questioned by taxonomists (Filgueiras, 2003; Peichoto and Rúgolo, 2012; Welker and Longhi-Wagner, 2012). Molina (1981, under *Erianthus*) accepted two varieties for this species (*E. asper* var. *asper* and *E. asper* var. *brasilianus*), which are differentiated by the length of the spikelets and the indumentum of the axis of the inflorescence. However, these traits are not good taxonomic characters because they vary within plants and populations, and the two varieties are currently considered synonyms of *Saccharum asperum* (Filgueiras, 2003; Peichoto and Rúgolo, 2012; Welker and Longhi-Wagner, 2012). The morphology of the specimens of the two clades from our analysis does not correspond to the two varieties accepted by Molina (1981). *Saccharum asperum* occurs from Colombia and Venezuela to Argentina and Uruguay (Molina, 1981; Filgueiras, 2003). It is morphologically distinct from the other species of the genus in South America by the entirely glabrous glumes, in both sessile and pedicelled spikelets. The other species have pilose glumes, at least in the pedicelled spikelets (Welker and Longhi-Wagner, 2012) (Table 1, Fig. 1).

***Hybridization in* Saccharum s.l.—**Hybridization plays a significant role in the evolutionary history of sugarcane and relatives. Interspecific and intergeneric hybrids have been documented involving *Saccharum s.l.*, *Miscanthus*, and *Sorghum* (Hodkinson et al., 2002; Nair et al., 2005; Aitken et al., 2007). Welker and Longhi-Wagner (2012) suggested that the specimens identified as *Saccharum* aff. *villosum* might be natural hybrids between *S. villosum* and *S. angustifolium*, based on the intermediate morphology of those plants. The specimens of *S.* aff. *villosum* present leaves typical of *S. villosum* (lanceolate leaf blades with the midvein narrower than the lateral portion of the blade and inconspicuous in the upper portion of the leaf), but the blades are narrower than usual for the species, with width similar to those of *S. angustifolium* (Welker and Longhi-Wagner, 2012) (see Table 1 and Fig. 1). The lower bootstrap support for most nodes when these specimens were included in the phylogeny, compared to the tree without *S.* aff. *villosum*, is consistent with the hypothesis of hybrid origin for these specimens (Funk, 1985; McDade, 1992). However, as pointed out by McDade (1992), the disturbance to cladistic relationships in trees caused by hybridization is higher if the samples are hybrids between distantly related parents. This could explain the similar topologies of our trees, with and without *S.* aff. *villosum*, because the probable parents of the hybrids (*S. villosum* and *S. angustifolium*) are closely related species.

The three distinct paralogues per sample of *Saccharum* aff. *villosum* in the phylogenetic tree suggest that these plants are probably hexaploid, in contrast to the two paralogues of the specimens of *S. villosum* and *S. angustifolium*, which are probably tetraploids or triploids. The ploidy level inferred by the number of paralogues is in agreement with recent cytogenetic studies of these specimens, which confirm that *S.* aff. *villosum* presents a higher ploidy level than the other two species (C.A.D. Welker, unpublished data). The presence of only two paralogues for the specimen Welker 630, contrasting with the other accessions of *S.* aff. *villosum*, is not good evidence of a distinct ploidy level, because our PCR-based approach may not have uncovered all paralogues of this sample. The third paralogues (called C or D in Fig. 4) of *S.* aff. *villosum* specimens are probably recombinant copies that resulted from the hybridization events, because they share some synapomorphic bases with paralogues A and some with paralogues B. Because stop codons or other frameshifts are not present, which would inactivate the proteins, they do not seem to be pseudogenes (Zheng and Gerstein, 2007).

The paralogues of the specimens of *Saccharum* aff. *villosum* fell in many distinct clades along the phylogeny, both in genomes A and B, confirming the reticulate history of these plants. The topology of the paralogues of the specimen Welker 538 clearly demonstrates that it is a hybrid between *S. villosum* and *S. angustifolium*, because one paralogue is grouped in the *S. villosum* clade and other paralogue is in the *S. angustifolium* clade (Fig. 4). The higher ploidy level of *S.* aff. *villosum* compared to both putative parental species suggests interspecific hybridization followed by duplication of genomes (allopolyploidy), or hybridization involving an unreduced gamete. It is

well known that polyploidy can restore fertility to sterile hybrid lineages after hybridization (McDade, 1992). Many independent allopolyploid events in the tribe Andropogoneae were documented by Estep et al. (2014).

The evolutionary history of the plants identified as *S.* aff. *villosum* and their parents seems to be complex, with different contributions from both *S. villosum* and *S. angustifolium* in the formation of the hybrids. Hexaploid specimens with paralogues genetically more similar to *S. villosum*, and other specimens more similar to *S. angustifolium*, are consistent with this interpretation. Our results indicate that these plants with intermediate morphology are natural hybrids, and that hybridization has probably occurred more than once; the exact mechanism of formation is unclear. Additional molecular and cytogenetic studies, including fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) analyses, may bring new insights into the evolutionary dynamics of these taxa and elucidate the different genome contribution of the parents in the formation of these hybrids.

***Concluding remarks*—**The allopolyploid origin of *Saccharum* was demonstrated in this study using evidence from nuclear genes. Our phylogenetic analyses indicate that *Saccharum s.l.* is polyphyletic and *Tripidium* should be recognized as a distinct genus, following Grassl (1972, under *Ripidium*). However, no strong evidence was found to support the segregation of *Erianthus* from *Saccharum s.l.* The results also indicate that all South American taxa of *Saccharum s.l.* are polyploid, based on the number of paralogues in the trees. *Saccharum angustifolium, S. asperum,* and *S. villosum* proved to be distinct species. The occurrence of natural hybrids between *S. villosum* and *S. angustifolium* was also documented. Better understanding of the phylogenetic relationships of *Saccharum* and relatives may be useful for sugarcane breeders to identify potential taxa for interspecific and intergeneric crosses in the genetic improvement of sugarcane.

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APPENDIX 1. Species names, voucher specimens, and GenBank accession numbers for sequences included in this study. NA (not available).

Species	Voucher	*apo*1	*d*8	*ep2-ex*7	*ep2-ex*8	*rep*1
Andropogon eucomus Nees	Malcomber et al. 3089 (MO)	KM578363	KM578119	KM577921	KM577706	KM578555
Andropogon virginicus L.	Kellogg 1240 (MO)	KM578449	KM578209	KM578001	KM577789	KM578635
Andropterum stolzii (Pilg.) C. E. Hubb.	Malcomber et al. 3091 (MO)	KM578417	KM578168	KM577978	KM577764	KM578609
Apocopis siamensis A. Camus	Teerawatananon & Sungkaew 975 (THNHM)	KM578503	KM578287	KM578058	KM577852	NA
Arthraxon lanceolatus (Roxb.) Hochst.	Teerawatananon & Sungkaew 720 (THNHM)	NA	KM578290	KM578061	KM577854	KM578689
Arthraxon prionodes (Steud.) Dandy	Kellogg PI 659331 (MO)	NA	KM578256	KM578036	KM577831	KM578672
Bothriochloa barbinodis (Lag.) Herter	Kellogg PI 204138 (MO)	KM578453	KM578212	NA	KM577794	KM578638
Bothriochloa laguroides (DC.) Herter	Kellogg PI 283006 (MO)	KM578462, KM578463	KM578223, KM578224	NA	NA	KM578648, KM578649
Capillipedium assimile (Steud.) A. Camus	Teerawatananon & Sungkaew 791 (THNHM)	KM578504	KM578291	KM578062	KM577855	KM578690
Chasmopodium caudatum (Hack.) Stapf	Kellogg Kew MSB 184054 (MO)	KM578498	KM578275	KM578046	KM577842	KM578683
Chionachne koenigii (Spreng.) Thwaites	Kellogg Chio-6-D-93 (MO)	KP243072	KP233123	KP242922	KP242976	KP243025
Chrysopogon gryllus (L.) Trin.	Kellogg PI 250984 (A/GH)	KM578372, KM578373	NA	KM577928, KM577929	KM577714, KM577715	KM578561, KM578563
Chrysopogon serrulatus Trin.	Kellogg PI 219580 (A/GH)	KM578434	KM578184	KM577988	KM577769	KM578619
Coix lacryma-jobi L.	Kellogg PI 320865 (MO)	KM578425	KM578175	NA	NA	KM578613
Cymbopogon distans (Nees ex Steud.) Will. Watson	Kellogg PI 271552 (MO)	KM578378	KM578126	KM577939	NA	KM578569
Cymbopogon flexuosus (Nees ex Steud.) Will. Watson	Kellogg PI 209700 (A/GH)	KM578385	KM578128	KM577940	KM577719	KM578570
Dichanthium annulatum (Forssk.) Stapf	Kellogg PI 240155 (A/GH)	NA	KM578135	KM577945	KM577725	KM578575
Diheteropogon amplectens (Nees) Clayton	Kellogg RF 1819 (MO)	KM578432	KM578183	KM577987	KM577768	KM578616
Diheteropogon hagerupii Hitchc.	Kellogg Kew MSB 254456 (MO)	KM578548	KM578355	KM578110	KM577907	KM578735
Dimeria fuscescens Trin.	Teerawatananon & Sungkaew 830 (BKF, THNHM)	KM578511, KM578513	KM578300, KM578301	KM578069, KM578070	KM577862, KM577863	NA
Dimeria ornithopoda Trin.	Teerawatananon & Sungkaew 685 (BKF, THNHM)	KM578514	KM578305	NA	KM577864	NA
Eriochrysis pallida Munro	Malcomber et al. 3086 (MO)	KM578393	KM578137	KM577947	KM577728	NA
Germainia capitata Balansa & Poitr.	Teerawatananon & Sungkaew 834 (THNHM)	KM578515	KM578312	KM578075	KM577870	NA
Heteropogon triticeus (R. Br.) Stapf ex Craib	Teerawatananon & Sungkaew 733 (THNHM)	KM578516	KM578314	KM578076	KM577874	KM578703
Hyparrhenia rufa (Nees) Stapf	Kellogg PI 206889 (A/GH)	KM578396	KM578140	KM577948	KM577732	KM578578
	Teerawatananon & Sungkaew 735 (THNHM)	KM578523	KM578319	KM578080, KM578081	KM577875, KM577876	KM578708, KM578710
Imperata cylindrica (L.) P. Beauv.	Kowarat 108 (THNHM)	KM578524	KM578321	KM578082	KM577877	KM578712
Ischaemum rugosum Salisb.	Kellogg Kew MSB 183574 (MO)	KM578551	KM578356	KM578113	KM577910	NA
Iseilema macratherum Domin	Snow et al. 7239 (A/GH)	KM578440	KM578192	KM577992	KM577775	KM578625
Microstegium vimineum (Trin.) A. Camus	Kellogg VA-2 (MO)	NA	NA	KM578051	KM577846	KM578685

APPENDIX 1. Continued.

Species	Voucher	*apo*1	*d*8	*ep2-ex*7	*ep2-ex*8	*rep*1
Miscanthus sinensis Andersson	Kellogg PI 668403 (MO)	KM578443, KM578444	KM578199, KM578201	KM577993, KM577994	KM577779, KM577781	NA
Pogonatherum crinitum (Thunb.) Kunth	Teerawatananon & Sungkaew 865 (THNHM)	NA	NA	KM578088	KM577885	KM578715
Polytoca wallichiana (Nees ex Steud.) Benth.	Teerawatananon & Sungkaew 683 (THNHM)	KP243073	KP233124	KP242923	KP242977	KP243026
Polytrias indica (Houtt.) Veldkamp	Kellogg 1264 (MO)	NA	KM578208	KM578000	KM577788	KM578633
Pseudosorghum fasciculare (Roxb.) A. Camus	Teerawatananon & Sungkaew 698 (THNHM)	NA	KM578329	KM578089	KM577886	KM578716
Saccharum angustifolium (Nees) Trin.	Longhi-Wagner & Welker 10656 (CTES, ICN)	KP243042, KP243043	KP233105	KP242902, KP242903	KP242943, KP242944	KP242993, KP242994
	Welker 344 (ICN)	KP243035, KP243036	KP233100	NA	KP242935, KP242936	KP242987, KP242988
	Welker 498 (ICN)	KP243037	KP233101, KP233102	KP242896, KP242897	KP242937, KP242938	KP242989
	Welker 628 (CTES, ICN)	KP243038, KP243039	KP233103	KP242898, KP242899	KP242939, KP242940	KP242990
	Welker 650 (CTES, ICN)	KP243040, KP243041	KP233104	KP242900, KP242901	KP242941, KP242942	KP242991, KP242992
Saccharum arundinaceum Retz.	Teerawatananon & Sungkaew 864 (THNHM)	NA	KM578332	KM578090	KM577888	KM578720
Saccharum asperum (Nees) Steud.	Longhi-Wagner & Welker 10673 (CTES, ICN)	KP243050, KP243051	NA	NA	KP242951, KP242952	KP243001, KP243002
	Welker 366 (CTES, ICN)	KP243044	KP233106	KP242904	KP242945, KP242946	KP242995, KP242996
	Welker 435 (ICN)	KP243046, KP243047	KP233109	NA	KP242947, KP242948	KP242997, KP242998
	Welker & Peichoto 583 (CTES, ICN, K, SI)	KP243048, KP243049	KP233110	KP242905, KP242906	KP242949, KP242950	KP242999, KP243000
Saccharum ecklonii (Nees) Steud.	Kellogg PI 410159 (MO)	KM578467, KM578468	KM578229, KM578230	KM578012, KM578013	KM577807, KM577810	KM578654, KM578656
Saccharum giganteum (Walter) Pers.	Layton & Zhong 161 (MO)	KP243052, KP243053	KP233111, KP233112	NA	KP242953, KP242954	KP243003, KP243004
Saccharum narenga (Nees ex Steud.) Wall. ex Hack.	Teerawatananon & Sungkaew 783 (THNHM)	KM578528, KM578529	KM578334, KM578337	KM578092, KM578094	KM577891, KM577892	KM578722, KM578725
Saccharum officinarum L.	Welker s.n. (MO)	KP243055, KP243056	NA	KP242907, KP242908	KP242956, KP242957	NA
Saccharum ravennae (L.) L.	Vela s.n. (MO)	KM578491	KM578269	KM578042	KM577837	KM578681
Saccharum villosum Steud.	Longhi-Wagner & Welker 10570 (CTES, ICN)	KP243064, KP243065	KP233118	KP242917	KP242966, KP242967	KP243014, KP243015
	Longhi-Wagner & Welker 10611 (CTES, ICN)	KP243070, KP243071	NA	NA	KP242974, KP242975	KP243023, KP243024
	Welker 539 (CTES, ICN)	KP243057, KP243058	KP233113, KP233114	KP242909, KP242910	KP242958, KP242959	KP243006, KP243007
	Welker 547 (CTES, ICN)	KP243059, KP243060	KP233115	KP242911, KP242912	KP242960, KP242961	KP243008, KP243009
	Welker & Peichoto 560 (CTES, ICN)	KP243061	KP233116	KP242913, KP242914	KP242962, KP242963	KP243010, KP243011
	Welker 651 (CTES, ICN)	KP243062, KP243063	KP233117	KP242915, KP242916	KP242964, KP242965	KP243012, KP243013
Saccharum villosum Steud. ("wide leaf blades")	Welker 396 (CTES, ICN)	NA	KP233119, KP233120	KP242918, KP242919	KP242968, KP242969	KP243016, KP243017
	Welker 477 (CTES, ICN)	NA	KP233121	NA	KP242970, KP242971	KP243018
	Welker & Peichoto 575 (CEN, CTES, ICN, K)	KP243068, KP243069	KP233122	KP242920, KP242921	KP242972, KP242973	KP243021, KP243022
Saccharum aff. *villosum* Steud.	Welker 502 (CTES, ICN)	NA	NA	KP242887, KP242888	KP242924, KP242925	KP242978, KP242979, KP242980
	Welker 538 (CTES, ICN)	KP243027	KP233094, KP233095	KP242889, KP242890, KP242891	KP242926, KP242927, KP242928	KP242981, KP242982
	Welker & Peichoto 556 (CTES, ICN)	KP243028, KP243029	KP233096, KP233097, KP233098	KP242892	KP242929, KP242930, KP242931	KP242983
	Welker & Peichoto 584 (CORD, CTES, ICN)	KP243030, KP243031, KP243032	NA	KP242893, KP242894, KP242895	KP242932	KP242984
	Welker 630 (CTES, ICN)	KP243033, KP243034	KP233099	NA	KP242933, KP242934	KP242985, KP242986

APPENDIX 1. Continued.

Species	Voucher	*apo*1	*d*8	*ep2-ex*7	*ep2-ex*8	*rep*1
Schizachyrium brevifolium (Sw.) Nees ex Buse	Teerawatananon & Sungkaew 750 (THNHM)	KM578530	NA	KM578097	KM577893	NA
Schizachyrium sanguineum (Retz.) Alston	Teerawatananon & Sungkaew 751 (THNHM)	KM578532, KM578533	KM578339, KM578340	KM578099, KM578100	KM577894	NA
Sorghastrum elliottii (C. Mohr) Nash	Kellogg Kew MSB 491101 (MO)	KM578552	NA	KM578114	KM577911	KM578737
Sorghastrum nutans (L.) Nash	Kellogg PI 315744 (A/GH)	NA	NA	KM577963	KM577751	KM578591
Sorghum bicolor (L.) Moench	Kellogg PI 156549 (A/GH)	KM578410	KM578151	KM577964	KM577752	KM578593
	Ortiz & Gomez K-1996-1544 (K)	KM578412	KM578153	KM577966	KM577754	KM578595
Thelepogon elegans Roth	Teerawatananon & Sungkaew 697 (THNHM)	KM578539	NA	KM578103	KM577895	KM578728
Themeda arundinacea (Roxb.) A. Camus	Teerawatananon & Sungkaew 739 (THNHM)	NA	KM578349	KM578104	KM577897	NA
Tripsacum dactyloides (L.) L.	Kellogg 1261 (A/GH)	KM578413	KM578154	KM577967, KM577968	KM577755, KM577756	KM578596, KM578597
Zea mays L.	Cultivar B73 (genome sequence)	NA	GRMZM2G360081, GRMZM2G109966	GRMZM2G024973, GRMZM2G144744	GRMZM2G098859, GRMZM2G414043	GRMZM2G110242, GRMZM2G064628